Optimizing reproduction in the Tasmanian echidna *Tachyglossus aculeatus setosus*: the influence of an obligatory hibernation period and intense sexual conflict

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania
September 2013
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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government’s Office of Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Statement of Publication and Co-authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

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This thesis is based on field data collected by me in the period of January 2008 to December 2010, but also includes some data collected by me in 2007 as well as data collected as part of a long time project on echidna reproductive biology. Data I collected in 2007 is included in chapter 2 Cool sex? Hibernation and reproduction overlap in the echidna and chapter 5 Maternal care in the Tasmanian echidna. Data from 2007 in chapter 2 Cool sex? Hibernation and reproduction overlap in the echidna includes two observations previously reported in my honours thesis (Morrow 2007). A small number of additional data collected by other researchers is included in chapter 2 Cool Sex? Hibernation and reproduction overlap in the echidna, Chapter 3 Reproductive tactics of the Tasmanian echidna and Chapter 5 Maternal care in the Tasmanian echidna.
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Abstract

The echidna is a solitary, seasonally breeding monotreme mammal with a mating system characterized by high levels of intra-male competition for access to receptive females. Throughout Australia the breeding season follows a period of inactivity which ranges from shallow bouts of torpor to prolonged deep hibernation. In this thesis I investigated how the Tasmanian echidna optimizes its reproduction around an obligatory hibernation period and in the presence of intense sexual conflict.

The bradymetabolic (slowing of metabolism) effect of hibernation was exploited by both sexes to optimize their reproduction. I found that testes recrudescence (defined as an increase in testes volume and density) was initiated prior to males entering hibernation, a strategy not seen in any other hibernating mammal. This strategy can be linked to the low energy and density diet and requirement to hibernate to maximise energy-savings, and to the large relative size of echidna testes reflecting a mating system with intense levels of intra-male competition. It took approximately two months at euthermic body temperatures from the initiation of recrudescence in December for echidna testes to reach 75% of peak size. Therefore if testes recrudescence did not occur prior to entering hibernation, hibernation would be restricted to a one and a half month period to allow mating in June.

Male echidnas initiated mating activity by locating hibernating females and entering their hibernacula. This strategy was common in my study population and males that remained with a female in her hibernaculum for 13 hours or more gained a copulation opportunity. However, all females that mated or were disturbed by males prior to July 27 re-entered hibernation. This indicates that mating often occurred earlier than optimal for female reproductive success. Many of the females that re-entered hibernation after mating were pregnant. Pregnant females entered hibernation only in early pregnancy: hibernation extended the gestation period and hence allowed females to delay egg-laying. Females timed their reproduction so that they emerged from their 37 day period of nursery burrow confinement as ecosystem productivity increased. Hibernation therefore allows successful reproduction in a population where there is asynchronous timing of optimal mating between males and females.

This thesis explores the influence of hibernation on sexual conflict, demonstrates the numerous interactions that can occur between hibernation and reproduction and shows that the bradymetabolic property of hibernation is exploited by both male and female echidnas to optimize reproductive fitness.
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Chapter 1: General Introduction
Chapter 1. General Introduction

The battle of the sexes is no myth. Success at sexual reproduction is at the heart of the evolutionary process. But greater success for her often means less success for him. The upshot? An eternal war – and an astounding diversity of strategies

Olivia Judson, ‘Dr. Tatiana’s sex advice to all creation’ 2002

In all dioecious species males and females share the common goal of reproducing and passing on their genes. However, differences in energetic investment in reproduction between the sexes result in conflict (sexual conflict), in which both sexes manipulate and exploit their mate(s) to maximise their own fitness (Bateman 1948, Trivers 1972, Parker 1979, Birkhead 2000, Wedell et al. 2006). For individuals of both sexes, reproductive success is dependent on body condition (Kimber et al. 2009), the behaviour of mates and ecological variables which influence encounter rates (Emlen and Oring 1977, Kokko and Rankin 2006, Klug 2011). Importantly, in seasonally breeding species reproductive success depends on timing reproduction at the optimal time of year when climate and food availability are favourable for offspring survival (Lincoln and Short 1980, Bronson 1985). Sexual conflict evolves within the constraints set by the environment and the species’ mating system, while mating systems evolve in response to sexual conflict (Stumpf et al. 2011). Hence there are complex interactions between ecology and the environment, mating systems and sexual conflict (see Fig. 1.1). To understand how an individual optimizes its reproduction, the inter-relationships between ecology and the environment, mating systems and sexual conflict must be examined.
1. Sexual conflict

Sexual conflict arises due to the different fitness optima of each sex (Parker 1979). Differences between the sexes are rooted in anisogamy (differences in gamete size) which result in competing phenotypic optima for each sex and distinct sexual roles (Bateman 1948, Trivers 1972, Pizzari and Snook 2003). Anisogamy results in the total investment in gametes expended by females per mating event being larger than that of males (Kokko and Jennions 2008). In addition, females typically provide more prolonged parental care than males, which delays females’ return to receptivity for further mating (Kokko and Jennions 2008). Although the potential reproductive rate (the average lifetime number of offspring) of males usually exceeds that of females (Clutton-Brock and Vincent 1991), in populations with an even sex ratio the average reproductive rate will be the same for both sexes (Wedell et al. 2006, Parker and Birkhead 2013). Individual male success is dependent on the number of females inseminated while female reproductive success is limited by the number of viable eggs (Bateman 1948) or neonates produced. This means that males potentially
show a greater variance in reproductive success than females (Wade and Shuster 2004). While average reproductive rate will be the same for both sexes (Wedell et al. 2006, Parker and Birkhead 2013), females typically have a lower optimal mating rate than males (Arnqvist and Rowe 2005). Differences in optimal mating rates between the sexes biases the operational sex ratio (OSR): the ratio of the number of sexually receptive females to sexually active males (Emlen and Oring 1977) and skews in the OSR result in males competing against one another for access to receptive females. Consequently females are typically the more limited and, hence, “choosier sex”, and are expected to maximise their fitness with low mating rates while males maximise their fitness with high mating rates (Arnqvist and Rowe 2005).

Sexual conflict is inevitable and ubiquitous (Hosken and Stockley 2005). All reproductive interactions between the sexes including mate choice, mating rates, mating duration and parental investment imply a conflict (Trivers 1972, Parker 1979, Gavrilets et al. 2001, Pizzari and Snook 2003, Wedell et al. 2006) However, conflict will not occur under strict monogamous conditions where there is no prospect of re-mating even after one partner dies (Hosken et al. 2001, Wedell et al. 2006) or if an individual only breeds once in its lifetime (semelparity). Sexual conflict provides the potential for each sex to reach its preferred optimum at the expense of the other sex (Chapman 2006), resulting in the evolution of a variety of behavioural, anatomical and physiological traits related to reproduction (Stumpf et al. 2011).

1.1 Forms of sexual conflict

There are two main forms of sexual conflict: intralocus conflict and interlocus conflict. Intralocus conflict occurs when alleles at the same locus in males and females have different benefits to the
bearer depending on its sex. In contrast, interlocus conflict occurs when there is conflict between alleles at two or more different interacting loci in males and females (Arnqvist and Rowe 2005).

1.1.1 Intralocus sexual conflict

Autosomal allelic variation at a locus can affect traits in both sexes (Parker 2006). Intralocus conflict results from the different selection pressures faced by males and females and occurs when the same allele has opposite effects on the fitness of each sex (Arnqvist and Rowe 2005, Wedell et al. 2006). Intralocus conflict has the potential to limit adaptive evolution in both sexes as genes at relevant loci can be pulled in opposite directions by antagonistic selection between the two sexes, where the trait is selected for by the one sex which benefits, but selected against by the sex for which the trait is disadvantageous (Arnqvist and Rowe 2005). Hence, intralocus conflict may hinder adaptive evolution in both sexes because selection acting on one sex specifically can constrain evolution of the other (Parker and Partridge 1998). Examples of intralocus conflict include: sexual size dimorphism at fledging in collared flycatchers Ficedula albicollis (Merilä et al. 1997); bill width, bill depth and tail length in serins Serinus serinus (Björklund and Senar 2001); immune defence and orange throat colouration in the side-blotched lizard Uta stansburiana (Svensson et al. 2009); fitness in the red deer Cervus elaphus (Foerster et al. 2007) and the expression of sexually antagonistic alleles (Rice 1992) as well as adult fitness variation (Chippindale et al. 2001) in the fruit fly Drosophila melanogaster.

1.1.2 Interlocus sexual conflict

Interlocus conflict occurs when there is conflict over the outcome of male-female interactions. Reciprocal adaptation and counter-adaptation between the sexes can shift the outcome towards the
reproductive optima of one sex and this sexually antagonistic coevolution can result in irresolvable evolutionary chases or “arms races” (Parker 1979, Gavrilets et al. 2001). Interlocus conflict can occur at any phase of reproduction: mate acquisition, mating rates, re-mating, fertilization and parental investment (Chapman et al. 2003, Arnqvist and Rowe 2005), and can lead to the evolution a variety of adaptations in either sex that bias the outcomes of interactions of the bearer in their favour (Arnqvist and Rowe 2005). In this thesis I have concentrated on only one major influence on sexual conflict, the conflict over mating and re-mating, however, sexual conflict also occurs over parental investment (Trivers 1972, Clutton-Brock 1991), genomic imprinting (see Wedell et al. 2006 and references therein), limited resources and mate cannibalism (Parker 1979, 2006).

1.1.2.1 Conflict over mating and re-mating

When and with whom to mate are important decisions for an individual. Mating incurs a variety of costs: increased energy expenditure (Rowe et al. 1994); decreased foraging time (Schlupp et al. 2001); increased risk of injuries (Parker 1979, Crudgington and Siva-Jothy 2000, Blanckenhorn et al. 2002, Stockley 2002, Eady et al. 2007); increased stress levels and increased predation risk (Rowe et al. 1994, Shine et al. 2000) and increased risk of disease transmission and parasites (Sheldon 1993, Abbot and Dill 2001). An individual’s decisions on whether to mate and with whom therefore reflect a balance between the costs and benefits of copulation. Differences in reproductive optima between the sexes often result in conflict over mating. Although females typically have a lower optimal mating rate than males (Arnqvist and Rowe 2005), females from a wide variety of animal taxa are promiscuous. Female promiscuity creates conflict over re-mating, as even if a male copulates with a female he may not necessarily sire her offspring. Hence, mating conflict may be pre-copulatory regarding mating decisions, or post-copulatory regarding sperm use.
Sperm competition, where ejaculates from different males compete against one another within the female tract for fertilization of the ova (Parker 1970), is central to conflict between males and females over re-mating.

Females can gain a variety of benefits from mating with multiple males: genetic inbreeding avoidance (Stockley et al. 1993, Tregenza and Wedell 2002); reduced genetic incompatibility (Zeh and Zeh 1997); compensation for low-quality partners (Kempenaers et al. 1992); promotion of sperm competition which may enhance the viability or competitiveness of offspring (good genes hypothesis) and/or increase survivorship amongst offspring from increased genetic diversity (genetic diversity hypothesis); fertility insurance (Krokene et al. 1998); reduced harassment (Rowe et al. 1994, Knott et al. 2010); increased conception rates (Beatty 1960, Hoogland 1998); increased litter/clutch size (Hoogland 1998); nuptial gifts which can be prey, nutritional benefits associated with receiving ejaculates, somatic gifts where the female eats part of the male’s body or body products, or suicide gifts where the female consumes the male (reviewed in Simmons and Parker 1989, Vahed 1998, 2007); territories and nest sites (Møller and Thornbill 1998); increased parental care if males help rear offspring with all females which whom they copulate (Hartley et al. 1995); and reduced risk of infanticide (Hrdy 1979, Agrell et al. 1998, Knott et al. 2010, Stumpf et al. 2011). For females the primary benefit from multiple mating is the ability to influence paternity of young. This is particularly important as a female may be coerced to mate with a non-preferred male as rejecting mates can result in attacks and assaults, abortion, loss of reproductive performance (Clutton-Brock and Parker 1995 and references therein) and even death (Enders 1952, Le Bouef and Mesnick 1990, Olsson 1995). Females have evolved a number of counter-adaptive post-copulatory choice mechanisms to manipulate paternity. These include a variety of cryptic
female choice mechanisms such as selective sperm use by differential storage of sperm from different males (Hellriegel and Bernasconi 2000, Snow and Andrade 2005) as well as physically manipulating ejaculates by ejecting sperm in a process known as sperm dumping (Pizzari and Birkhead 2000, Snook and Hosken 2004, Burger 2007).

In species that exhibit sperm competition males have evolved a variety of anatomical, physiological and behavioural adaptations to increase their paternity success. These include the evolution of large testes which increase the rate of sperm production (Harvey and Harcourt 1984, Møller 1989, Hosken et al. 2001) and have been described as infallible predictors of mating systems (Short 1997), releasing large ejaculates which contain large numbers of sperm to displace rival sperm and increase the chance of fertilization (Parker 1998) and a variety of sperm characteristics which increase sperm velocity, viability and motility (Gage and Freckleton 2003, Snook 2005, Gomendio et al. 2006). Males may also attempt to postpone the female’s next mating opportunity to reduce the possibility of sperm competition by deliberately injuring the female so that continual sexual activity is painful (Parker 1979, Crudgington and Siva-Jothy 2000, Blanckenhorn et al. 2002, Stockley 2002, Eady et al. 2007), reducing female sexual receptivity through substances transferred within ejaculates (Johnstone and Keller 2000 and references therein, Wedell et al. 2006) and the formation of copulation plugs which prevent the female from re-mating (Parker 1970). Behavioural responses by males to reduce sperm competition include reducing parental care if their mate is promiscuous (Hoogland 1998, Møller 2000) as well as physical interventions by males by either guarding mates (Parker 1970, Allen et al. 1994) or punishing unfaithful females (Clutton-Brock and Parker 1995, Valera et al. 2003). It is also important to recognise that females may also want to prevent males re-mating in species where males provide parental care (Wedell et al. 2006). Hence
conflict over mating and re-mating occurs in both sexes: the resulting mating system dynamics and
behaviours we see are the result of a balance between costs and benefits of mating in both sexes.

2. The short – beaked echidna as a study species for studying sexual conflict
The short-beaked echidna *Tachyglossus aculeatus*, is one of five extant species of monotreme (egg-
laying mammals). The echidna is an ideal species for studying sexual conflict over mate choice and
mating decisions because there is no conflict over which sex invests more in the raising of
offspring as males provide no form of parental care, there is no genomic imprinting (John and
Surani 2000, Killian *et al.* 2001) and no evidence of mate cannibalism or limited resources. Sexual
conflict in the echidna is therefore isolated to conflict over mating.

The echidna is also a suitable species in which to study how species optimize their reproduction in
response to their environment and within the constraints of sexual conflict. The echidna has a near
ubiquitous distribution throughout Australia and occurs in coastal regions of New Guinea (Augee *et
al.* 2006). Echidnas are highly seasonal throughout their range with distinct changes in activity and
body mass throughout the year (Nicol and Andersen 2007a). The echidna’s primarily
myrmecophagous diet governs much of its life history and ecology. Echidnas eat both ants and
termites but diet varies, with more termites consumed in hot arid regions and a greater proportion
of ants consumed in wetter regions (Griffiths 1978), although echidnas also eat scarab beetles
(Smith *et al.* 1989, Harrison 1997, Sprent 2012) as well as moth larvae (Sprent 2012). Despite this
variable diet, the density and energy content of prey items is low, and this contributes to echidnas
having the metabolic attributes of a protoendotherm (Grigg *et al.* 2004): low basal (Nicol and
Andersen 2007a) and field metabolic rates (Schmid *et al.* 2003). Echidnas also share many
characteristics with other myrmecophagous species including low intraspecific aggression, a
solitary lifestyle, low reproductive rate and an extended period of maternal care (see Laurie and
Seidensticker 1977). These qualities along with female echidnas on average breeding only every
second year while males breed in three out of four years (Nicol and Morrow 2012), result in a
skewed OSR, a characteristic of species with intense sexual conflict (Stumpf et al. 2011) which in
the echidna is manifested in high levels of intra-male competition for mates.

Echidnas are seasonal breeders throughout Australia. They breed in winter after undergoing a
period of inactivity which ranges from shallow bouts of torpor (Rismiller and McKelvey 1996) to
prolonged deep hibernation (Beard et al. 1992, Beard and Grigg 2000, Nicol and Andersen 2002).
In many populations mating behaviour is characterised by intense intra-male competition for access
to receptive females, resulting in the formation of mating groups in which females are accompanied
by one or more males. There is, however, a large amount of variation in courtship patterns between
geographical subspecies: Kangaroo Island echidnas (T. a. multiaculeatus) form large mating
groups termed ‘mating trains’ where a single female is pursued by up to 11 males (Rismiller 1992,
Rismiller and McKelvey 2000) with males pursuing the female for up to 5 weeks (Rismiller and
McKelvey 1996); mating groups involving 3-6 animals have also been documented in the Northern
Territory, Victoria (Griffiths 1978) and Canberra (Griffiths et al. 1969). In contrast echidnas from
south-east Queensland (T.a aculeatus), Mt Kosciusko (T. a. aculeatus) and Tasmania (T.a. setosus)
are rarely seen in train formation and their breeding seasons follow a period of deep prolonged
hibernation. Mating occurs immediately (Mt Kosiusko) (Beard et al. 1992) or one to three weeks
(south-east Queensland) (Beard and Grigg 2000) after females emerge from hibernation, while in
Tasmania females may mate before hibernation is terminated (Morrow 2007, discussed below).
Mating group size also differs between geographical regions. In south-east Queensland and Mt Kosciuszko only single pairs have been documented (Beard et al. 1992, Beard and Grigg 2000), although this may be due to small sample sizes (Morrow et al. 2009), while in Tasmania mating groups involving five animals have been observed (Morrow et al. 2009).

Echidnas typically only lay a single egg, although twins have been reported (Griffiths 1978, Pierce et al. 2006). Males provide no form of parental care and thus the male echidna’s reproductive strategy is not constrained by having to provide for offspring. In contrast female echidnas provide prolonged parental care including a long lactation period which is a significant energetic cost that is under substantial selective pressure (Stumpf et al. 2011). As males provide no parental care, no pair-bonding post-mating and no nuptial gifts or territories, it is likely that females base mate choice on genetic benefits to offspring. The lack of male parental care, skewed OSR and lower female reproductive rate suggest that intense pre-copulatory sexual conflict characterises echidna mating systems throughout the species’ range.

