Advances in Assisted Reproductive Techniques for the Conservation of Australian Carnivorous Marsupials

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DECLARATION

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SYNOPSIS

In Australia almost 40% of the carnivorous marsupials, or dasyurids, are threatened. Assisted reproductive techniques (ART), especially genome resource banking, have the potential to contribute to the conservation of these species by reducing the loss of genetic diversity. This project aimed to advance the knowledge of ART in dasyurids by focusing on the long term preservation of male and female gametes and establishing protocols for the production of mature oocytes for use in future ART. These studies used the fat tailed dunnart (*Sminthopsis crassicaudata*) as a model dasyurid and replicated many of the findings on threatened dasyurids.

Dasyurid spermatozoa had a relatively unstable acrosome which lacked acrosomal membrane disulphide stabilisation. There was no evidence that *S. crassicaudata* spermatozoa were susceptible to high concentrations of cryoprotectants, but spermatozoa frozen with up to 40% glycerol using a rapid freezing protocol were not viable. Nonetheless the morphology and acrosomal integrity of frozen spermatozoa was normal and there was no evidence of DNA damage. The lack of success with cryopreservation is likely to be an artifact of cold shock, which was observed in *S. crassicaudata* and had not previously been described in any other marsupial. This susceptibility to low temperature can be overcome by slow cooling spermatozoa to 0 ºC at 0.5 ºC minute $^{-1}$ with up to 20% egg yolk, and it is likely that this finding will result in successful sperm cryopreservation in the near future. Freeze drying spermatozoa represents an additional strategy for long term sperm preservation and freeze dried *S. crassicaudata* spermatozoa had normal morphology and nuclear integrity.

In this study preserved dasyurid spermatozoa were immotile and non-viable but had no nuclear damage, suggesting that fertilisation may be achieved with intracytoplasmic sperm injection (ICSI). As ICSI requires a large number of mature oocytes to be collected, a reliable timed ovarian stimulation protocol was established in *S. crassicaudata*. This protocol enabled the collection of up to 28 oocytes which were either mature, or able to be cultured to the first polar body stage within 48 hours. Despite the success of induced ovulation, methods for preservation of the female gamete are essential to genome resource banking. This study also described a protocol for the enzymatic dissociation of dasyurid ovarian tissue allowing collection of high quality individual preantral follicles. The oocytes inside these follicles were able to be vitrified without any loss of viability and short term *in vitro* culture of immature follicles repaired the small amount of vitrification-induced damage to the surrounding granulosa cells.

This collection of studies describes progress in genome resource banking for spermatozoa and oocytes from dasyurids and the development of protocols allowing the collection of a large number of oocytes for use in fertilisation experiments. These advances provide a solid and comprehensive framework for continuing the study of dasyurid ART which is timely due to the urgent need for genome resource banking in several threatened dasyurid marsupials.

ABBREVIATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
ART	Assisted reproductive techniques
CL	Corpus luteum
CEC	Cornified epithelial cells
cm	Centimetres
CO_2	Carbon dioxide
DiC_8	1,2-dioctanoyl-*sn* glycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
dUTP	2'-Deoxyuridine 5'-Triphosphate
DNA	Deoxyribonuclease
DTT	Dithiothreitol
eSG	Equine serum gonadotrophin
FSH	Follicle stimulating hormone
FCS	Fetal calf serum
g	Gram
g	Relative centrifugal force
G	Glycerol
GnRH	Gonadotrophin hormone releasing hormone
GOC	Granulosa cell-oocyte complexes
GV	Germinal vesicle stage
GVBD	Germinal vesicle breakdown stage
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hCG	Human chorionic gonadotropin
ICSI	Intracytoplasmic sperm injection

i.p.	Intraperitoneal
ITS	Insulin-Transferrin-Selenium
IU	International units
IVF	*In vitro* fertilisation
KCl	Potassium chloride
kg	Kilogram
L	Litre
LH	Luteinising hormone
LN	Liquid nitrogen
m	Metres
mBBr	Monobromobimane
min	Minutes
mL	Millilitres
mm	Millimetre
mm^2	Millimetres squared
mM	Millimolar
M	Molar
mOsm L^{-1}	Milliosmoles per litre
NaCl	Sodium chloride
nL	nanolitre
nm	Nanometres
nM	Nanomolar
O_2	Oxygen
P	Probability
PB1	First polar body stage
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide

PMSG	Pregnant mare serum gonadotrophin
PVA	Polyvinyl alcohol
qBBr	Monobromotrimethylammoniobimane
ROC	Receiving operator characteristic
sec	Seconds
SEM	Standard error of the mean
SUZI	Subzonal insemination
TBS	Tris buffered saline
TCF	Tris-Citrate Fructose buffer
TEM	Transmission electron microscopy
Tris	Tris(hydroxymethyl)aminomethane
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
µg	Microgram
µM	Micromolar
µL	Microlitre
µm	Micrometre
vs	Versus
v/v	Volume per volume
w/v	Weight per volume
χ^2	Chi square statistic
π	Pi or 3.142
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to
~	Approximately
ºC	Degrees Celsius