2.1 Reproductive anatomy of the echidna: evidence for post-copulatory conflict

The anatomy of the echidna suggests that sexual conflict may not be restricted to pre-copulatory mechanisms: various features of males and females indicate that post-copulatory conflict (conflict over re-mating) is also present in echidna mating systems. One of the primary driving forces of sexual conflict is the competition between the sexes to control paternity (Stumpf et al. 2011). In males this is manifested in physiological adaptations which enhance fertilization success in sperm competition (discussed above). Many features of echidna morphology indicate that there is a high
likelihood of sperm competition: lack of sexual size dimorphism; large testes; large initial segment of the epididymis; long and elaborate penis; sperm bundles and the long oviducts.

2.1.1 Lack of sexual size dimorphism, large testes and a large initial segment of the epididymis

In species in which males cannot monopolize females and prevent them from breeding with additional males, males have evolved features to enhance their reproductive success. These features include large testes (Harvey and Harcourt 1984, Kappeler 1997, Short 1997, Zenuto et al. 1999, Schwab 2000) and large epididymides. Large testes produce greater volumes of ejaculate (Hosken and Ward 2001), produce more sperm per ejaculate (Møller 1989) and have a higher sperm production capacity per unit of testes tissue (Harvey and Harcourt 1984). The epididymis is specialized for sperm maturation and storage, and a large epididymis increases the quantity and quality of sperm in ejaculates as well as allowing males to have enhanced control on the volume and number of sperm released per ejaculate (Jones et al. 2007). The echidna shows only slight sexual size dimorphism (Nicol 2013) which means that males cannot monopolize females. Male echidnas possess large testes, larger than predicted for their body size (Kenagy and Trombulak 1986, Jones et al. 1992, Rose et al. 1997) with testes reported to peak between 1 – 1.3% of a reproductively active male’s body mass during the breeding season (Taggart et al. 1998, Jones et al. 2007). The echidna also has a large initial segment of the epididymis (location of spermatozoa maturation) that contains a greater proportion of spermatozoa than that of the epididymis of scrotal mammals (Djakiew and Jones 1981, Jones 1999, Jones et al. 2007). The relative size of testes and length of epididymis reflect the intensity of sperm competition (Birkhead 2000) and hence both
features in the echidna, along with the lack of sexual size dimorphism, support the presence of post-copulatory conflict in the form of sperm competition.

2.1.2 Penis length

Long penises have been suggested to be indicators of sperm competition as long penises could optimize the location of ejaculates within the female reproductive tract (Parker 1984, Ramm 2007) although the correlation between penis length and the presence of sperm competition is not supported across all taxa (Gomendio et al. 1998). The echidna has as elaborate penis, the glans is grooved and gives the impression of being bifid and each portion exhibits a pair of bulbous rosettes (Griffiths 1968, 1978). When erect, the penis is approximately one quarter of a sexually mature male’s body length (Johnston et al. 2007). The echidna penis is only used for mating; urine passes through the urogenital sinus (Home 1802, Griffiths 1968). Therefore the structure has not been restricted by the requirement to excrete urine, and likely represents the optimal structure for fertilization success. Prior to ejaculation in the latter stages of erections, two of the four terminal rosettes of the penis retract so that sperm is deposited directly adjacent to female oviductal ostia (Johnston et al. 2007). This precise positioning of the sperm high in the female reproductive tract may enhance male fertilization success. Penile anatomy in the echidna is therefore further circumstantial evidence of sperm competition.

2.1.3 Sperm bundles

The competitiveness of ejaculates and hence fertilization success in mating systems with female multiple-mating is dependent on sperm velocity, viability and motility (Gage and Freckleton 2003, Snook 2005, Gomendio et al. 2006). Echidnas have a form of sperm co-operation where 100 or
more spermatozoa bind together at the acrosome to form a bundle prior to leaving the epididymis (Jones et al. 2007). Sperm bundles increase sperm velocity to three times the velocity of individual sperm and increase persistence in vitro (Jones et al. 1992) and it has even been suggested that proteins used to form sperm bundles are likely to be sperm competition proteins (Jones et al. 2007). Sperm conjugation in diving beetles provides positional advantages for fertilization within the female reproductive tract (Higginson et al. 2012) and hence echidna sperm bundles have also been suggested to be a male strategy to store sperm in the female reproductive tract (Johnston et al. 2007). The proposal of sperm bundles as a form of storage of sperm in the female echidna tract in plausible, particularly as sperm bundles increase persistence in vitro, as females lack sperm storage structures (Griffiths 1978) which would enhance sperm survival (Gomendio and Roldan 1993).

2.1.4 Oviduct length

Female reproductive tract structure can influence the outcome of sperm competition (Higginson et al. 2012). Long convoluted oviducts are thought to provide an additional challenge to sperm in reaching and fertilizing the ovum, and hence assist sperm selection by females (Anderson et al. 2006). Oviduct length is positively correlated with testes size, ejaculate volume and the volume of the sperm midpiece in mammals (Anderson et al. 2006) suggesting that females have evolved in response to sperm competition (Stumpf et al. 2011) and providing further evidence that the female reproductive tract is not a passive arena for sperm competition (Anderson et al. 2006). Echidna oviducts are long (Griffiths 1968, 1978) and may assist females with sperm selection. Additionally there is also a positive correlation between sperm conjugates and female reproductive tracts, with conjugation declining by elongation of the female reproductive tract (Higginson et al. 2012),
suggesting that the long oviducts of female echidnas may be a counter-adaptation to sperm bundles, allowing females to have greater control of paternity of offspring.

2.2. Reproductive behaviour of the echidna: evidence of post-copulatory conflict

Male reproductive behaviours can indicate the presence of sperm competition. Mate guarding is a common behaviour utilized by males in mating systems with sperm competition as it delays or prevents females re-mating and hence offers individual males a greater chance of achieving paternity of offspring. Mate guarding behaviour has been documented in Tasmanian echidnas (Morrow et al. 2009) and individual female echidnas have been observed in multiple mating groups in a single breeding season (Morrow 2007). Using a method to sample the female reproductive tract (described in chapter 2) I have demonstrated that female echidnas will mate with multiple males (Morrow 2007 discussed below) in a single breeding season, indicating a large potential for sperm competition.

The reproductive behaviour and morphology of echidnas therefore suggests a mating system with high levels of pre- and post-copulatory sexual conflict. In this study I examined reproductive biology in a wild population of Tasmanian echidnas in the context of sexual conflict. Tasmania is the southern-most extent of echidna’s range and therefore the area where seasonal effects are likely to be the greatest. Hence, this study offers the opportunity to explore how the environment influences reproduction and how Tasmanian echidnas optimize their reproduction around an obligatory hibernation period and in the presence of sexual conflict.
2.3 The Tasmanian subspecies *T. a. setosus*

Reproduction in the Tasmanian echidna (*T.a. setosus*) follows a period of deep prolonged hibernation where body temperatures (*T<sub>b</sub>*) drop to within 1°C of substrate temperature (Nicol and Andersen 2007b), when substrate temperatures are below 16 and above 4°C (unpublished obs.). Males enter hibernation in late January to early February and terminate hibernation in late May in a breeding year. Females enter hibernation approximately one month after males and in reproductive years emerge from June onwards. As in all hibernators, the period of reduced *T<sub>b</sub>* is broken by periods of spontaneous arousals in which body temperatures return to normothermic levels (Nicol and Andersen 2002). My study population has been the focus of physiological and behavioural observations since 1996 and while reproduction has been found to closely follow female’s emergence from hibernation (Nicol *et al.* 2005), in 2007 I observed two females in mating groups at sub-euthermic *T<sub>b</sub>* (3cm cloacal temperatures of 14.1 and 20.6°C). In both cases the female was found with a single male (*T<sub>b</sub>* of males was between 31-32°C) in close proximity to her previous hibernaculum. While the males appeared to be attempting to mate with the torpid females, both females were sluggish (Morrow 2007). One week after being observed in these mating groups both females re-entered hibernation and I was able to recover sperm from their reproductive tracts (using methods described in chapter 2), demonstrating that females in this population may re-enter hibernation after mating (Morrow 2007). These females were then found in further mating groups with different males and I was able to recover fresh sperm from the reproductive tracts of both females demonstrating that females in this population may be promiscuous. These observations provide evidence for sperm competition and hence post-copulatory conflict as well as conflict over the timing of reproductive events in this study population.
3. Thesis objectives

In this thesis I investigate how male and female wild Tasmanian echidnas optimize their reproduction around an obligatory hibernation period and in the presence of sexual conflict. Tasmania is the southern-most extent of the echidna’s range and my study population is the only population in which males have been found in mating groups with torpid females and females have been demonstrated to be promiscuous. Studying this population of Tasmanian echidnas therefore offers a unique opportunity to examine sexual conflict over mating and re-mating as well as over the timing of reproduction. The aim of this thesis is to contribute to our understanding on the interactions between hibernation and reproduction and the trade-off decisions utilized by each sex to optimize their reproduction.

3.1. Thesis structure

This thesis is structured as four inter-related scientific papers. Two chapters (chapter 2 and chapter 5) have been published while the remaining chapters have been written in a format to facilitate publication in the near future. As each chapter represents a series of stand-alone manuscripts some repetition is unavoidable and there is stylistic variation throughout this thesis.

Chapter 2: In this chapter I investigate how male echidnas locate torpid females, how common mating groups involving torpid females are in my study population and whether pregnant females re-enter hibernation.

Chapter 3: In this chapter I examine the strategies utilized by males and females to optimize reproduction and build on observations made in chapter 2. A particular focus of this chapter is how
females respond to the early mating strategy utilized by males. Hormonal changes that occur throughout pregnancies, when females re-enter hibernation and at the timing of egg-laying are also investigated. This chapter also discusses whether females mate at torpid Ts, why females may re-enter hibernation after mating and why females continue to mate while pregnant.

Chapter 4: In this chapter I examine how males time their reproduction around an obligatory hibernation period. This chapter focuses on the timing of testes recrudescence relative to the hibernation period, however, this chapter also examines seasonal changes in crural glands, plasma testosterone concentrations and body mass to give an overall picture on how male echidnas optimize their reproduction in response to environmental variables as well as intra-male competition for mates.

Chapter 5. In this chapter I examine the extent of post-gestation maternal care in the echidna and relate my findings to maternal energy expenditure and female reproductive strategies for timing reproduction to the optimal time of year.

Chapter 6. This thesis concludes with a general discussion which synthesises how Tasmanian echidnas optimize their reproduction around an obligatory hibernation period as well as in the presence of sexual conflict, and discusses possibilities for future research.
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Chapter 2: Cool Sex? Hibernation and reproduction overlap in the Tasmanian echidna *Tachyglossus aculeatus setosus*
Chapter 2. Cool Sex? Hibernation and reproduction overlap in the Tasmanian echidna *Tachyglossus aculeatus setosus*


Abstract

During hibernation there is a slowing of all metabolic processes, and thus it is normally considered to be incompatible with reproduction. In Tasmania the egg-laying mammal, the echidna (*Tachyglossus aculeatus*) hibernates for several months before mating in mid-winter, and in previous studies we observed males with females that were still hibernating. We monitored the reproductive activity of radio-tracked echidnas by swabbing the reproductive tract for sperm while external temperature loggers provided information on the timing of hibernation. Additional information was provided by camera traps and ultrasound imaging. More than a third of the females found in mating groups were torpid, and the majority of these had mated. Some females re-entered deep torpor for extended periods after mating. Ultrasound examination showed a developing egg in the uterus of a female that had repeatedly re-entered torpor. The presence of fresh sperm in cloacal swabs taken from this female on three occasions after her presumed date of fertilization indicated she mated several times after being fertilized. The mating of males with torpid females is the result of extreme competition between promiscuous males, while re-entry into hibernation by pregnant females could improve the possibility of mating with a better quality male.
Chapter 2: Cool Sex? Hibernation and reproduction overlap

Introduction

Hibernation has been documented in species from a wide range of mammalian orders [1], and although originally thought to be an adaptation to the cold, hibernation is now considered to be an energy conserving strategy which different species employ in a range of ecological circumstances [2]. Hibernation is characterized by a reduction in body temperature (T\textsubscript{b}) which typically falls to within 1˚C of ambient, and a very substantial, but size dependent, reduction in metabolic rate [1]. Because metabolic processes are slowed during hibernation it is generally considered to be incompatible with reproduction: hibernation prevents spermatogenesis in males [3], slows fetal development, delays parturition [4] and inhibits lactation [5].

Among Australian mammals many dasyurid marsupials enter daily torpor during pregnancy [6], but bats are the only mammalian group in which reproduction and deep, extended torpor (i.e. hibernation) are known to overlap. In temperate zone bats, which show an extensive period of winter torpor, the reproductive cycle is interrupted by hibernation [7]. A number of strategies, including sperm storage and delayed ovulation, allow gestation to be initiated on arousal from hibernation in spring although gametogenesis occurs in summer [7,8]. However, the only species known to enter deep, prolonged torpor while pregnant is the North American hoary bat (Lasiurus cinereus) - in extreme spring weather conditions pregnant females showed bouts of deep torpor lasting up to 5.6 days [9].

The short-beaked echidna Tachyglossus aculeatus is distributed throughout southern and eastern New Guinea, mainland Australia, Tasmania, Kangaroo Island, and smaller offshore islands. It is the most common of the egg-lying mammals and is in fact the most widespread native Australian
mammal [10]. Throughout their range echidnas show some degree of seasonal inactivity. In Tasmania (subspecies *T. a. setosus*) reproductively active males hibernate from mid February to mid June, while reproductively active females hibernate from early March until mid July [11,12]. By contrast reproductively active adults of the Kangaroo Island subspecies (*T. a. multiaculeatus*) show reduced activity and only intermittent bouts of torpor between April and June [13]. Courtship behaviour also appears to differ between the two areas [14]. Kangaroo Island echidnas have been described as forming mating “trains” of up to 11 individuals with a period of competition between males and courtship lasting between 14-44 days [15]. After mating there is a gestation period of 22-24 days, after which the female normally lays a single egg [12,15].

Since 1996 we have been studying a population of echidnas in the Tasmanian southern midlands, and on several occasions during the course of this study we found males with females which were torpid, or which subsequently re-entered hibernation. In order to examine more closely the relationship between hibernation and reproduction in Tasmanian echidnas we conducted a detailed investigation of radio-tracked echidnas during the 2007 and 2008 mating seasons.

**Material and methods**

*Ethics Statement*

This work was carried out under permit from the Tasmanian Department of Primary Industries, Water & Environment, and the University of Tasmania Animal Ethics Committee, and complies with the Tasmanian and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).
**Chapter 2: Cool Sex? Hibernation and reproduction overlap**

*Study site and animals*

Fieldwork was conducted on a 12km² site on a grazing property in the southern midlands 50km north of Hobart, Tasmania [12]. Between 1996 and 2006 we had tagged 180 echidnas in this area with passive implantable transponder (PIT) tags (LifeChip, Destron-Fearing, St. Paul, MN, USA). At the start of 2007 six echidnas (3M, 3F) already had tracking transmitters (Bio Telemetry Tracking, South Australia) glued to the spines. Over the two years of this study we found 70 animals (26M, 31F, 13 juveniles), 37 of which had been tagged previously. Forty-eight were found while slowly driving around the property in a 4WD vehicle, 22 (5F, 17 M) were found in mating groups when we were tracking other animals. All new animals were tagged, and tracking transmitters attached to 14 females and 9 males. A small temperature logger (iButton, DS1922L, Maxim Integrated Products, Inc. Sunnyvale, California, resolution 0.5°C), was glued to the tracking transmitter. The camera trap was an Olympus digital camera (model C-120) with a passive infrared motion detector (Archipelago Consulting Ltd, Westerway, Tasmania, Australia), which we were able to position above a female echidna while she was hibernating in a hollow tree, and later in a tree stump.

*Internal temperature logger data*

Details of loggers and surgical procedures are described in Nicol et al. [17], and ten of the data points shown in Figure 2.3 were published in that study while nine data points were from loggers that were part of that study but downloaded subsequently.
Recovery of sperm from the female reproductive tract

Urogenital smears were collected by inserting a soft flexible catheter (6 mm diameter) into the cloaca while the female was anaesthetized under light isoflurane anaesthesia. The lubricated catheter was inserted approximately 5 cm into the urogenital tract, bringing the tip into close proximity to the opening of the paired uteri. A nylon bristled DNA buccal cell collection brush (MasterAmp™ Buccal Swab Brush, Epicentre Technologies, Madison WI, USA) was then advanced through the catheter until the bristles were just beyond the end of the catheter. The brush was then withdrawn from the catheter and wiped across a microscope slide. Slides were then air-dried and stained (Rapid Diff, Australian Biostain Pty. Ltd., Victoria, Australia). The appearance of spermatozoa recovered from the female reproductive tract changed quite significantly over several days of repeated sampling. Sperm sampled immediately after mating had at least 4 curves along their length. With increasing time in the female tract the sperm had fewer and fewer curves, and after 4 to 5 days began to break into fragments.

Ultrasonography was carried out in the field, using a MyLab30CV portable ultrasound with linear probe (Esaote, Genova, Italy). For ultrasound examination echidnas were lightly anaesthetized with isoflurane-oxygen, and placed on their back.

Results/Discussion

Over the two mating seasons we found 26 mating groups. The most common number of males in a group was one (15 mating groups), but on three occasions in 2008 females were found with four males. In ten of the mating groups the female was torpid and reacted slowly to stimuli; body temperatures of these torpid females ranged from 10 to 29°C. All males in mating groups were
active and had normal (euthermic) body temperatures (ca 32˚C [16]). Of five torpid females checked, four had sperm in their reproductive tract. Four of the females found torpid in mating groups were radio tracked and were observed to re-enter hibernation; three of these were later found in mating groups while euthermic and had fresh sperm recovered from their tracts.

One female (echidna 5D5E) was studied intensively in 2008, using a combination of field observations (including cloacal swabbing), camera traps, an external temperature logger and ultrasound. This showed that while hibernating she was visited by a male at least twice without mating occurring. In one of these events (July 6) a camera trap showed that a male was with her in her hibernaculum for 13 hours, while the temperature logger showed that she did not rewarm significantly. She subsequently mated five times between July 11 and 28 before entering a nursery burrow on August 7. During the period of mating she repeatedly re-entered torpor with a minimum \( T_b \) of about 10˚C. Maximum length of these torpor bouts was only about 12 hours, but she was being frequently disturbed by us. On July 15 female 5D5E was with male 5036, had fresh sperm in her tract, and had a temperature of less than 20˚C. (A fault in the temperature probe prevented us from measuring her \( T_b \) more accurately). An ultrasound scan showed an egg in her uterus (Figure 2.1). A second scan 5 days later confirmed the presence of the egg, and showed fresh sperm again. Nine days before entering the nursery burrow she was with the same male, had fresh sperm in her tract, but was torpid with a temperature of 26.6˚C.

We have shown previously that internal body temperature loggers allow accurate timing of reproductive events [17], and as seen in Figure 2.2, this information can also be obtained from external temperature loggers. Figure 2.3 shows the time between final arousal from hibernation and
egg-laying, as determined from these internal and external logger records for 21 reproductive events from 13 females, during this and previous studies. (Data from female 5D5E for 2008 have not been included as she was so frequently disturbed). The majority of eggs are laid between 20 and 24 days after the final arousal from hibernation. As the gestation period reported for echidnas is also 20-24 days [12,15], this would indicate that most females become pregnant immediately after arousal from hibernation, or are already pregnant at their final arousal, as was the case for female 5D5E in 2008. Figure 2.3 shows that in some cases the time from final arousal to egg-laying is so short (e.g. female 0118) that there must have been considerable development of the egg before the final arousal. As seen in Figure 2.2, this female appears to have become pregnant during an extended euthermic period, and was probably euthermic for 8-9 days before re-entering hibernation for eight days, with T_b falling to about 7˚C.

This is only the second account of a mammal entering deep torpor, or hibernation, when pregnant. In hoary bats it has been suggested that hibernation is used during harsh weather to delay parturition and thus lactation, which is more energetically expensive than pregnancy [9]. While this will be a significant consideration in hoary bats where the total litter mass is about 30% of maternal mass [18], the single newly hatched echidna young at about 0.5 g [19] will be less than 0.02% of maternal mass. In a previous study we found no measureable increase in field metabolic rates of lactating females with young aged 45-65 days [20], and thus the newly hatched baby will be an insignificant energy drain, at least initially. Furthermore, during the 10-11 days of egg incubation and first 30 days of lactation, Tasmanian echidnas are in a closed nursery burrow [12], and protected from the weather. Thus it seems unlikely that pregnant female echidnas enter deep torpor to postpone the energetic costs of lactation.
Chapter 2: Cool Sex? Hibernation and reproduction overlap

In our study area reproductively active males finished hibernation between May 10 and August 5 ($n = 7$), while the final arousal from hibernation of reproductively active females was between June 7 and September 3 ($n = 23$) [12]. As males are ready to mate about 30 days after the end of hibernation [12], many will be seeking matings while some females are still hibernating. Male mating activity lasts for about 60 days, and although we could not continually observe the animals, males seemed typically to stay with a female for up to seven days, with some males leaving and joining new groups, while others stayed in close proximity to the female for longer periods. Males were observed with up to four females during a breeding season, while females mated more than once, often with more than one male. Outside the mating season, echidnas are solitary and home ranges of males are typically twice that of females (Nicol SC, Vanpé C, Sprent JA, Morrow G, Andersen NA, unpublished observations). The mating trains noted on Kangaroo Island are also clearly a manifestation of intense competition between males, and, as the Tasmanian females are clearly promiscuous, the observation that female Kangaroo Island echidnas only mate once [15] is likely to be incorrect. Thus the echidna mating system appears to be characterised by roving promiscuous males [21] which guard promiscuous females before and after mating.

When females are polyandrous or promiscuous there is selection for male traits favoured by cryptic male choice, or traits that increase competitiveness during sperm competition [22]. Male traits that potentially increase fertilization success include genital morphology, sperm size and morphology, and copulatory and post-copulatory behaviour [22]. The male echidna has an elaborate penis which has a quadripartite anemone-like appearance [23], and ejaculates its sperm in bundles [24]. Sperm bundles are very likely to be an adaptation for sperm competition as spermatozoa in larger bundles
show greater progressive motility than single spermatzoa or smaller sperm bundles [25]. Another trait that should increase competitiveness during sperm competition is large testes, and monotremes have larger testes relative to body size than marsupials, primates or avian species [26].

We suggest that there is extreme competition between echidna males, which in the Tasmanian subspecies leads to males mating with torpid females. A male finding a hibernating female, and repeatedly mating with her, and then guarding her, would have a high probability of successful paternity. Our observations raise the possibility that the echidna is an induced ovulator - in induced ovulators copulation initiates ovulation, and in some species multiple matings are required to initiate ovulation [27]. It is not clear whether females must rewarm before mating can occur, but even if they do it would seem unlikely that they could exert a pre-copulatory choice. If torpid females do not have any pre-copulatory choice this would provide strong selection for the female to mate again - to ‘trade up’ - if she subsequently encounters a better quality male [22], which in turn would select for mate guarding by males. It is also not clear why some females should re-enter torpor after mating. For female 0118 (Figure 2.2) the successful mating was not particularly early – it would have been in the middle of the normal mating season [12]. Re-entering torpor would be expected to prolong the gestation period, and for those egg-laying events which occurred less than 20 days after the final arousal from hibernation (Figure 2.2) the time from mating, as estimated from temperature records was 26–30 days (average 28.1, n = 4). As females will mate when pregnant it is possible that a female which has been mated while torpid, and thus had no pre-copulatory choice, may extend her gestation period by re-entering hibernation to increase the possibility of being found by a more desirable male, allowing her the option of abandoning the first pregnancy.
This study raises a number of questions that should be testable when we have sufficient microsatellites to establish paternity [28]. What determines successful male parentage? Do males which mate with the largest number of females dominate paternity, or do females, despite mating with several males, have preferences? It is possible that successful male parentage is related to major histocompatibility complex (MHC) compatibility. Investigation of this aspect of mate choice is dependent on the development of MHC typing for the species.

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Figure 2.1. Ultrasound image showing an egg in the uterus of echidna 5D5E on July 23 2008.

Fertilization probably occurred on July 9, but she had fresh sperm in her reproductive tract and was also torpid. Distance between the two markers showing the structure within the egg is 0.35 cm.
Figure 2.2. External temperature logger record from female echidna 0118. She entered hibernation in March, and her final arousal was on July 27 (arrow 2), when temperature variability increased. The subsequent reduction in variability (arrow 3) is associated with entry into the nursery burrow and egg-laying. Periodic arousals can be seen between April and July. In mid-July she shows an extended arousal (July 10 – 21), and we presume fertilization occurred at the time indicated by arrow 1.
Figure 2.3. Time between final arousal from hibernation and egg-laying for 23 reproductive events from 13 echidnas, as estimated from internal (red circles), and external (blue circles) temperature loggers. The reported gestation period for echidnas is 20 - 24 days [15]. Although three animals (3A61, 4057, 2753) were active for periods of up to 3 weeks before becoming pregnant, the majority of points lie between 20 and 24 days after the end of hibernation. In these cases the females must have become pregnant nearly immediately after the final arousal from hibernation, or were already pregnant. Echidna 0118 must have become pregnant during the previous euthermic period (see Figure 2), as was probably also the case for three of 5D5E’s pregnancies.
References


Chapter 3: Reproductive tactics in the Tasmanian echidna: balancing the competing demands of hibernation and successful reproduction
Chapter 3. Reproductive tactics in the Tasmanian short-beaked echidna

*Tachyglossus aculeatus setosus*: balancing the competing demands of hibernation and successful reproduction

**Abstract**

Differences in energetic investment in reproduction between the sexes often result in sexual conflict, in which each sex manipulates and exploits their mate(s) to maximise their own fitness. In our population of Tasmanian echidnas conflict is primarily over the control of paternity, and this conflict is exacerbated by high levels of female promiscuity and a skewed operational sex ratio that results in intense intra-male competition for mates. Male echidnas initiated mating activity by locating hibernating females. If a male located a hibernating female and remained in her hibernaculum with her for 13 hours or more he gained a copulation opportunity. All females that mated or were disturbed by males prior to July 27 re-entered hibernation. Many of the females that re-entered hibernation after mating were pregnant. Pregnant females only entered hibernation in early pregnancy, when plasma progesterone concentrations were low. By re-entering hibernation pregnant females extended their gestation period and delayed egg-laying. This allowed females to time their emergence from the period of maternal nursery burrow confinement with increases in ecosystem productivity. Pregnant females continued to attract males and mate even in the late stages of pregnancy. This may have occurred simply because mating was less costly than resisting persistent males but could also be a strategy to create confusion over the paternity of offspring and reduce the risk of infanticide. The ability of pregnant females in this population to re-enter
hibernation without adversely affecting their young allows successful reproduction in a population where there is asynchronous timing of optimal mating between males and females.

Introduction

Hibernation and daily torpor are two common strategies employed by animals to survive periods of low energy availability. Hibernation, a seasonally regulated and controlled reduction of body temperature (\(T_b\)) and metabolic rate (Geiser 2004, 2011), reduces energy expenditure (Heldmaier and Ruf 1992, Geiser 2011) and thus allows animals to tolerate periods of food and/or water shortages and survive adverse environmental conditions. Although popularly considered a response to extreme cold, hibernation and daily torpor may also occur in temperate and tropical mammals in response to reduced food availability and low rainfall (Dausmann et al. 2005, Kobbe and Dausmann 2009, Stawski et al. 2009, Geiser and Brigham 2012). Hibernation and daily torpor therefore offer many benefits for a variety of animal taxa. However, because a significant amount of the year may be spent in the torpid state, hibernators have a shortened period for activities which in non-hibernators are spread over the whole year.

The systemic down-regulation of metabolic processes that occurs during hibernation inhibits the function of the endocrine system and the reproductive organs (Hoffman 1964, Hudson and Wang 1979, Barnes et al. 1986) and hence reproduction and hibernation have traditionally been considered mutually exclusive processes. However, a number of species including birds, reptiles and examples from all three subclasses of mammals enter torpor and hibernation at various stages of reproduction. Broad-tailed and Anna’s hummingbirds (Selasphorus platycercus and Calypte anna) may enter torpor during egg incubation (Calder and Booser 1973, Vleck 1981), while female
vespertilionid and rhinolophilid bats use torpor to extend the viability of stored sperm prior to fertilization (Oxberry 1979). While entering hibernation or torpor while pregnant may seem counter-intuitive due to the potential effects to developing embryos; mulgaras Dasycercus cristicauda (Geiser and Masters 1994, Körtner et al. 2008), a variety of bat species (Geiser et al. 2001, Chruszcz and Barclay 2002, Turbill and Geiser 2006, Willis et al. 2006, Dzal and Brigham 2013), Tasmanian snow skinks Niveoscincus microlepidotus (Olsson and Shine 1998), stripe-faced dunnarts Sminthopsis macroura (Geiser et al. 2005), tenerecs Geogale aurita (Racey and Stephenson 1996), European hedgehogs Erinaceus europaeus (Fowler 1988) and grey mouse lemurs Microcebus murinus (Canale et al. 2012) may enter hibernation or torpor during their gestation period and this phenomenon is also observed in the short-beaked echidna Tachyglossus aculeatus (Geiser and Seymour 1989, Morrow et al. 2009, Morrow and Nicol 2009, see chapter 2).

The short-beaked echidna is the most widespread monotreme (egg-laying mammal), occurring from the tropics of New Guinea to the temperate island of Tasmania (Augee et al. 2006). In Tasmania, breeding follows the annual hibernation period, during which $T_h$ drops to within 1°C of substrate temperature (Nicol and Andersen 2007), when substrate temperatures are below 16 and above 4°C (unpublished obs.). Sexually mature males and females do not breed every year, with females breeding on average only every second year while males breed in three out of four years (Nicol & Morrow 2012). Males enter hibernation in summer during late January to early February and in their breeding years they terminate hibernation in early to late May (autumn). Females enter hibernation approximately one month after the males, and in their reproductive years, they emerge from June onwards (austral winter). In our study population of echidnas in the Tasmanian midlands, male echidnas have been found to enter females’ hibernacula while females are still
hibernating, and sperm has been recovered from the reproductive tracts of torpid females (see chapter 2, Morrow et al. 2009, Morrow and Nicol 2009). Females have been found to re-enter hibernation after mating (see chapter 2, Morrow et al. 2009, Morrow and Nicol 2009). The gestation period of the echidna is reported to be 20 - 24 days (Beard et al. 1992, Beard and Grigg 2000, Rismiller and McKelvey 2000), but in our population, the time between the final arousal from hibernation and egg-laying is often significantly less than this because many females are pregnant before the final termination of hibernation (chapter 2, Morrow and Nicol 2009).

To understand why female echidnas in our study population utilize the strategy of re-entering hibernation while pregnant we need a better overall understanding of their reproductive physiology. We still lack vital information on many aspects of reproduction in female echidnas including the frequency and duration of the oestrous cycle as well as the timing of fertilization (Griffiths 1978, Rismiller and McKelvey 2000), and it is uncertain whether both uteri and ovaries are functional (see Semon 1894, Flynn and Hill 1939). In a number of domestic and wild mammalian species including eutherian mammals and marsupials (D'Souza 1978, Sinha Hikim et al. 1991, Asa et al. 1992, Bekyürek et al. 2002, Durrant et al. 2003, Hesterman et al. 2008), insights into ovarian cycles and pregnancy have been obtained from vaginal cytology used in conjunction with hormone analysis. As the echidna lacks a vagina, we investigated whether comparable information could be obtained from female echidnas by collecting cell smears from the urogenital sinus. We used urogenital smears in conjunction with camera traps, external temperature loggers, hormone analysis and ultrasonography to examine female reproductive physiology and gain insights into reproductive tactics in wild Tasmanian echidnas. By using this combination of techniques we aimed to determine whether both uteri and ovaries are functional, why some pregnant females re-enter
hibernation, what effect hibernation has on plasma progesterone concentrations and how Tasmanian echidnas balance the competing demands of hibernation and successful reproduction.

**Methods**

**Field Site**

This study was conducted on a 12 km$^2$ site (42°28′S, 142°14′E) in the Tasmanian Midlands approximately 50 km north of Hobart, Tasmania. The study site is part of a grazing property and consists of native and improved pasture as well as remnant *Eucalyptus amygdalina* woodland. The site has variable topography with altitudes ranging from 200 to 400 m above sea level, and mean daily minimum and maximum temperatures are -1°C to 10°C in winter and 7°C to 23°C in summer (Australian Bureau of Meteorology).

**Animals and radiotelemetry**

Over the duration of this study (2008 – 2010) 18 reproductively mature females were monitored, 11 of these females for multiple breeding seasons, resulting in the accumulation of 30 individual years of data (reproductive and non-reproductive years). Data collected from individuals during non-reproductive years has been excluded from this study. All echidnas were fitted with passive implantable transponder (PIT) tags (LifeChip, Destron-Fearing, St Paul, MN, USA) on their right ventral side under light isoflurane anaesthesia. Each tag has a unique 10-digit hexadecimal or 14 digit numeric code, but for simplicity individuals are referred to by the last four digits of their tag. Transmitters (Bio Telemetry Tracking, South Australia) were attached between spines on the lower back of animals using two-component epoxy glue. All females also had small external temperature loggers (iButton, DS1922L, Maxim Integrated Products Inc., Sunnyvale, California, resolution
0.5°C) attached to their transmitters. These loggers were programmed to record temperature at one hour intervals and could be downloaded in the field. Reproductive events of echidnas can be identified from changes in body temperature, which can be recorded using internal or external temperature loggers (Nicol and Andersen 2006, Nicol and Andersen 2008, Morrow and Nicol 2009, Morrow and Nicol 2012, see chapters 2 & 5). Body temperatures of females found in mating groups were verified using a thermocouple probe inserted at least 4cm into the cloaca.

**Blood samples**

Blood samples (approximately 1ml) were collected from the rostral sinus while animals were under light isoflurane anaesthesia using 21G needles and plastic syringes. All samples were collected within an hour of animal capture and were stored on ice until centrifuged at 13000 rpm for 10 minutes to separate plasma. Plasma was then stored at -20°C until analysis. Plasma progesterone was measured via radioimmunoassay (RIA) using methods described in Nicol et al. (2005). The sensitivity of the assay was 6pg progesterone (~0.15 ngml⁻¹ plasma). Assay accuracy and precision were monitored by including commercially available human control serum (CON 6, DPC) in each assay. All samples collected from the same individual, even if collected over several years, were analysed in a single assay.

**Camera traps**

Infrared motion-triggered cameras (Scoutguard SG550, HuntingCamOnline, Gadsden, SC, USA) were placed over females’ hibernacula. Time stamps on captured images and data from the females’ external temperature loggers were used to determine when males entered females’
hibernacula and when females entered and emerged from hibernation (Morrow and Nicol 2009, chapter 2).

**Ultrasonography**

We used ultrasonography to confirm the presence of uterine eggs. This allowed us to identify a distinct physiological state (see below) for comparison of urogenital cell compositions between early and late pregnancy. Examining eggs *in utero* also allowed us to determine whether both uteri and ovaries in the echidna are functional. Ultrasonography (ESAOTE Caris Plus, Germany) was performed in the field using a LA523 linear probe (13-4 MHz) while echidnas were under light isoflurane anaesthesia. Females were examined by ultrasonography after sperm were recovered from their reproductive tracts and they had started to develop a rudimentary pouch. The animal was placed in dorsal recumbency for the procedure and the hair on either side of the pouch was removed with electric clippers. As young cling to their mother’s hair within the pouch while feeding, hair on the swollen edges of the pouch and within the pouch was not removed.

**Urogenital smears**

Urogenital epithelial cells and spermatozoa were recovered from the female reproductive tract by taking a urogenital smear. A 6 mm diameter soft flexible catheter was inserted into the cloaca while females were under light isoflurane anaesthesia and advanced 5cm up the reproductive tract. This brought the end of the catheter close to the opening of the two uteri. A nylon-bristled DNA buccal cell collection brush (MasterAmp™ Buccal Swab Brush, Epicentre Technologies, Madison, WI, USA) was then gently advanced up the catheter until some resistance was felt when the brush made contact with the wall of the uteri opening. This was verified by ultrasonography during one
procedure. The brush was then gently rotated 90° and back before being retracted into the catheter, and the catheter removed from the tract with the brush still inside. By leaving the brush inside the catheter, material collected from the opening of the two uteri was not contaminated with faecal material. The brush was then withdrawn from the catheter and wiped across a microscope slide. Slides were air-dried and stained with Rapid Diff (Australian Biostain Pty Ltd, Australia).

Urogenital epithelial cells were classified as partly cornified superficial cells (PCS), anucleated superficial cells (ANS), intermediate cells (I), parabasal cells (P) or neutrophils (N). The presence of spermatozoa, mucus or bacteria was also recorded. To examine the proportion of different cells present in each urogenital smear, a field of view was chosen at random and the first 100 cells were counted. If there were fewer than 100 cells present in the field of view, a new field of view was randomly selected and sampling continued until 100 cells were counted. Smears were scored at 100X magnification.

Blood samples and urogenital smears were collected throughout the year from females being radio-tracked. We aimed to collect one set of samples (blood sample and urogenital smear) per month from females fitted with radio-transmitters, but it was not possible to collect and sample all individuals in each month. The frequency of sample collection increased during the breeding season: we aimed to collect blood samples and urogenital smears every two days during the breeding season to determine the estimated day of fertilization, examine changes in urogenital cell compositions throughout pregnancies, pinpoint changes in plasma progesterone throughout pregnancies and determine when peak plasma progesterone concentrations occurred. Only females with two or more samples collected within a year were included in analyses. We did not disturb
females in June until a male entered or investigated their hibernacula as we did not want to affect females’ behaviour or $T_b$ during the time in which males begin to locate hibernating females. Individual females varied in the timing of hibernation as well as in reproductive tactics utilized during the breeding season: some individuals did not enter re-enter hibernation post-mating, and those that did re-enter hibernation post-mating entered hibernation for different durations of time and at different reproductive states (some were pregnant while others were not). For statistical analysis, smears collected from females were categorised into one of eight physiological states (see Table 3.1). If a female had more than one urogenital smear collected within a category, a mean urogenital cell composition value was used for analysis.

*Estimated fertilization dates*

In echidnas egg-laying and hatching are evidenced by a distinct change (trough or peak) in the temperature record from the external loggers (Morrow and Nicol 2012, chapter 5). The day of fertilization was estimated by counting 23 euthermic days back from egg-laying. This estimate could be confirmed by the presence of sperm recovered from the urogenital tract on, or within two days of, the estimated date.

*Statistics*

All data are represented as means ± standard deviations (S.D.) unless indicated otherwise. Student’s unpaired $t$-test was used for comparison between plasma progesterone concentrations of females that re-entered hibernation while pregnant and females that mated and re-entered hibernation but were not pregnant. Statistical analyses were performed using the software package Statistica 6.1 (Statsoft, Tulsa, Oklahoma). We used linear mixed effects models (Pinheiro and Bates
to detect differences in urogenital cell compositions between different physiological states (see Table 3.1) using the ‘nlme’ package (Pinheiro et al. 2013) in R (R Core Team, 2012). The modelling procedure followed that of Zuur et al. (2009) using likelihood-ratio tests and inspection of residuals and diagnostics. The model had physiological state and year as fixed effects and animal ID as a random effect and were refitted using REML. The significance level was $p < 0.05$, $N =$ number of samples, $n =$ number of animals.
Table 3.1. Physiological states of female echidnas during breeding years. Samples collected between January and March during the period where females are intensively foraging to gain body condition prior to the upcoming hibernation period (see Nicol and Morrow 2012, Sprent et al. 2012) were classified as the pre-hibernation fattening (PHF) state. Samples collected during PHF were from females that had either lost their young the previous year or did not breed the previous year. Samples collected between March and June were from females in deep hibernation (HIB) and all but one sample were collected from females with $T_b < 20^\circ$C. Samples collected from females within 48 hours of a male entering their hibernaculum (verified from time stamps on camera trap recordings) were classified as the male entered hibernaculum (MEH) category. Samples categorised as egg *in utero* (EIU) were from females sampled on the day an egg was detected *in utero* via ultrasonography. If a female was sampled within three days of losing her young in the first week of lactation her smear was placed in the lost baby (LB) category and subsequent smears collected from these females were placed in the lost baby foraging category (LBF). Females that were sampled during their lactation period were divided into two separate categories: LACT1 if the female was sampled within two days of leaving her nursery burrow confinement period (Morrow and Nicol 2012, chapter 5) or LACT2 for all other samples collected during the lactation period.

<table>
<thead>
<tr>
<th>Physiological state</th>
<th>Time of year</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHF Pre-hibernation fattening</td>
<td>Jan - Mar</td>
</tr>
<tr>
<td>HIB Hibernation</td>
<td>Mar – Jun</td>
</tr>
<tr>
<td>MEH Male entered hibernaculum *</td>
<td>Jun – Jul</td>
</tr>
<tr>
<td>EIU Egg <em>in utero</em> #</td>
<td>Aug – Sept</td>
</tr>
<tr>
<td>LB Lost baby in first week of lactation period</td>
<td>Aug – Sept</td>
</tr>
<tr>
<td>LACT1 Samples collected within the first two days of a female leaving her nursery burrow</td>
<td>Sept – Oct</td>
</tr>
<tr>
<td>LACT2 Samples collected during late lactation</td>
<td>Oct- Dec</td>
</tr>
<tr>
<td>LBF Lost baby, foraging</td>
<td>Aug – Dec</td>
</tr>
</tbody>
</table>

* recorded by camera traps

# verified using ultrasonography
Chapter 3: Reproductive tactics

Results

Males seeking out and entering female hibernacula

Camera traps outside the hibernacula of female echidnas showed that during breeding years males entered the hibernaculum before the female had emerged from hibernation (n = 7). The mean time between a female’s last periodic arousal to euthermia during her hibernation period to a male entering her hibernaculum was 11.9 ± 6.6 days, range 2 - 19 days (n = 7). Average hibernation bout length during June and July is 15.0 ± 7.1 days and average minimum T_b is 8.4 ± 1.4°C (Nicol and Andersen 2000). External temperature loggers verified that males were locating females that were in deep hibernation (T_b < 10°C). Generally only one male at a time entered a female’s hibernaculum, although one female which was undisturbed for the first three weeks of monitoring had two males enter her hibernaculum within an hour of one another. The urogenital tracts of three females were sampled less than 13 hours after a male had entered their hibernacula and while the females were still within their hibernacula with the males. In these three females T_b was between 12 – 27.8°C, it was difficult to insert the catheter into the cloaca and no sperm were recovered from the females’ reproductive tracts, indicating that mating had not occurred. Two females found within their hibernacula with males more than 13 hours after males had entered their hibernacula (24 and 35 hrs) had T_b s of 29 and 30°C respectively: sperm were recovered from the reproductive tract of both females.

The presence of a male within a female’s hibernaculum did not always lead to an arousal from hibernation. External temperature records from two females demonstrated that the females did not re-warm after a male entered their hibernacula even in one case where a male remained with one of the females for 13 hours (see Fig 3.1). The presence of males within these two females’ hibernacula could be detected by a 5 - 8°C rise in temperature on the female’s external temperature loggers.
which coincided with time stamps on camera trap recordings, but temperatures recorded on the external temperature loggers returned to soil temperatures immediately after the males left. When the reproductive tracts of these females were sampled shortly after the male’s visits it was difficult to insert the catheter and no sperm were recovered from the urogenital smears of either female.

Cameras were set up over the hibernacula of two reproductively mature females which did not breed. Males did not investigate the hibernaculum belonging to one of the females, however, males investigated female 6865’s hibernaculum on eight separate occasions over three consecutive non-breeding years. Despite the fact that males found and entered 6865’s hibernaculum she did not mate: we collected a urogenital smear after each male had investigated her hibernaculum, but no sperm were recovered from her reproductive tract.

*Mating groups*

Seventy-one mating groups were observed over the three years of this study. For 61 of these groups, the identity and $T_b$ of all animals within the group could be recorded. In 36% of these groups the female $T_b$ was < 28°C when she was found and sperm were recovered from the reproductive tract of 74% of these females. All males found in mating groups were at euthermic $T_b$ (31 – 32 °C). All females that mated (confirmed via the recovery of sperm from their reproductive tract) and were subsequently monitored became pregnant. Pregnant females continued to attract mates: males located the hibernacula of pregnant females that re-entered hibernation, with all pregnant females that re-entered hibernation resuming euthermia after males entered their new hibernaculum. Males also continued to pursue pregnant females that did not re-enter hibernation after mating. Fresh sperm were recovered from the urogenital tract of 54% of pregnant females and
females continued to mate even in late pregnancy. One female was found in a mating group only three days prior to her constructing and entering a nursery burrow and a urogenital smear demonstrated the presence of fresh sperm.

_Females re-entering hibernation after mating_

All females observed in mating groups prior to July 27 re-entered hibernation. Of the 11 females that re-entered hibernation after being observed in mating groups, eight had sperm in their reproductive tracts when sampled after re-entering hibernation, while three did not. It was difficult to insert a catheter into the cloacas of the three females that did not have sperm recovered from their reproductive tracts. After the initial disturbance from males and resulting copulations, females remained active for a period of \( 5.8 \pm 4.0 \) days \((n = 5)\) before re-entering hibernation. All females rewarmed to euthermic \( T_b > 30^\circ C \) before re-entering hibernation. Five of the eight females that re-entered hibernation post-mating were pregnant. Pregnant females re-entered hibernation after \( 2.4 \pm 2.1 \) days \((n = 5, \text{range } 1 - 6 \text{ days})\) of gestation. The mean period for which pregnant females re-entered hibernation was \( 7.5 \pm 6.7 \) days \((n = 4, \text{range } 3-13 \text{ days})\). Pregnant females that re-entered hibernation had a mean \( T_b \) of \( 10.7 \pm 3.8 \) °C, range \( 7.6 - 23.7^\circ C \) \((N = 1613, n = 4)\) over the duration of their hibernation bout. One pregnant female re-entered hibernation for 50 days (see below) but as she was an outlier she was excluded from analysis. There was a close relationship between gestation length and the period of post-fertilization hibernation: gestation was extended by one day for every day in hibernation. There was no relationship between the date of fertilization and the length of post-fertilization hibernation but the latest date at which a pregnant female re-entered hibernation was 27 July (Nicol and Morrow 2012).
Female 5D5E (2009) hibernated for 50 days while pregnant (see Fig. 3.2). Her estimated fertilization date was July 15 when she was observed in a mating group with two males and sperm were recovered from her urogenital tract. She re-entered hibernation on July 22 and hibernated until September 10: a camera trap over her hibernaculum for this period demonstrated that she emerged alone on 11 September and that no male entered or disturbed her hibernaculum. 5D5E was monitored closely upon emergence from post-mating hibernation: she was not found in any further mating groups and no sperm were recovered from her reproductive tract. Furthermore all radio-tracked males in our population had ceased mating activity by late August of that year of study. On September 18 female 5D5E had a swollen pouch, and on September 29 an egg was detected in utero via ultrasonography. This means that egg-laying occurred 80 days after estimated fertilization and 22 days after her final arousal from hibernation.

Plasma progesterone concentrations of females sampled within three days of re-entering hibernation were $1.68 \pm 0.9 \text{ ngml}^{-1}$ ($n = 4$) for pregnant females and $0.75 \pm 0.6 \text{ ngml}^{-1}$ ($n = 2$) for females that re-entered hibernation but were not pregnant. The composition of cells recovered in urogenital smears collected within three days of re-entering hibernation was significantly different between pregnant and non-pregnant females: pregnant females had a significantly smaller proportion of PCS and ANS cells in urogenital smears than non-pregnant females (PCS: $F_{1,4} = 9.24, p < 0.05$; ANS: $F_{1,4} = 17.1, p < 0.05$).

*Plasma progesterone throughout pregnancies*

In pregnant echidnas plasma progesterone concentrations rose after the estimated date of fertilization and peaked at levels of $6.81 \pm 1.92 \text{ ngml}^{-1}$ (range $3.9 - 10.6 \text{ ngml}^{-1}$, $n = 10$) (see Fig. 3.3).
The plasma progesterone concentrations of females that did not re-enter hibernation post-mating peaked 17.2 ± 2.4 days after the estimated time of fertilization. Two females that re-entered hibernation while pregnant also had peak plasma progesterone concentrations occurring within this period. However, entering hibernation while pregnant delayed the timing of plasma progesterone peak concentrations compared with females that did not re-enter hibernation while pregnant (see Fig 3.4). One female (5D5E 2009) re-entered hibernation for 50 days after fertilization occurred, and her peak progesterone (3.9 ng/ml) occurred 76 days after the estimated date of fertilization (see Fig 3.2).

Plasma progesterone profiles of those females that re-entered hibernation while pregnant varied amongst individuals because females re-entered hibernation at different stages of their pregnancies, and for different durations of time (see Fig. 3.3.). As a general trend, plasma progesterone concentrations remained stable when females re-entered hibernation and only began to increase when females resumed euthermic $T_b$. However, in one female, plasma progesterone concentration increased while she was hibernating, and initially fell when she became euthermic, before increasing a few days later (see Fig. 3.3 c).

Eggs were detected in utero on average 2.25 ± 1.26 days (range 1-4 days, $n = 4$) prior to egg-laying. Plasma progesterone concentrations at the time an egg could first be detected in utero ranged from 1.2 to 5.7 ng/ml (mean 3.6 ± 2.1 ng/ml, $n = 5$). Plasma progesterone was measured in all of these animals 2 – 9 days before the egg could be detected. There was a significant correlation between the difference in plasma progesterone concentrations between the paired measurements and the timing of the first sample ($\text{prog 1} – \text{prog 2} = -1.03t + 6.53$, where $t$ is the
time in days between sample 1 and sample 2, $r^2 = 0.086$, $p < 0.05$). These results are consistent with plasma progesterone reaching peak concentrations several days before an egg could be detected in utero and then declining before egg-laying (see Fig. 3.3). The plasma progesterone concentration of a female with a freshly laid egg in her pouch was $1.2 \text{ngml}^{-1}$ (see Fig. 3.3 d).

Eggs were detected in both right and left uteri: four females had an egg in the left uterus, and two females had an egg in the right uterus. One female that bred in consecutive years had an egg in her left uterus in 2008 and in the right in 2009.

Changes in urogenital smear cell compositions throughout the female cycle

Neither year nor physiological state had a significant effect on the proportion of any of the cell types recovered in urogenital smears. Many smears could not be scored as cells recovered on the smear were obscured by mucus or bacteria. Mucus and bacteria occurred in smears throughout different stages of the female cycle.

Discussion

By using an innovative combination of techniques: urogenital smears; hormone analysis; camera traps; external temperature loggers and ultrasonography we have revealed new insights into female echidna reproductive physiology. Physiology is one of the primary determinants of behaviour, and an understanding of physiology provides the basis for an explanation of why females in our population utilize particular reproductive tactics. Ultrasonography allowed us to determine which of the two uteri contained an egg. This has been debated since the discovery that echidnas were oviparous, as only the left uterus and ovary are functional in the oviparous platypus Ornithorhynchus anatinus (Griffiths 1978). Semon (1894) claimed that while eggs will form in
both the left and right ovaries of the echidna, the egg only becomes ripe on the left ovary, with fertilization only occurring in the left oviduct, although Flynn and Hill (1939) collected 134 uterine eggs from echidnas and found that 67 were in the left uterus and 67 in the right. By using the non-lethal technique of ultrasonography we could follow individual females through several breeding years, and as well as confirming the observations of Flynn and Hill (1939) that both uteri and ovaries are functional in the echidna, we have shown that in consecutive breeding years females may alternate which uterus contains the egg.

While ultrasonography was successful, urogenital smears provided the best insights into female reproductive strategies. The presence of sperm in these smears allowed us to determine whether mating had occurred, and this has provided new insights into female echidna behaviour. Although urogenital cytology could not be used to identify different stages of the female echidna reproductive cycle, there were significant differences in cell compositions of smears collected from the urogenital sinus of pregnant and non-pregnant females within three days of the females re-entering hibernation. This, along with pregnant females having higher plasma progesterone concentrations than non-pregnant females within three days of re-entering hibernation, provides further evidence that pregnant female echidnas re-enter hibernation in our study population and that Tasmanian echidnas do not simply have a shorter gestation length than their mainland conspecifics.

Why should Tasmanian echidnas re-enter hibernation while pregnant? The majority of mammals which enter hibernation or daily torpor during pregnancy are small insectivorous or nectarivorous species. As nectar and insect abundance fluctuate it has been suggested that torpor during gestation in these species is linked to restricted food abundance or unpredictable or adverse environmental conditions (Geiser and Masters 1994, Geiser et al. 2001, Willis et al. 2006, McAllan et al. 2012).
While the lack of food resources is often a stimulus for pregnant mammals to enter hibernation, males in our population can forage and increase their body mass even at lowest ecosystem productivity (Nicol and Morrow 2012, chapter 4). Therefore it is not a lack of food that stimulates pregnant female echidnas in our population to re-enter hibernation.

We have previously suggested that pregnant females may re-enter hibernation to have an opportunity to ‘trade up’ and mate with another male of higher genetic quality (chapter 2, Morrow & Nicol 2009), but, pregnant females do not abandon their pregnancies after re-entering hibernation. On the contrary, by re-entering hibernation, pregnant females in our population extend their gestation period and hence delay the timing of egg-laying. A similar strategy is utilized by pregnant hoary bats *Lasiurus cinereus*, which may enter torpor in adverse environmental conditions during pregnancy to slow embryonic growth and delay parturition until environmental conditions are optimal for lactation and offspring survival (Willis *et al.* 2006). It is often assumed that something as fundamental as the optimal timing of reproduction will not differ between males and females (Ball and Ketterson 2008). However, differences in breeding roles between the sexes, and hence different selective pressures and evolutionary interests mean that this is not always the case.

The optimal timing of mating does not always coincide with the optimal timing for subsequent birth (Sandell 1990) or egg laying, and the male assessment for the optimal time of breeding is likely to reflect the influence of sexual selection on male breeding success and fitness (Ball and Ketterson 2008). For males, initiating breeding early may increase the opportunities for copulations with additional females (Ball and Ketterson 2008) and intra-sexual competition has been demonstrated to force earlier reproductive readiness in male mammals when reproductive behaviours are reliant on gonadal hormones (Prendergast 2005). In contrast breeding early may
compromise female reproduction through exposing mother and offspring to suboptimal environmental conditions (Ball and Ketterson 2008). The optimal timing of reproduction is crucial for offspring survival and therefore reproduction should be adjusted to the time of year when environmental conditions are favourable (Lincoln and Short 1980, Johansson and Rowe 1999, Varpe et al. 2007). Females have a central role in determining optimal breeding times (Caro et al. 2009): females from a wide variety of species exert some control over the timing of parturition by either storing sperm (Racey 1979, Birkhead and Møller 1993), delaying implantation by embryonic diapause (Sandell 1990, Ptak et al. 2012), varying gestation lengths using compensatory mechanisms (Scott et al. 2008) or through entering hibernation or torpor during pregnancy which extends the gestation period (Olsson and Shine 1998, Willis et al. 2006, Morrow and Nicol 2009, Geiser and Brigham 2012, Morrow and Nicol 2012, Dzal and Brigham 2013). Maternal thermoregulation should therefore be under direct positive selection via its influence on offspring survival (Wapstra et al. 2010). By re-entering hibernation while pregnant, female echidnas in our study population delay the timing of egg-laying. Because the newly hatched young are so small (less than one gram), the energetic costs during early lactation are very low (Morrow and Nicol 2009, chapter 2), but the importance of delaying egg-laying is that it ensures that the high energetic costs of mid- and late lactation occur when ecosystem productivity, and thus food availability, is high (Morrow and Nicol 2012, chapter 5, Nicol and Morrow 2012). As all female echidnas in our population that mate prior to July 27 re-enter hibernation this suggests that delaying egg-laying is crucial for successful reproduction if females are fertilized early in the breeding season. How female echidnas judge whether to remain euthermic or re-enter hibernation after mating is likely to be related to photoperiod, rather than the weather at the time, as photoperiod is used by a wide
variety of seasonal breeding mammals to time annual breeding efforts (see Bronson 1985, Paul et al. 2008).

Although we have previously suggested that females that males located in hibernacula do not have any pre-copulatory choice (chapter 2, Morrow and Nicol 2009), sperm were not recovered from the reproductive tracts of any of the three females sampled less than 13 hours after males entered their hibernaculum. This indicates that male echidnas cannot force copulations with torpid females and that mating only occurs after females reach euthermic $T_b$s. Why would females mate with a male that initiates mating activity earlier than optimal for subsequent egg-laying? Females may mate to simply avoid continued harassment and hence mating may be occurring due to male coercion, however, as many females become pregnant from early matings this suggests that females may benefit from becoming fertilized by an early-mating male. Male echidnas presumably locate hibernating females through olfactory cues (Harris et al. 2012) and attractants such as pheromones may allow females to make indirect mate choice as choice is restricted to those males that can detect and locate the source of the attractant quickly (Wiley and Poston 1996). It has been suggested that indirect mate choice may allow less costly assessment of competitive and quality mates than direct mate choice in situations where females have limited opportunities to interact with males before mating (Wiley and Poston 1996). This means that pheromones may play a very important role in female mate choice in our study population and explain why females often become fertilized from matings that occur earlier than is optimal for the subsequent timing of egg-laying.
Pregnant female echidnas that re-entered hibernation had higher plasma progesterone concentrations than non-pregnant females that re-entered hibernation post-mating indicating that plasma progesterone begins to increase early in pregnancy. However, as reported for the stripe-faced dunnart (see Geiser et al. 2005, Menkhorst et al. 2009, McAllan et al. 2012), female echidnas only re-entered hibernation at an early stage of pregnancy, when plasma progesterone levels were still low compared with peak concentrations. For those species for which information is available it appears that females only enter hibernation or daily torpor when plasma progesterone concentrations are low. Natal clinging bats *Miniopterus schreibersii natalensis* and Gould’s wattled bat *Chalinolobus gouldii* enter torpor while storing sperm during the period of delayed implantation when plasma progesterone concentrations are only slightly elevated (van der Merwe and van Aarde 1989, Hosken et al. 1996). In contrast pregnant southern snow skinks *Niveoscincus microlepidotus* enter hibernation in the latter stages of pregnancy when embryos are at full size, but plasma progesterone concentrations levels decrease prior to entering hibernation (Girling et al. 2002) so that females enter hibernation when plasma progesterone is already low. The stage of gestation at which pregnant females may enter hibernation or torpor varies with species: Pipstrelle bats only enter torpor during early stages of gestation (Racey and Speakman 1987); western long-eared bats *Myotis evotis* and mulgaras *Dasycercus cristicauda* enter torpor throughout their pregnancies (Geiser and Masters 1994, Chruszcz and Barclay 2002, Körtner et al. 2008) and blossom bats *Syconycteris australis* and grey mouse lemurs *Microcebus murinus* may enter torpor during the latter stages of pregnancy (Geiser et al. 2001, Canale et al. 2012). Unfortunately, none of these studies collected plasma progesterone data from these pregnant females while the animals were in hibernation or daily torpor. There is therefore a large gap in our knowledge and understanding of the physiological mechanisms that control entry into hibernation and daily torpor in pregnant
females, however, like snow skinks, stripe-faced dunnarts and many bat species, pregnant echidnas appear to only enter hibernation when plasma progesterone concentrations are low, implying that only some stages of pregnancy are compatible with hibernation.

Although we have previously reported that females in our population mate with more than one male (Morrow et al. 2009, Morrow and Nicol 2009, see chapter 2), it is surprising that females continue to attract mates and copulate while pregnant. Mating is a costly activity (reviewed in chapter 1) and as echidnas do not abandon pregnancies they are not mating with multiple males to select higher quality sires for their offspring. Females also do not receive direct benefits from mating such as nuptial gifts, territories or parental care from mating partners. Why do females continue to mate while pregnant? Pregnant females may continue to mate simply to avoid male harassment. However, why males would be attracted to a pregnant female is puzzling: females typically produce only one egg, although twins have been reported (Griffiths 1978, Pierce et al. 2006); and locating, courting and then mate-guarding a pregnant female is costly in both time and energy for a male and limits further copulation opportunities. As mating with pregnant females offers no benefits to male echidnas this suggests that males may not be able to detect when females are pregnant. Concealed ovulation and continued mating while pregnant has been documented in primates (Hrdy 1979, Stumpf et al. 2011) and carnivores (Wedell et al. 2006) as a strategy to minimize the risk of infanticide by causing confusion over paternity. Is it possible that female echidnas are concealing pregnancies as a strategy to create confusion over paternity? Preliminary analysis of female echidna chemical profiles indicates that that there is no identifiable change in signals after females become pregnant (R. Harris, pers comm.), male echidnas have been known to kill young in captivity (P. Rismiller, pers comm. and T. Sinander, pers comm.) and males in our
study population were captured on camera traps entering two female’s nursery burrows, which resulted in the loss of young (Harris and Nicol unpublished obs.). The highest rates of young echidna mortalities occur during the first few weeks of lactation when young are in the burrow with their mother (Morrow and Nicol 2012, chapter 5) and it is possible that some of these mortalities may be linked to males investigating the nursery burrow particularly as many of the nursery burrows belonging to females that lost their young were found disturbed (unpublished obs.). Mating with multiple males by pregnant Tasmanian female echidnas may therefore be a strategy to conceal their pregnancy state and reduce the risk of infanticide. While the ability of females to conceive again closely after losing young is considered a pre-adaptation for the evolution of infanticide through sexual selection (Hrdy 1979), seasonally breeding females are more likely to breed in the following year if they lose their young (Borries 1997, Wolff and Macdonald 2004). This means that while infanticide may not offer a male a chance of siring young in that season, it may offer males future mating opportunities (Borries 1997). Female Tasmanian echidnas are unable to raise offspring to weaning in consecutive years, but will breed in consecutive years if they lose their young in the first year (Morrow and Nicol 2012, chapter 5) and one of the females that lost her young after males entered her nursery burrow in 2012 produced a second baby in the same breeding season (Harris and Nicol unpublished obs.).

Differences between male and female evolutionary interests and selective pressures in my study population result in sexual conflict, particularly over which sex has control of paternity and the timing of mating events. The ability of pregnant females to re-enter hibernation without adversely affecting their young allows successful reproduction in a population where there is asynchronous timing of optimal mating between males and females.
Figure 3.1. Temperature record from an external temperature logger attached to female 5D5E in 2008. Periodic arousals from hibernation are marked with a star. During hibernation logger temperatures normally change slowly, while during active periods the logger responds to variations in ambient temperature, and may warm rapidly if exposed to the sun. Arrow 1 indicates when a male entered 5D5E’s hibernaculum (verified by a camera trap) and she rewarmed and was active for several days before re-entering hibernation. Urogenital smears collected from 5D5E on the day that the male entered her hibernaculum and several days after this event indicated that she did not mate with this male as no sperm were recovered. Arrow 2 indicates when a male entered 5D5E’s new hibernaculum (verified from a camera trap). Despite this male remaining with 5D5E for 13 hours, she did not arouse from hibernation and the temperature recorded by the external logger fell immediately after the male left her hibernaculum. Arrow 3 indicates when 5D5E entered her nursery burrow. The grey triangle shows when sperm were first recovered from 5D5E’s urogenital tract. The estimated day of fertilization is represented by a black triangle.
Fig. 3.2. External temperature record and plasma progesterone concentrations for female 5D5E in 2009. Day of estimated fertilization was calculated by counting 23 euthermic days back from egg-laying. Estimated fertilization dates were then checked and correlated with when 5D5E was observed in mating groups and when sperm were recovered from her urogenital tract. The grey bar represents when 5D5E re-entered hibernation. The black triangle represents when an egg was detected in utero via ultrasonography. Egg-laying evidenced by a distinct 5°C change in her external temperature logger record is indicated by a grey triangle.
Fig. 3.3. Plasma progesterone profiles for four individual females in our population. The two individuals on the left (a and c) re-entered hibernation while pregnant, while the two individuals on the right (b and d) did not re-enter hibernation after becoming fertilized. Day of estimated fertilization was calculated by counting 23 euthermic days back from egg-laying. Estimated fertilization dates were checked and correlated with when females were observed in mating groups and when sperm were recovered from the urogenital tract. Grey bars in (a) and (c) represent when females re-entered hibernation. Black triangles represent when eggs were detected in utero via ultrasonography. Egg-laying evidenced by a distinct 5°C change in external temperature logger records is indicated by a grey triangle. The female in (d) had a blood sample collected when she had a freshly laid egg in her pouch.
Fig. 3.4. Plasma progesterone concentrations (N = 108, n = 10) plotted against time since estimated fertilization. Day of estimated fertilization was calculated by counting 23 euthermic days back from egg-laying. Estimated fertilization dates were then checked and correlated with when females were observed in mating groups and when sperm were recovered from the urogenital tract. Females that re-entered hibernation while pregnant (n = 4) are represented by grey triangles, females that did re-enter hibernation but were not pregnant (n = 3) or did not re-enter hibernation after mating (n = 3) are represented by black squares.
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Chapter 4: Seasonal changes in plasma testosterone concentrations, testicular and crural gland size in wild Tasmanian echidnas
Chapter 4. Seasonal changes in plasma testosterone concentrations, testicular and crural gland size in wild Tasmanian echidnas *Tachyglossus aculeatus setosus*

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**Abstract**

The short-beaked echidna *Tachyglossus aculeatus* is a seasonally breeding animal with a near ubiquitous distribution throughout Australia. In Tasmania, breeding follows a period of deep prolonged hibernation during which body temperatures ($T_b$) drop to within 1°C of substrate temperature. The involution of gonads is often considered a prerequisite for entering hibernation, yet echidna testes are reported to reach 1% of an echidna’s body mass during the breeding season which in our study population begins approximately 30 days after males terminate hibernation. As hibernation typically suppresses reproductive function, this raises questions about the timing of testes recrudescence in the Tasmanian echidna. In this study, we measured plasma testosterone concentrations and used ultrasonography to measure testicular and crural gland volume through the annual cycle in a population of wild Tasmanian echidnas. Testes were at their minimum size in November; testes recrudescence (defined as an increase in testes volume and density) occurred prior to entry into hibernation and in the absence of an increase in plasma testosterone concentrations, and testes were maintained at 75% of peak testes volume throughout the
hibernation period. Crural glands, which are secondary reproductive structures in the echidna, exhibited a circannual pattern of recrudescence and involution. Recrudescence of crural glands occurred after males emerged from hibernation, and was positively correlated with rising plasma testosterone concentrations. We suggest that the unusual strategy of testes recrudescence occurring prior to hibernation in the Tasmanian echidna is linked to maximising energy-savings and optimizing reproduction around an obligatory hibernation period.

**Introduction**

Hibernation (multi-day torpor) places constraints on the endocrine system and function of reproductive organs in hibernating mammals [1-3]. As many crucial reproductive functions are reliant on hormone-mediated processes which are normally depressed at lower body temperatures [see 4], reproduction and hibernation have traditionally been considered mutually exclusive processes. However many endothermic animals including birds and examples from all three subclasses of mammals enter torpor and hibernation during their reproductive period [see 5, 6, 7, 8, chapter 2 & 3, 9-12].

The short-beaked echidna (*Tachyglossus aculeatus*) is a myrmecophagous monotreme with a near ubiquitous distribution throughout Australia. It is a seasonal breeder, breeding in winter following a period of inactivity which ranges from short periods of torpor (Kangaroo Island) [13] to prolonged deep hibernation in echidnas from Mount Kosciuszko, south-east Queensland and Tasmania [14-16]. Tasmania represents the southern-most extent of the echidna’s range and therefore the region where seasonal effects are likely to be the greatest. In Tasmania, the breeding season extends from mid June to mid September following a period of deep hibernation during which body temperature
(T\textsubscript{b}) falls to within 1°C of substrate temperature [17], when substrate temperatures are below 16 and above 4°C (unpublished obs.). Sexually mature males and females do not breed every year, and females breed less frequently than males [18]. Males enter hibernation in late January to early February and in breeding years terminate hibernation in early to late May. As in all hibernators, the period of reduced T\textsubscript{b} is broken by periods of spontaneous arousal, during which body temperatures return to normothermic levels [16]. Arousals are, however, generally less than 24 hours in duration [19] and hence echidnas spend the majority of their hibernation season with T\textsubscript{b} below 10°C. The involution of gonads is often considered a prerequisite for entering hibernation [2, 20], and growth of gonads is inhibited at the low temperature during torpor [4], yet echidna testes are reported to reach 1% of an echidna’s body mass during the breeding season [21] which in our study population begins approximately 30 days after males terminate hibernation [7]. As hibernation typically suppresses reproductive function, this raises questions about the timing of testes recrudescence in the Tasmanian echidna.

The overall aim of this study was to understand the temporal relationships between reproduction and hibernation in the male Tasmanian echidna. In order to address this aim, we investigated seasonal variations in key reproductive physiological and anatomical parameters: plasma testosterone concentrations, testis size and crural gland volume. It has already been established that male echidnas exhibit seasonal changes in plasma testosterone concentrations [22, 23] and testes mass and seminiferous tubule diameter peak during the breeding season [24]. However these studies have significant gaps in their data, with few samples collected during the period of inactivity that precedes reproduction. Furthermore, as echidnas are testicond, testes mass and
Chapter 4: Seasonal changes in male physiology

Seminiferous tubule data collected by Griffiths [24] were collected via lethal sampling: annual changes in testes size within individuals have never been documented.

Males of many species exhibit characteristic changes in secondary reproductive structures that are driven by seasonal variations in circulating testosterone concentrations. In male echidnas, the crural glands have been considered vestigial [25]. However Griffiths [26] noted that these glands reach two cm in diameter during the breeding season, and Carmichael and Krause [27] demonstrated seasonal changes in gland histology. We have observed that during the breeding season a milky fluid can be expressed from the spur canal by pressing on the base of the spur and compressing the ampulla (unpublished obs.). The spur is part of a gland-spur apparatus located at each of the hind limbs consisting of a keratinous spur on the ankle joint with a central canal connected to a duct which leads to the crural gland, located in the popliteal fossa [28].

We investigated the relationships between plasma testosterone concentrations, testes and crural gland volume throughout the year in a wild population of echidnas in the Tasmanian Midlands. Testes and crural gland volumes were measured via ultrasonography: ultrasonography has previously been used in echidna research to determine sex [29] and reproductive status in captive male individuals [30] but has not previously been used to monitor seasonal changes in organ size in wild echidnas.
Methods

Field site

This study was conducted on a 12 km² site (42°28’S, 142°14’E) in the Tasmanian Midlands approximately 50 km north of Hobart, Tasmania. The study site is part of a grazing property and consists of native and improved pasture as well as remnant *Eucalyptus amygdalin*a woodland. The site has variable topography with altitudes ranging from 200 to 400 m above sea level, and mean daily minimum and maximum temperatures are -1°C to 10°C in winter and 7°C to 23°C in summer (Australian Bureau of Meteorology).

Animals and radiotelemetry

Over the duration of this study (2008 – 2010), 14 reproductively mature males were monitored, nine of these males for multiple breeding seasons, resulting in the accumulation of 28 individual years of data (breeding and non-breeding years). All data collected during non-breeding years has been excluded from this study. All echidnas were fitted with a passive implantable transponder (PIT) tags (LifeChip, Destron-Fearing, St Paul, MN, USA) on their right ventral side under light isoflurane anaesthesia. Each tag had a unique 10-digit hexadecimal or 14 digit numeric code, but for simplicity individuals are referred to by the last four digits of their tag. Transmitters (Bio Telemetry Tracking, South Australia) were attached between spines on the lower back of animals using two-component epoxy glue. Small external temperature loggers (iButton, DS1922L, Maxim Integrated Products Inc., Sunnyvale, California, resolution 0.5°C) were attached to their transmitters. These loggers recorded temperature at one hour intervals and could be downloaded in the field. When the echidna is hibernating the logger falls to substrate temperature and the record is
clearly distinguishable from that of an active animal, allowing precise determination of the data and time of entry into and arousal from hibernation [8, 31, chapter 2].

**Blood samples**

Blood samples (approximately 1ml) were taken from the rostral sinus while animals were under light isoflurane anaesthesia using 21G needles and plastic syringes. All samples were collected within an hour of animal capture and were stored on ice until centrifuged at 13000 rpm for 10 minutes to separate plasma. Plasma was then stored at -20°C until analysis. Plasma testosterone was measured via radioimmunoassay (RIA) as described in Nicol et al. (2005). The sensitivity of the assay was 6 pg authentic testosterone (~0.15 ng ml⁻¹ plasma). Assay accuracy and precision were monitored by including three levels of commercially available human control serum (CON 6, DPC) in each assay. All samples collected from the same individual, even if collected in several years, were analysed in a single assay.

**Ultrasonography**

Ultrasonography was performed in the field using an ESAOTE (Caris Plus, Germany) ultrasound unit with a LA523 linear probe (13-4 MHz) while echidnas were under light isoflurane anaesthesia. Echidnas were placed in dorsal recumbency and hair removed from the cloaca to the lower chest and the inside of their hind legs from the spur to the point where the leg joins the body using animal clippers. Conductance gel was then applied directly onto the animal.

The dimensions of testes and crural glands were measured by capturing images when the organs appeared at their maximum size on the screen and using the calliper system integrated into the
ultrasound machine. Each testis and crural gland was measured twice, with two different images used to measure dimensions. The volume of testes and crural glands was calculated according to the volume of a simple rotation ellipsoid accommodating the symmetrical shapes of the organs [as seen in 32, 33]. Total testes and crural gland volume was calculated by adding the average left and right dimensions together.

It was not always possible to visualize the right testis using ultrasonography due to interference from ingesta-filled intestinal loops; this was, however, only problematic during the post-breeding phase when males were feeding most heavily. There was no significant difference in volume between the right and left testis ($t_{108} = 1.37, p = 0.17$), and therefore when the right testis was not visible, measurements calculated for the left testis were doubled to determine total testes volume.

Testis density

We measured testes density using autopsied specimens collected opportunistically through the study. Animals used for autopsies were from different regions of Tasmania: five were road-kill echidnas, and two echidnas died of natural causes at our study site. Only males determined to be adults by the presence of adult male spurs were included in the analysis. Density was calculated by removing the testes and measuring their mass and volume. Also, testis mass was expressed as a percentage of body mass.

Frequency of sample collection

Blood samples were collected between 2008 and 2010, while ultrasound images were taken from June 2009 until December 2010. Samples were collected throughout the year from males that were
being radio-tracked. Body mass was measured each time a blood sample and/or ultrasound was performed using portable weighing scales (OHAUS NavigatorXT NVT10001, Ohaus Corporation, USA, resolution < 1 gram). We aimed to collect at least one set of samples (blood sample and ultrasound data) per month from all males fitted with radio-transmitters, but it was not always possible to collect and sample all individuals in each month. Opportunistic samples were also collected from males found in mating groups that were not fitted with radio-transmitters. Only males with two or more samples collected within a year were included in analyses. If a male either lost his transmitter or his transmitter failed and that male was subsequently not observed in a mating group, samples collected from that individual were discarded as it could not be determined whether samples were collected during a breeding year. If more than one blood sample or ultrasound was collected from an individual in one month, mean monthly values for each variable were used for analysis.

**Statistics**

We calculated monthly means ± standard deviations for each variable. Differences in testes and crural gland volume, plasma testosterone concentrations and body mass between months were analysed using linear mixed effects models [34] using the ‘nlme’ package [35] in R [36]. Response variables were log-transformed to improve normality when required (plasma testosterone and crural gland volume only), and the modelling procedure followed that of Zuur et al. 2009 [37] using likelihood-ratio tests and inspection of residuals and diagnostics. Initially, a full model with random effects for animal ID, month and year, and fixed effects for month and year was fitted, but only random effects for animal ID were retained (all p for ‘year’ and ‘month’ random effects > 0.10). Models were refitted using Maximum Likelihood to simplify the main effects [37], and ‘Year’ was
eliminated from all models (all \( p > 0.10 \)). The final models included month as a fixed effect and animal ID as a random effect and were refitted using REML. The significance level was \( p < 0.05 \).

\[ N = \text{number of samples}, \; n = \text{number of animals}. \]

**Results**

Over the duration of this study we collected a total of 196 blood samples from males during breeding years which yielded 104 mean monthly testosterone samples from 13 individuals. Some individuals were sampled over multiple years, giving a total of 22 breeding years of data. Seventy-five mean monthly testes and 70 mean monthly crural gland volumes were collected via ultrasonography from 10 individual males. Ultrasounds were performed on several individuals for multiple years, resulting in 15 breeding years of ultrasound data.

*Frequency of breeding*

During the period 2008 – 2010, all males monitored except for male 6C6C (discussed below) mated during at least one year of the study, with the majority of males (75%) mating during at least two breeding seasons over the course of this study. Nine males were monitored for consecutive breeding seasons. One male mated every year of the three year period. Due to transmitter loss and/or failure, other males monitored over the duration of this study have some missing data and we do not always know if an individual male participated in mating groups.

One male (6C6C) did not mate over the three year duration of this study. This male was at least five years of age at the beginning of the study and possessed two adult male spurs. However 6C6C was never observed to participate in mating, although he was observed with a female in February.
(well outside of the breeding season) and ultrasonography revealed that this male possessed only a left testis.

**Testosterone**

Plasma testosterone concentrations from the 13 males sampled during breeding years varied significantly throughout the year. Plasma testosterone concentrations were significantly higher in June than in May (p < 0.001), with testosterone concentrations increasing after males terminated hibernation in May. Plasma testosterone concentrations peaked in June (0.92 ± 0.14 ngml⁻¹, n = 8) just prior to the breeding season and decreased progressively throughout the breeding season (Fig. 4.1). Plasma testosterone decreased significantly from June to July (p < 0.05), from July and August (p < 0.001) and from August to September (p < 0.001). The highest plasma testosterone concentration measured was 3.30 ngml⁻¹ from an individual sampled four hours after he located a female within her hibernaculum: a camera-trap set up over the female demonstrated that mating had not yet occurred. Testosterone concentrations were lowest in March (0.13 ± 0.03 ngml⁻¹, n = 4).

Plasma testosterone was low at the end of hibernation but increased after arousal, reaching peak levels after approximately 30 days of euthermia (see Fig. 4.2.). The mean time from a male’s termination of hibernation to his being observed in a mating group was 34 ± 6.5 days, range 32 - 44 days, n = 5.

**Testes**

Total testes volume varied significantly with month. Testes volume during the entire period from
February to August was significantly larger than testes volume in January (p < 0.05). Testes recrudescence was initiated in December after the summer solstice with testes volume increasing throughout January and February. Testes volume was significantly larger in February than January (p < 0.01). Mean hibernation entry date was 17 February ± 3.61 days (n = 14) [18], and testes were at 75% of peak volume in March shortly after males entered hibernation. Total testes volume did not change significantly during hibernation and testes volume during hibernation was not significantly different from peak testes volume. Total testes volume peaked in June (18.99 ± 4.94 cm$^3$, n = 6) just prior to the breeding season and decreased throughout the breeding season until the testes reached minimum size in November (2.17 ± 1.11 cm$^3$, n = 7). Testes volume decreased significantly between July and August (p < 0.01), August and September (p < 0.0001) and September and October (p < 0.01). Fig. 4.3 shows ultrasound images of the left testis from one individual during the breeding season and in December prior to the initiation of recrudescence.

Testis density varied throughout the year. Testis density was greatest during the breeding season (1.73 ± 0.32 g cm$^{-3}$) and lowest (1.15 ± 0.02 g cm$^{-3}$) during the post-breeding period, when the testes were regressed in size. Testis density increased after recrudescence was initiated in late December so that testes density during hibernation (1.42 ± 0.62 g cm$^{-3}$) was greater than during the post-breeding period.

Total testes mass peaked at 0.84 ± 0.05 % of body mass during June and July and was lowest in November and December (0.06 ± 0.04 %). Five males sampled between June and August had testes greater than 1% of their body mass. The greatest testes mass relative to body mass was from
one individual sampled in July with testes at 1.16% of body mass. Testes mass as a % of body mass was lowest in October and December (0.02 % of body mass).

**Crural glands**

There was a significant variation in crural gland volume with month. Recrudescence did not occur until after the termination of hibernation. There was a significant increase in crural gland volume between May and June (p < 0.01) with crural gland volume increasing after males terminated hibernation in May. Crural gland volume peaked in July (3.45 ± 1.15 cm³, n = 13) during the breeding season and decreased in volume as the breeding season continued (see Fig. 4.1). There was a significant decrease in crural gland volume between August and September (p < 0.001).

Crural glands were only enlarged during the breeding season (June – August), and from October to May were their minimal size. There was a significant positive relationship between crural gland volume and testosterone concentration (y = 1.46 + 1.05x, r² = 0.179, p < 0.0005, where x is plasma testosterone concentration). Fig. 4.3 shows ultrasound images of crural glands from one individual during the breeding and non-breeding periods.

**Body mass**

To assess changes in body mass, an individual’s lifetime average adult mass was calculated from data collected between 1996 and 2012. Body mass is represented as % of average mass over an individual’s lifetime (long term mean masses were calculated for echidnas for which we had more than three years of data, and the measured mass converted to a percentage of the long-term mean mass). There was a significant variation in body mass throughout the year. Body mass in May, July, August and September was significantly less than body mass in January (p < 0.05). Males
reached maximum body mass in December (108.7 ± 1.99 % of adult average mean mass, \( n = 4 \)). Body mass decreased significantly between February and March (\( p < 0.05 \)) and continued to decrease throughout the hibernation period. When males ended hibernation (May) they were below their average adult body mass (95.0 ± 0.40 % of adult mean mass, \( n = 4 \)); body mass increased back to their adult average in June (see Fig. 4.1). However, males lost weight during the breeding season, with body mass decreasing significantly between June and July (\( p = 0.0195 \)) and males at minimum body mass (92.7 ± 1.9 % of adult mean mass, \( n = 12 \)) in August at the end of the breeding season. At the termination of the breeding season males began to forage extensively and body mass increased significantly between September and October (\( p < 0.01 \)) and October and November (\( p = 0.05 \)).

**Discussion**

This study has provided new insights into the relationship between hibernation and reproduction in Tasmanian male echidnas. The involution of gonads is often considered a prerequisite for entering hibernation [2, 20], however, in our echidna population, testes recrudescence began after the summer solstice (late December) so that males entered hibernation in February with testes that were already 75% of peak testes volume. This unusual pattern of testes recrudescence being initiated prior to the hibernation period in our population can be related to the diet and resulting lifestyle as well as the mating system. The diet of Tasmanian echidnas is primarily ants [38], a food with relatively low nutritional content [39] and available at low densities. Consistent with a low energy myrmecophagous diet the echidna has many of the metabolic attributes of a protoendotherm [40]: a low basal [41] and field metabolic rate [42], and hence a low energy lifestyle. The low energy diet and resulting lifestyle mean that Tasmanian echidnas utilize strategies to maximise energy savings and this is achieved through hibernation [16].
In our population there is a highly skewed operational sex ratio (OSR): the ratio of the number of sexually receptive females to sexually active males, resulting from females breeding less frequently than males [18]. In any given breeding season the OSR in our study population is 1.55 males: 1 female. This skewed OSR has resulted in intense intra-male competition for access to receptive females and this competition is further exacerbated by high levels of female promiscuity (see chapter 3). Consistent with a mating system experiencing intense sperm competition, males in our population have large testes relative to body size. Testes require two to three months at euthermic $T_b$ to reach 75% of peak size and as intra-male competition for females is intense, it is important that males emerge from hibernation in a reproductive state that allows them to mate as soon as possible. Testes recrudescence being initiated prior to males entering hibernation in our population allows males to maximise energy-savings gained through hibernation while also emerging from hibernation in a state which allows males to mate shortly after resuming euthermic $T_b$.

Testes recrudescence prior to entering hibernation has been documented in reptiles such as the horned lizard *Phrynosoma cornutum, P. douglassi, P. modestum* [43] and the sagebrush lizard *Sceloporus graciosus* [44], but is highly unusual for a mammal. Testes recrudescence prior to hibernation has been reported in the golden hamster *Mesocricetus auratus* [45] and the arctic ground squirrel *Spermophilus parryii* [46] but later studies on both species found that testes recrudescence only occurred after hibernation was completed [47, 48]. While few species enter hibernation with enlarged testes, small amounts of testicular growth and testes recrudescence in the latter stages of hibernation (during periodic arousals) have been reported in golden mantled-ground squirrels *Spermophilus lateralis* [1], woodchucks *Marmota monax* [49], European hedgehogs
Why do we not see a strategy of testes recrudescence being initiated prior to entering hibernation in hibernating mammals other than the echidna? For many other hibernating animals it is simply not necessary to utilize this strategy as they have smaller testes relative to their body mass, and presumably smaller testes require less time to recrudesce. For example woodchucks *M. monax* are comparable to the echidna in body weight with average peak body mass $5.0 \pm 0.1$ kg [53] but their testes are significantly smaller in volume than that of the echidna, with marmot testis volume peaking at $4.8 \pm 0.4$ cm$^3$ in animals kept in captive conditions and $3.6 \pm 0.2$cm$^3$ in animals captured at the beginning of the breeding season [49]. The large relative size of echidna testes and the consequential time required at euthermic $T_b$ for recrudescence, along with the necessity of hibernation to maximise energy-savings, has resulted in testes recrudescence being initiated prior to entering hibernation in our population.

Seasonal breeding animals restrict breeding to optimal times of the year and in males the most common inhibition of reproduction is gonadal retrogression [54]. The maintenance of mature-size testes is more energetically expensive than the smaller incremental investment required for testes growth [55], and hence the majority of seasonally breeding species undergo 40 – 90% atrophy in testis mass from the breeding to non-breeding period, with apoptosis being an integral process in normal testicular function [54]. The percentage of basal metabolic rate (BMR) to maintain testes is roughly equal to the percentage of body mass comprised by the testes, however, for species with large testes relative to body mass, the maintenance of gonadal tissue (testes and epididymis) can be as much as 5-10 % of BMR [55]. In our population mean testes volume was 0.84% of body mass during the breeding season, with some males having testes greater than 1% of their body mass.
Chapter 4: Seasonal changes in male physiology

during the breeding season, and therefore testes maintenance is a substantial energetic cost for echidnas. Testicular atrophy post-breeding in echidnas therefore results in significant energy savings.

Testes recrudescence in the Tasmanian echidna is initiated after the summer solstice: presumably the long photoperiod stimulates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus and stimulates the secretion of follicle-stimulating hormone (FSH). While testosterone is required to maintain germ cells within the testis and for spermatid maturation [54], it is not required for the initiation of recrudescence in the echidna as testes volume and density increased prior to plasma testosterone concentrations increasing. Hence seasonal increases in testes volume and plasma testosterone concentrations are not synchronized in the Tasmanian echidna. A similar pattern of testes recrudescence in the absence of an increase in plasma testosterone concentration is seen in the Djungarian hamster *Phodopus sungorus* [56]. Spermatogenesis and spermatid maturation is reliant on testosterone [57], and therefore it is likely that spermatogenesis is not initiated until male echidnas resume euthermic $T_b$ as plasma testosterone concentrations only increased after males terminated hibernation. However, Griffiths [24] found mainland echidnas in April and May with large testes exhibiting spermiogenesis. Therefore echidnas may not be reliant on increased concentrations of plasma testosterone for the early stages of spermatogenesis. It is possible that the echidnas sampled by Griffiths [24] were from a region where echidnas do not enter deep hibernation, as testes size did not increase until April. Whether spermiogenesis is initiated prior to males entering hibernation in our study population cannot be determined without the use of invasive techniques, although the increase in testes density prior to entering hibernation suggests an increase in seminiferous tubule diameter and the maturation of Sertoli cells.
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The pattern of testes recrudescence occurring in late December after the summer solstice in our population of wild Tasmanian echidnas is in sharp contrast to the study conducted on captive echidnas from Queensland in which testes volume only began to increase in late autumn [30]. Testes recrudescence being initiated in late autumn has also been reported in echidnas from coastal Victoria (M. Augee, pers comm.). Not all Queensland echidnas hibernate or undergo short bouts of torpor prior to the reproductive period. Echidnas from south-east Queensland have reproduction following a period of deep hibernation [14] while in arid areas of Queensland hibernation is facultative [40] and there is no information on whether they enter torpor in late summer or autumn in arid areas [58]. Animals used in the Johnston et al. [30] study were from south-east Queensland and Coffs Harbour, but these animals do not hibernate when held in captive conditions (S. Johnston, pers comm.). In subspecies which do not undergo hibernation prior to the breeding season it is likely that testes recrudescence is initiated in April/May (late autumn) so that males are in breeding condition in June as reported by Griffiths [24] and Johnston et al. [30]. Maintenance of large testes is energetically expensive [55], and therefore testes should only be at an increased size for the minimum time requirement. In contrast, in our population of echidnas in Tasmania, testes are enlarged for six months of the year but for three of these months males are in deep hibernation when metabolic rates of all tissues are reduced. Variations in the timing of testes recrudescence in echidnas from different geographical regions demonstrate the adaptive plasticity of the annual life cycle in this widely distributed species.

Although crural glands in the echidna have been describes as vestigial [25], consistent with the Krause [28] study we have demonstrated that the echidna crural glands exhibit a distinct circannual
pattern of recrudescence and involution. Recrudescence of crural glands in Tasmanian echidnas occurs after males emerge from hibernation, and crural glands are at maximal size during the breeding season: crural gland size therefore appears to be correlated with plasma testosterone concentrations. A correlation between testosterone and crural gland size has also been demonstrated in the platypus *Ornithorhynchus anatinus* [59]. The function of crural glands in the echidna remains unknown, but we suggest that they may have a role in chemical communication. Camera-trap footage of echidnas in mating groups shows that males spend a considerable amount of time grooming themselves (using their grooming claws on their hind legs), and it is therefore possible that males are spreading crural gland secretions on themselves, and possibly females, as a form of communication. The spurs of both male platypus and echidnas are canalized by a small human-hair width channel which terminates at a small orifice located on the side at the tip of the spur with the central canal connected to a duct which leads to the crural gland [28]. Because crural glands only become active during the breeding season, the presence or absence of this fluid within the male spur provides a useful reproductive indicator in the field.

The Tasmanian echidna is an atypical hibernator, utilizing the unusual strategy of initiating testes recrudescence prior to entering hibernation to optimize reproduction around an obligatory hibernation period. This strategy can be linked to the low energy and density diet and requirement to hibernate to maximise energy-savings and to the large relative size of echidna testes reflecting a mating system with intense levels of intra-male competition. By initiating testes recrudescence prior to entering hibernation, males ensure that they emerge from hibernation in a state which allows them to mate within 30 days of terminating hibernation. Hence, the initiation of testes
recrudescence prior to entering hibernation in our echidnas allows males to maximise the time that they can hibernate and hence maximize energy-savings.

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Fig 4.1. Seasonal changes in testes volume (a), crural gland volume (b), body mass (c) and plasma testosterone (d) in breeding male echidnas. The gray background represents samples collected while animals were hibernating while the diagonal-lined background represents samples collected during the breeding period. Body mass is represented as % of average mass over an individual’s lifetime (long term mean masses were calculated for echidnas for which we had more than three years of data, and the measured mass converted to a percentage of the long-term mean mass).
**Fig. 4.2.** Plasma testosterone concentration (ngml$^{-1}$) and days since the final termination of hibernation (represented by a gray vertical line) for five breeding males (N = 90). When males terminated hibernation, testosterone concentrations were low, but increased with increasing days of euthermic $T_b$. 
**Fig 4.3.** Ultrasound images of left testis in December prior to testes recrudescence being initiated \((a)\), left testis in June at the beginning of the breeding season \((b)\), right crural gland in December \((c)\) and right crural gland in July \((d)\) from individual 7A59. All images were taken at the same scale.
Chapter 4: Seasonal changes in male physiology

References


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Chapter 5: Maternal care in the Tasmanian echidna
Chapter 5.

Maternal care in the Tasmanian echidna *Tachyglossus aculeatus Setosus*

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Chapter 6: General Discussion
Chapter 6. Optimizing reproduction in the Tasmanian echidna: the influence of an obligatory hibernation period and intense sexual conflict

Hibernation and daily torpor are common strategies utilized by animals from the poles to the tropics to survive periods of low energy availability. Although the primary function of hibernation and torpor is often considered to be the reduction in energy expenditure associated with the regulated and controlled reduction of body temperature ($T_b$) (see Heldmaier and Ruf 1992, Carey et al. 2003, Humphries et al. 2003, Geiser 2004, Geiser 2011), a recent review identified many additional functions: enhanced fat storage; reduced water loss; survival during prolonged droughts; the coexistence of competing species; reduced parasite loads; the separation of male and female reproductive cycles and the facilitation of timing reproduction to the optimal time of year (Geiser and Brigham 2012). While hibernation and torpor offer multiple benefits for animals, the resulting period of reduced activity can have a large effect on the timing and duration of the breeding season as well as reproductive behaviour and physiology. In this thesis I have examined how the Tasmanian echidna optimizes its reproduction around an obligatory hibernation period as well as in the presence of intense sexual conflict.

The timing and duration of the echidna breeding season throughout Australia is remarkably consistent (Morrow et al. 2009), and while hibernation reduces the active period for Tasmanian echidnas, it does not shorten the period over which mating occurs. Hibernation does however limit the opportunities for interactions between the sexes prior to reproduction. In Tasmania, male echidnas enter hibernation in late January to early February and females enter hibernation in mid to late March. This means that interactions between the sexes cease in January, five months prior to
the breeding season. This is in stark contrast to non-hibernating subspecies of echidna, which are able to interact throughout the autumn period prior to breeding in winter. In species in which females have limited opportunities to interact with males prior to breeding, pheromones have been suggested to play an important role in female mate choice (Wiley and Poston 1996). This is because pheromones restrict female mate choice to those males that can detect and locate the source of the attractant quickly which may allow less costly assessment of competitive and quality mates than direct mate choice (Wiley and Poston 1996). In my population male echidnas presumably locate hibernating females through olfactory cues (Harris et al. 2012). Pheromones may play a very important role in mate choice in these female echidnas and help explain why females often become fertilized from matings that occur earlier than optimal for the subsequent timing of egg-laying (see chapter 3). It is not yet understood what triggers the production of signals which attract males to females’ hibernacula and when this change occurs (Harris et al. 2012): this large gap in our knowledge of echidna mate choice and physiology requires further research.

The slowing of metabolism (bradymetabolic) property of hibernation is often considered inhibitory for reproduction. However, bradymetabolism can assist reproduction by allowing the separation of male and female reproductive cycles (Parker 1970, Racey 1979, Krutzsch et al. 1982, Birkhead and Møller 1993, Geiser and Brigham 2012). This is an important feature of hibernation as the optimal timing of mating can differ between males and females (Ball and Ketterson 2008). All females in my population that mated or were disturbed by males prior to July 27 re-entered hibernation (chapter 3), indicating that mating is often occurring earlier than may be optimal for female reproductive success. Re-entering hibernation extends the gestation period and hence delays egg-laying. By extending the gestation period and delaying egg-laying, female echidnas in my
population time their reproduction so that they emerge from their 37 day period of maternal nursery burrow confinement when ecosystem productivity is increasing and therefore optimal for lactation (Morrow and Nicol 2012, chapter 5, Nicol and Morrow 2012). Females from a wide variety of species can exert some control over the timing of parturition or egg-laying by storing sperm (Racey 1979, Birkhead and Møller 1993), delaying implantation by embryonic diapause (Sandell 1990, Ptak et al. 2012), varying gestation lengths using compensatory mechanisms (Scott et al. 2008) or, as in the echidna, by entering hibernation or torpor during pregnancy thereby extending the gestation period and slowing offspring development until conditions are optimal for mothers and young (Racey 1973, Racey and Swift 1981, Geiser 1996, Olsson and Shine 1998, Willis et al. 2006, Morrow and Nicol 2009, Geiser and Brigham 2012, Morrow and Nicol 2012, chapter 2, 3 & 5, Dzal and Brigham 2013).

The bradymetabolic property of hibernation can also be advantageous for male reproduction. Male echidnas in my study population initiate testes recrudescence prior to entering hibernation and maintain their testes at 75% of peak volume during the hibernation period (see chapter 4). This strategy allows male echidnas to reach breeding condition within 30 days of resuming euthermic $T_b$. There is a selective advantage for males to be in reproductive condition or being close to reproductive condition should the possibility of mating arise (Bronson 1985) and by initiating testes recrudescence prior to hibernation, thus exploiting the bradymetabolic property of hibernation, male echidnas in my population are able to maintain their testes at an activated state while simultaneously utilizing hibernation as an energy-saving strategy. While no other hibernating mammal has been shown to initiate testes recrudescence prior to entering hibernation (discussed in chapter 4), and hibernation is often viewed as inhibitory for reproduction, males from a variety of
species have evolved solutions for timing reproduction around hibernation and utilizing hibernation to optimize reproduction. The European hedgehog *Erinaceus europaeus*, woodchuck *Marmota monax* and golden mantled ground squirrel *Spermophilus lateralis* initiate testicular activity during the latter stages of hibernation (Dutourné and Saboureau 1983, Baldwin *et al.* 1985, Barnes *et al.* 1986) and the hedgehog has an additional feature: a seminiferous epithelium cycle that is not affected by the bradymetabolic property of hibernation (Dutourné and Saboureau 1983). As testes recrudescence is initiated during hibernation in these three species, peak spermatogenic activity is achieved shortly after resuming euthermic $T_b$, an essential requirement as breeding occurs shortly after hibernation is terminated (Wimsatt 1969, Baldwin *et al.* 1985 and references therein, Barnes *et al.* 1986). A variety of reptiles also exploit the bradymetabolic qualities of reduced $T_b$ to optimize their reproduction. Breeding seasons following immediately after the emergence from winter dormancy periods are seen in the red-sided garter snake *Thamnophis sirtalis parietalis* and the Tasmanian snow skink *Niveoscincus ocellatus*. Although peak mating in the snow skink occurs in autumn prior to the dormancy period and females can store sperm, both species have a dissociated cycle: sperm production occurs in summer while breeding occurs after the winter dormancy period. In both species testes begin to decrease in volume prior to entry into hibernation and males store their sperm in their epididymis, and use the stored sperm for mating upon emergence (Garstka *et al.* 1982, Jones *et al.* 1997, Shine 2012). This means that sperm stay viable throughout the dormancy period. Lower temperatures prolong sperm viability and facilitate sperm storage (Krutzsch *et al.* 1982) and therefore these two species of reptile exploit their obligatory winter dormancy period to assist their reproduction as well as compensate for the asynchronous reproductive cycles of males and females. Male echidnas from my population may initiate spermatogenesis prior to entering hibernation: testes density increases prior to males entering
hibernation (see chapter 4), which suggests an increase in seminiferous tubule diameter and the maturation of Sertoli cells. However, as plasma testosterone levels do not begin to increase until male echidnas attain euthermic $T_b$ (chapter 4), and spermatogenesis is reliant on testosterone (McLachlan et al. 1996), sperm production prior to hibernation in the Tasmanian echidna is unlikely unless increased plasma testosterone concentrations are not required for the early stages of spermatogenesis in echidnas. To determine when spermatogenesis is initiated in Tasmanian echidnas, future work should involve performing testis aspiration on individuals prior to entering hibernation, during hibernation and shortly after hibernation is terminated.

Although the involution of gonads has been considered a prerequisite for entering hibernation (Hoffman 1964, Darrow et al. 1988) both male and female echidnas enter hibernation when gonads are in a semi-active state. Tasmanian male echidnas enter hibernation with testes that have already undergone at least two months of recrudescence (chapter 4), and females presumably enter hibernation with developed follicles as they can be fertilized within a few days of emerging from hibernation (see chapters 2 & 3). Female echidnas may also re-enter hibernation during early pregnancy (chapter 2 & 3) while the corpus luteum is active. However, at the commencement of hibernation both plasma testosterone and progesterone concentrations are low in males and females respectively (see chapters 3 & 4). Relatively few studies have examined plasma progesterone concentrations in pregnant females that enter hibernation or torpor (discussed in chapter 3), but there is more information available regarding plasma testosterone concentrations and hibernation. Arctic ground squirrels Spermophilus parryii have high testosterone concentrations prior to entering hibernation and it has been suggested that the function of this is to stimulate growth of muscles which are then catabolised during hibernation (Boonstra et al. 2011). However, the arctic
ground squirrel is atypical: the more general pattern of low plasma testosterone concentrations prior
to entering hibernation and during hibernation are reported in male little brown bats *Myotis
* *lucifugus lucifugus* (Gustafson and Shemesh 1976), vizcachas *Lagostomus maximus maximus*
(Fuentes *et al.* 1991), Tasmanian snow skinks *Niveoscincus ocellatus* (Jones *et al.* 1997) and
golden mantled ground squirrels *Spermophilus lateralis* (although testosterone begins to increase
during brief periodic arousals towards the end of hibernation) (Barnes *et al.* 1988). In addition,
testosterone-induced inhibition of torpor has been reported in Turkish hamsters *Mesocricetus
brandti* (Hall and Goldman 1980), European hamsters *Cricetus cricetus* (Darrow *et al.* 1988) and
golden hamsters *Mesocricetus auratus* (Janský *et al.* 1984). Low plasma testosterone
concentrations appear to be a prerequisite for entering hibernation in most species studied. Male
echidnas in my population therefore follow the typical testosterone pattern for hibernating species.

In my population the male echidna strategy of locating hibernating females (see chapter 2 & 3,
Morrow and Nicol 2009) is likely driven by intra-male competition for access to mates. Male
echidnas in mating groups spend the majority of time jostling with other males in an attempt to get
as close to the female as possible (unpublished obs.) and it is highly likely that many males within
such mating groups miss out on mating opportunities. It is therefore in a male echidna’s best
interest to avoid this competition and locate a female that is not in a mating group. All males that
located a hibernating female, entered her hibernaculum and remained with her for 13 hours or more
mated with that female. Entering a female’s hibernaculum is therefore a highly successful strategy
for a male to gain a copulation opportunity (see chapter 3). Thirteen hours is sufficient time for a
female to rewarm to euthermic $T_b$ after being disturbed from deep hibernation (Nicol and Andersen
2008), and hence I can assume that females have some pre-copulatory choice. Is the choice simply
to mate to avoid further harassment? Determining whether females make strategic decisions about mating or are being coerced requires knowledge of whether females avoid matings when they are highly costly or less beneficial (Rowe et al. 1994, Parker 2006, Wedell et al. 2006). It is difficult to determine whether mating occurred as a result of female preference for a male or from male pressure and intimidation (Stumpf et al. 2011, Shine 2012, Parker and Birkhead 2013). It is important to distinguish between female preference and resistance (Kokko 2005, Brennan and Prum 2012) as otherwise forced copulations are considered forms of female choice (Brennan and Prum 2012). As I have not evaluated the cost of mating for females in my study population and I do not know whether females avoid matings in different situations, I cannot determine whether female echidnas mate with males that enter their hibernaculum through preference for that male (mate choice) or to avoid continued harassment. However, as female echidnas often become fertilized from matings that occur earlier than is optimal for the subsequent timing of egg-laying (see chapter 3), mating with a male that initiates breeding early in the season may offer genetic benefits for a female’s offspring.

There are often gaps in sexual conflict studies regarding the costs of female manipulation on males (Vahed 2007, Madjidian and Karlsson 2012, Makowicz and Schlupp in press). During the breeding season male echidnas spend the majority of their time locating females, competing with other males for access to females, copulating, and then mate guarding. Courtship and feeding are incompatible activities and result in males being at minimal body mass at the end of the breeding season (see chapter 4). However, many of the females that males spend time courting and competing for are already pregnant (see chapter 3). Pregnant females do not abandon their pregnancies after encountering new males (see chapter 3) and therefore male echidnas are often expending energy,
sperm reserves and time mating and guarding females with which they have no chance of siring young. How much a male invests in courtship should be dependent on probable returns (successful matings) relative to energetic costs of copulation (Shine 2012). As courting a pregnant female offers no benefit to a male and reduces his opportunities to mate and court other females, this indicates that males cannot detect when females are pregnant. Is it possible that female echidnas in my population conceal their pregnancy state? Concealing pregnancy state is a clear manipulation of males by females and this strategy is utilized by many primates and carnivores to confuse paternity and reduce the risk of infanticide (see Hrdy 1979, Wedell et al. 2006, Stumpf et al. 2011).

While infanticide may appear unlikely in the echidna, in 2012 males were documented entering females’ nursery burrows in my study population, resulting in the death of young (Harris and Nicol unpublished obs., see chapter 3). One female that lost her young after males entered her nursery burrow then bred for a second time in the same season (Harris and Nicol unpublished obs., see chapter 3). It is therefore possible that while female echidnas may continue to mate while pregnant to simply avoid male harassment, this behaviour may also reduce the risk of infanticide. Regardless of the reason why pregnant females continue to mate, the continued harassment by males results in reduced foraging opportunities for both sexes and females experience the highest rate of mass loss per day during their gestation period (Morrow and Nicol 2012, chapter 5). The Tasmanian echidna is a capital breeder for the first part of lactation, feeding young on milk derived from fat reserves (Morrow and Nicol 2012, chapter 5). As fecundity is influenced by body mass across a range of animal taxa (Kimber et al. 2009) if females are accepting additional matings as a strategy to reduce the risk of infanticide the benefits gained from this behaviour must outweigh the high rates of mass loss and reduced body condition.
Sexual selection and sexual conflict are condition-dependent and a range of environmental and ecological factors including environmental variability (Magellan and Magurran 2006, Darden and Croft 2008, Reinhardt et al. 2008, Eldakar et al. 2010a, Eldakar et al. 2010b, Karlsson et al. 2010, Bokides et al. 2012), population structure and genetic relatedness (Eldakar et al. 2009, Rankin 2011), population density (Emlen and Oring 1977, Rowe et al. 1994, Kokko and Rankin 2006, Eldakar et al. 2010a), sex ratio (Emlen and Oring 1977, Rowe et al. 1994, Janowitz and Fischer 2012), colony size (Pitcher et al. 2005), predation risk (Rowe et al. 1994, Croft et al. 2006), and food abundance (Rowe et al. 1994), have been demonstrated to affect sexual conflict. As sexual conflict is condition-dependent we should expect differences in behaviours and strategies utilized between populations (Stumpf et al. 2011, Shine 2012) and this makes studying large-scale patterns of sexual conflict difficult (Eldakar et al. 2009, Fricke et al. 2009). In this thesis I have examined how a population of wild echidnas in Tasmania optimize their reproduction around an obligatory hibernation period as well as in response to intense sexual conflict. So how general are my results to all echidnas? I have shown that although the timing of echidna reproduction is remarkably consistent across Australia (Morrow et al. 2009), there are significant differences between geographical regions in mating behaviour (see chapters 2 & 3), the timing of testes recrudescence (see chapter 4), as well as patterns of maternal care and the duration of lactation (see chapter 5). My results show that Tasmanian echidna behaviour and physiology are an intense exaggeration of what is reported from other regions throughout Australia. There is therefore significant phenotypic plasticity in echidna behaviour and physiology between regions and echidnas use different strategies in different environmental conditions and regions to optimize their reproduction. The ability of a species to adapt the timing and duration of seasonal events such as reproduction may predict the resilience of species to environmental change (Sheriff et al. 2011): it is interesting to
note that not only does the echidna occur in all major habitats and is the most widespread native mammal in Australia (Augee et al. 2006) but it is also one of the species least affected by European settlement (McKenzie et al. 2007).

The obligatory hibernation period in the Tasmanian echidna has a large influence on reproductive behaviour and physiology. Despite the bradymetabolic quality of hibernation being often considered inhibitory for reproduction, both males and females in my study population use this feature of hibernation to their advantage. Hibernation in Tasmanian echidnas has a large influence in reducing conflict between the sexes over the optimal timing of mating and allows breeding to occur despite this asynchrony between the sexes. This study therefore contributes to our understanding of the numerous interactions between hibernation and reproduction as well as the influence of an obligatory hibernation period on sexual conflict. Sexual conflict studies are enhanced via coupling behavioural and genetic data together as it is then possible to assess the effectiveness of different mating strategies as well as the costs and benefits of sexually antagonistic selection on individual fitness (Stumpf et al. 2011). Echidna-specific microsatellites have recently been designed in our laboratory (unpublished) and future work will focus on which males achieve paternity, and the identity of males that females copulate with (through identifying which male’s sperm were recovered from the female reproductive tract). It is my hope that this study will stimulate further research into how other species utilize hibernation to optimize their reproduction as well as the influence of hibernation and torpor on sexual conflict.
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Chapter 6: General Discussion


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Appendix

Chapter 13
Sex and Seasonality: Reproduction in the Echidna (*Tachyglossus aculeatus*)

Stewart C. Nicol and Gemma E. Morrow

Abstract We studied seasonality in free-ranging echidnas (*Tachyglossus aculeatus*) in Tasmania near the most southern part of their range. Both sexes showed a large seasonal variation in body mass associated with hibernation and reproduction. Male echidnas entered hibernation in mid-February (late summer) and females 1 month later. Not all reproductively mature adults mated every year: in non-reproductive years both sexes hibernated for approximately 6 months, becoming active in spring when ecosystem productivity was increasing and reliable. In reproductive years, males aroused from hibernation in early winter, and sought out females. Matings began before females had completed hibernation, and females re-entered hibernation between matings and sometimes when pregnant. This timing of mating ensures that maximum growth rate of the young coincides with the period of greatest ecosystem productivity, while female torpor through the mating period minimizes energy expenditure during the time of lowest food availability.

13.1 Introduction

The short-beaked echidna (*Tachyglossus aculeatus*) is the most widespread native mammal in the Australasian ecozone. At the most northerly part of their range in New Guinea, echidnas occur within a few hundred kilometers of the equator, where seasonal changes in day length are minimal. For the most southerly populations in Tasmania (43° S) day length varies from 15 h in summer to 9 h in winter. Compared with equivalent northern hemisphere locations, seasonal
temperature variations in Tasmania are relatively small. At our Tasmanian field site (Lovely Banks, 42°27’S), mean summer maximum and minimum are 24 and 10°C, and winter maximum and minimum are 11 and 2°C. Despite these mild winters, echidnas in Tasmania and in parts of mainland Australia hibernate. In a previous study (Nicol and Andersen 2002) we investigated the timing of hibernation in Tasmanian echidnas, and compared them with data from other Australian sites. We suggested that echidna hibernation was part of low energy strategy that was of particular value in the Australian climate, which is characterized by high year-to-year rainfall variability, and very variable productivity. We also showed that reproductively active animals aroused from hibernation in mid-winter to mate, and suggested that this allowed young to be raised at the time of maximum food availability. At the time of this study, we had relatively little information on mating and lactation and in this chapter we review and update our previously published data, adding new information on reproduction and ecosystem productivity to provide a comprehensive overview of echidna seasonality at the southernmost part of their range, and its relationship to reproduction.

13.2 Materials and Methods

The study was carried out on a 12 km² site on a grazing property in the Tasmanian southern midlands 50 km north of Hobart, Tasmania. The study site consists of improved and native pasture with areas of Eucalyptus amygdalina woodland on sandstone (Harris and Kitchener 2005). The site has variable topography with altitudes ranging from 200 to 400 m above sea level with numerous sandstone outcrops, caves, creeks, and gullies. Echidnas found while driving slowly around the property were caught by hand, weighed, and scanned with a hand-held RFID reader (Destron Fearing, MN, USA). Echidnas that had not been previously tagged had a passive transponder tag (LifeChip, Destron Fearing, MN, USA) implanted subcutaneously. Sex was determined by the presence or absence of a spur on the ankle. Juvenile echidnas of both sexes have a sheath covered spur, which in males loses the sheath to become an adult spur, while females lose the spur entirely. Echidnas were deemed to be reproductively mature if they were found in a mating group, or by the presence of a palpable penis bulge in males, and in females by a developing pouch, and entry into a nursery burrow.

In any year up to 22 echidnas had a RF tracking transmitter (Bio-Telemetry Tracking, St Agnes, South Australia) glued to the spines of the lower back allowing them to be located using a hand-held receiver. Body mass, reproductive condition and any reproductive activity, evidence of hibernation, and other details of the location and animal activity were recorded in a database. Blood samples were taken from the rostral sinus for measurement of reproductive hormones. In the period 1996–2007, five male and 16 female echidnas were surgically implanted with temperature data loggers (Stowaway Tidbit, Onset Computer Corporation, MA, USA) and in the period 2007–2011, 21 echidnas (5 M, 5F) were
fitted with external temperature loggers (Thermochron iButtons, DS1922L; Maxim/Dallas Semiconductor, TX, USA) which were glued to the tracking transmitter. Times of initial entry into hibernation and final arousal were determined from the temperature records. Echidnas with implanted temperature loggers were considered to have entered hibernation when $T_b$ fell below 20°C and subsequent periods of euthermia did not exceed 2 days. For echidnas with external loggers entry was taken to be the time at which the daily variation in recorded temperature decreased to <5°C (see Fig. 13.2). For echidnas with internal loggers final arousal was considered to have occurred when $T_b$ remained above 30°C without further hypothermic bouts in that year. For echidnas with external loggers final arousal was considered to have occurred when normal daily temperature variation returned and persisted. Hibernation duration was the time between initial entry and final arousal.

From 2007 onwards reproductive status of echidnas was checked by taking cloacal swabs (Morrow and Nicol 2009). Microscopic examination of retrieved cells indicated reproductive status of females, and the presence of sperm showed recent mating. If the mating resulted in fertilization there were characteristic changes in reproductive tract cytology. The date of egg-laying was determined from the characteristic changes of the internal or external temperature loggers (Nicol and Andersen 2006; Morrow and Nicol 2009). For males, a swab of the penis was taken to check for the presence of viable sperm.

To allow comparison between echidnas of different body size long-term mean masses were calculated for echidnas for which we had more than 3 years of data, and the measured mass converted to a percentage of the long-term mean (mass%). Statistical analyses were performed using the software package Statistica 6.1 (Statsoft, Tulsa, Oklahoma). Values shown are means ± standard error.

### 13.3 Results

Between February 1996 and October 2011, 254 adult echidnas (115 female, 97 male) were tagged in the study area. Of the 50 echidnas tagged during the first year of the study, five were known to be alive in 2011, while 13 were known to be dead. Of the remainder, 11 were not seen after their first capture, while eight were seen in the last 5 years of the study. For all echidnas for which we had multiyear temperature records allowing unambiguous assessment of reproductive status, males were reproductively active in nine of 12 yearly records (75%), while females were reproductively active in 30 of 54 (55%) yearly records. Body mass of both sexes followed a strong seasonal cycle, with significant differences between masses in reproductive and non-reproductive years (Fig. 13.1). For both males and females there was highly significant effects of month (males: $F_{1,299} = 14.0, P < 0.0001$; females: $F_{1,374} = 23.5, P < 0.0001$) and reproductive status (males: $F_{1,299} = 11.1, P < 0.001$; females: $F_{1,374} = 15.5, P < 0.001$). The apparent increases seen in Fig. 13.1 in mass in August for non-reproductive females, and in
July, for non-reproductive males, were not statistically significant (Posthoc unequal N HSD test).

Main effects. ANOVAs for ID and reproductive status of males and females showed no effect of ID on date of entry, or final arousal, or duration of hibernation, so data from different years from individual animals were treated as being statistically independent. Factorial ANOVA for the effects of sex and reproductive status on entry date showed significant differences between males and females \((F_{1,60} = 20.9, \; P < 0.0001)\), but no effect of reproductive status
Mean entry day for males was 17 February ± 3.61, (n = 14), and for females 18 March ± 2.29, (n = 50). There were significant effects of both sex and reproductive status on final arousal date: sex $F_{1,70} = 26.3, P < 0.0001$; reproductive status $F_{1,70} = 122.6, P < 0.0001$. The earliest final arousal was for reproductive males (6 June ± 5.9, n = 13), then reproductive females (21 July ± 3.6, n = 32), non-reproductive males (29 August ± 10.3, n = 4), and then non-reproductive females (19 September ± 4.1, n = 25). Final arousal dates of non-reproductive males and females were not significantly different (Unequal N HSD $P = 0.45$). Duration of hibernation did not differ between sexes ($F_{1,53} = 1.26, P = 0.267$), but differed significantly with reproductive status ($F_{1,53} = 83.0, P < 0.0001$). Mean hibernation duration for reproductively active animals was 118.4 ± 4.0 days (n = 35) and for non-reproducing echidnas 186.1 ± 4.4 (n = 22). Example temperature records from reproductive and non-reproductive male and female echidnas are shown in Fig. 13.2. Before the start of the hibernation period a number of echidnas showed periods during which $T_0$ fell below 25°C. The average time of the first of these events was 25.6 ± 2.4 days before hibernation entry (n = 24, range 51–9.5). There was no difference in the relative dates for these prehibernatory events between males and females ($t_{27} = 0.58, P = 0.56$). For males, the minimum time between the end of hibernation and mating was 25 days, and the maximum was 85 days, giving a mating period of 40 days.

Mean fertilization date for females was July 22 ± 4.2 days (n = 13, range 1 July–4 October), and mean egg-laying date was August 16 ± 3.7 days (n = 29, range 24 July–28 August). There was a negative relationship between the number of days between final arousal date and the time from arousal to egg-laying ($y = 49.1 - 0.53x, r^2 = 0.28, F_{3,19} = 7.57, P < 0.02$, where x is day of the year), i.e., later arousing females had a shorter time before egg-laying. (Fig. 13.3a). In the period 2008–2011 five females re-entered hibernation after becoming pregnant, (mean duration of torpor 15.8 ± 6.3 days, range 3–50 days, median = 10 days). Data from these females, and from three others from previous years where the temperature records suggests post-fertilization hibernation are shown as shaded squares in Fig. 13.3a. Data from the intensively monitored animals (2008–2010) show a close relationship between gestation length and the period of post-fertilization hibernation ($y = 1.16x + 21.0, r^2 = 0.99, F_{1,9} = 972, P < 0.0001$; Fig. 13.3b). If the highest point on the graph was omitted the regression was very similar and still significant ($y = 0.90 + 21.7, r^2 = 0.99, F_{1,8} = 107, P < 0.0001$). There was no clear relationship between the date of fertilization and the length of post-fertilization hibernation, but the latest date at which a pregnant female re-entered hibernation was 25 July. Three females whose temperature loggers showed that they lost their egg or young during the period when the mother is confined to the nursery burrow re-entered hibernation. For two animals these hibernation periods were relatively brief (5 and 7 days in late September and early October), but the other animal re-entered hibernation on 31 July, and did not resume eutheria until 10 November, 102 days later.
Fig. 13.2 External iButton records from five adult echidnas during 2008. a Non-reproducing male, b reproducing male, c non-reproducing female, d reproducing female, e reproducing female which re-entered hibernation after fertilization. Gray line shows soil temperature at 20 cm recorded at Bureau of Meteorology station approximately 2 km from the field site. Triangles indicating observed matings for the male (b) and estimated fertilization dates for the females (d, e).
**13.4 Discussion**

Tasmanian echidnas are strongly seasonal. Both sexes show a significant annual cycle of body mass, related to hibernation and reproduction (Fig. 13.1). Figure 13.4 shows a graph of mean monthly C3 pasture productivity for the Lovely Banks field site, calculated using the pasture simulation model EcoMod (Johnson...
Fig. 13.4 Seasonality at the Tasmanian field site. Circles show estimated mean monthly C₃ productivity for pasture (Kg DM ha⁻¹ day⁻¹) at Lovely Banks based on climate data from the site during the course of this study. Bars show standard error. Solid line: soil temperature at a depth of 20 cm. This measure closely approximates the temperature that hibernating echidnas are exposed to (Nicol and Andersen 2007b). Short dashes: percentage of male echidnas that are euthermic, dotted line: percentage of euthermic female echidnas. Curves are logistic regressions fitted to data from this study. Gray bars: distribution of mating groups. Long dashed line: average growth curve for young. Pasture productivity modeling by Mike Perrin and Mark Hovenden (School of Plant Science, University of Tasmania). Growth of young from Nicol and Andersen (2002), and mating data from Morrow and Nicol (2009).

et al. 2008). This model uses climatic data from the site, including temperature, day length, rainfall, and evapotranspiration records for the last 15 years. Superimposed on this are curves showing the proportion of male and female echidnas that are active (data from this study), the occurrence of mating groups (data from Morrow et al. 2009), and the growth of young (data from Nicol and Andersen 2007a), as well as soil temperature at 20 cm. This temperature closely approximates the temperature that hibernating echidnas are exposed to (Nicol and Andersen 2007b). The diet of echidnas at this site consists almost entirely of ants (approximately two-thirds of dietary volume) and the underground larvae of cockchafer beetles or moths pasture grubs (Sprent, unpublished). Ant abundance is closely related to plant productivity (Siemann 1998), and C₃ productivity calculated in this model can be used as a proxy for abundance of potential prey items of echidnas. During the period January–April productivity is quite unreliable, and it is very low when echidnas begin entering hibernation, although temperatures are quite high. Prehibernatory hypothermic bouts occurred as early as January 7, but only occurred when there was a drop in ambient temperature, while stable torpor only occurred if Tₘ remained below 16–17°C, or temperature on the external logger remained below 14–15°C. These data imply that males are physiologically prepared for hibernation by mid-summer.

In non-reproducing years, echidnas hibernate for 6 months, arousing when ecosystem productivity begins to increase in spring. For these echidnas, activity is confined to the most productive time of the year rather than the warmest.
Reproductively active male echidnas finish hibernation two weeks before the winter solstice while ambient temperatures are still falling (Figs. 13.2b, 13.4). At this stage the testes are near their maximum size, having increased in size before entry into hibernation (Morrow unpublished). This prehibernatory testicular recrudescence is likely to be initiated by decreasing day length with the response mediated by melatonin (Scherbarth and Steinlechner 2010). Field observations show that some males leave the hibernaculum almost immediately after they become euthermic, and may feed extensively and increase in weight during the approximately 25 days before they begin mating. Males then seek out females, most of which are still hibernating. Mating occurs at the coldest, least productive time of the year, and for males the 40 day mating period appears to be the most energetically demanding part of the year, as this is when they show their maximum mass loss. As well as coping with low ambient temperatures, males compete with other males for matings. We have recorded up to four males in mating groups (Morrow et al. 2009), while on Kangaroo Island up to 11 males have been observed with a single female (Rismiller 1992). After mating, males may guard the female for several days, before looking for another mating opportunity, which means they will have few opportunities for feeding. By October, mating is over, their testes have regressed, and feeding and weight gain are maximal.

In this echidna population, the majority of females are still hibernating when found by males. The gestation period for echidnas is 20–24 days (Morrow et al. 2009) (but see below), but only eight of 28 egg-laying events (29%) occurred more than 24 days after the final arousal (Fig. 13.3a). In the period 2007–2010, when females were closely monitored during the mating period, none aroused from hibernation and moved from their hibernaculum before being mated. It is not clear what the $T_h$ of females is during these matings. All seem to coincide with periodic arousals, but the presence of a male in the hibernaculum could initiate rewarming. We never found fresh sperm in the tract of a female whose $T_h$ had not rewarmed above 15°C, although a male may have been with her for much or all of the rewarming period. We have found mating groups where the female had a deep cloacal temperature below 25°C and fresh sperm in her reproductive tract, and a female with a cloacal temperature of 26°C was observed being pursued by a group of males, so females may not be at the normal euthermic temperature of 32°C when mating occurs. Of eleven pregnancies only three females did not re-enter hibernation after mating, and of the eight that re-entered hibernation six were pregnant. The negative relationship between time from arousal and egg-laying and final arousal date (Fig. 13.3a) shows that late arousing females become pregnant very quickly, while very early matings are less likely to result in fertilization.

Although Rismiller and Mc Kelvey (2000) claim that females mate only once, cloacal swabbing for sperm shows that females mate many times with several males (Morrow et al. 2009; Morrow and Nicol 2009). Perhaps more surprisingly, females will continue to mate when pregnant, right up to the time that they enter the nursery burrow (Morrow unpublished). Up until late July pregnant females may re-enter hibernation. Figure 13.3b shows that the gestation period increases in direct relationship to the length of post-fertilization hibernation, indicating that embryonic
development is completely arrested during maternal torpor. All of this suggests that female echidnas would "prefer" to not come out of hibernation until spring, and only do so when they have adequate energy reserves and become pregnant.

Females minimize energy expenditure during winter by hibernating until disturbed by a male (or males), and re-entering hibernation after mating. If fertilization has occurred relatively early in the mating season (before 25 July) females will re-enter hibernation even if pregnant. After approximately 18 days of euthermic gestation, which may be broken by periods of hibernation, the female digs a nursery burrow and moves into it, blocking the entrance behind her, and laying her single egg into her pouch 3 days later (Morrow unpublished). Although they may re-enter torpor when pregnant, once the egg has been laid females maintain a much more constant $T_b$ than usual. Although mean $T_b$ does not vary significantly during the burrow period and immediately after (mean $T_b = 31.2 \pm 0.35, n = 36$), while the mother is incubating the egg the SD for $T_b$ is only 0.53, compared with 0.89 in the nursery burrow before egg-laying, 0.62 while the young is in the pouch, and 1.54 when the mother begins to leave the nursery burrow ($F_{3,32} = 33.7, P < 0.0001$, data from Nicol and Andersen 2006). Maintaining such a constant $T_b$ during egg incubation is likely to be very energetically expensive, and for an animal which is notable for the way it minimizes energy expenditure this indicates that at this stage of development of the young minimizing temperature variability is essential. The egg hatches after about 10–11 days of incubation, but the mother remains in the burrow for a total of approximately 37 days (Morrow et al. 2009), and she may have had virtually no opportunity to feed before entering the burrow. The lactation period for mothers at our field site is approximately 150 days (Morrow unpublished), with young being weaned in late December to early January. During these 150 days the young is completely dependent on the mother for food, and grows from approximately 0.6 g at hatching to about 1.5 kg at weaning (Fig. 13.4). This gives the mother <3 months after weaning the young to gain weight before entering hibernation. By comparison, Kangaroo Island echidnas, which mate at the same time as Tasmanian echidnas, but do not show deep prolonged hibernation have a lactation period of about 207 days (Morrow et al. 2009).

Although approximately only one in four young are raised to weaning female echidnas mate on average roughly every second year, with only females which are in good body condition before entering hibernation being likely to mate in the next winter. By contrast, only 25% of adult males hibernate through the mating season, implying that reproduction is less energetically demanding for males than for females. For males, maximum energy expenditure occurs over about 40 days during the middle of winter, when food availability is at its lowest. Food availability increases after the mating period, and males are able to attain maximum weight and be prepared for hibernation by January. Females attempt to minimize energy expenditure during the mid-winter mating period by re-entering torpor when possible. Males must be euthermic to seek out females, and compete with other males, but females can remain torpid until found by a male. Re-entering torpor when pregnant will delay egg-laying, but once the egg is laid the mother
must expend a considerable amount of energy to maintain a constant $T_b$ during this critical period. Figure 13.4 shows that by mating at the coldest time of the year echidnas ensure that maximum growth of young coincides with maximum ecosystem productivity. Despite the fact that Tasmanian winters are relatively mild, Tasmanian echidnas show a strongly seasonal pattern of energy expenditure consistent with their overall low energy life history.

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