SPECIES NAMES REFERRED TO IN THE TEXT

Scientific and Common Names

Antechinus stuartii	Brown antechinus
Ailuropoda melanoleuca	Giant panda
Bos gaurus	Gaur
Bufo marinus	Cane toad
Dasyuroides byrnei	Kowari
Dasyurus albopunctatus	New Guinea quoll
Dasyurus geoffroii	Western quoll or chuditch
Dasyurus hallucatus	Northern quoll
Dasyurus maculatus	Spotted tailed quoll
Dasyurus maculatus gracilis	Spotted tailed quoll (north QLD subspecies)
Dasyurus spartacus	Bronze quoll
Dasyurus viverrinus	Eastern quoll
Dicerorhinus sumatrensis	Sumatran rhinoceros
Didelphis virginiana	Virginian opossum
Equus ferus przewalskii	Przewalski's horse
Gazella dama mhorr	Mohor gazelle
Gymnogyps californianus	Californian condor
Lasiorhinus latifrons	Southern hairy-nosed wombat
Macropus eugenii	Tammar wallaby
Macropus giganteus	Eastern grey kangaroo
Monodelphis domestica	Grey short tailed opossum
Mustela nigripes	Black footed ferret
Ningaui timealeyi	Pilbara ningaui

Oryx tao	Scimitar-horned oryx
Panthera tigris	Tiger
Perameles nasuta	Long nosed bandicoot
Phascogale calura	Red phascogale
Phascogale tapoatafa	Brush tailed phascogale
Phascolarctos cinereus	Koala
Planigale ingrami	Long tailed planigale
Pongo pygmaeus	Orang-utan
Potorous longipes	Long footed potoroo
Pseudocheirus peregrinus	Ring tailed possum
Pseudantechinus mimulus	Carpentarian antechinus
Sarcophilus harrisii	Tasmanian devil
Sminthopsis crassicaudata	Fat tailed dunnart
Sminthopsis douglasi	Julia Creek dunnart
Sminthopsis macroura	Stripe faced dunnart
Thylacinus cynocephalus	Thylacine or Tasmanian Tiger
Trichosurus vulpecula	Brush tailed possum
Vombatus ursinus	Common wombat

PUBLICATIONS AND PRESENTATIONS

Peer-Reviewed Publications

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Czarny, N. A., Harris, M. S., De Iuliis, G. N. and Rodger, J. C. Acrosomal integrity, viability, and DNA damage of sperm from dasyurid marsupials after freezing or freeze drying. *Theriogenology*. **72,** 817-825.

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Presentations

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1: Introduction and literature review

1.1 INTRODUCTION

Australia has a unique faunal assemblage as a result of its historical isolation and the absence of large native eutherian predators. Instead Australia has its own marsupial carnivores, the dasyurids, which include species such as the iconic Tasmanian devil (*Sarcophilus harrisii*) and the imposing spotted tailed quoll (*Dasyurus maculatus*). As the largest native carnivores in Australia they are essential to a healthy ecosystem but many species are suffering declines due to habitat loss, disease or the effects of introduced species (Jones *et al.* 2003). While *in situ* conservation and captive breeding are contributing to the conservation of dasyurids (Morris *et al.* 2003), assisted reproductive techniques (ART), such as genome resource banking, are also a wise insurance policy to help preserve the genetic diversity, and avoid the disappearance, of highly threatened dasyurids (Holt 2001).

Compared to other marsupials, a few small dasyurids including the fat tailed dunnart (*Sminthopsis crassicaudata*), stripe faced dunnart (*Sminthopsis macroura*) and brown antechinus (*Antechinus stuartii*) are reasonably well studied along with the other marsupial models- the brush tailed possum (*Trichosurus vulpecula*), Tammar wallaby (*Macropus eugenii*) and American opossums (Tyndale-Biscoe and Renfree 1987). In addition to ecological studies, fundamental dasyurid reproductive studies describing endocrinology, sperm biology, oogenesis and embryology have been published (Selwood 1980; Hinds 1989; Taggart and Temple-Smith 1989; Kress *et al.* 2001). But in larger dasyurids the reproductive literature is restricted to descriptive studies regarding the female reproductive tissues and sperm morphology (Sandes 1903; Hill 1910; O'Donoghue 1911; O'Donoghue 1912; Hill and O'Donoghue 1913; Harding *et al.* 1982). Despite the availability of literature describing fundamental reproductive biology there are only a few investigations into the development of dasyurid ART (Rodger *et al.* 1992a; Selwood and VandeBerg 1992; Breed *et al.* 1994b; Taggart *et al.* 1996; Menkhorst *et al.* 2007).

This thesis describes the development of protocols for assisted reproduction contributing to the conservation of dasyurid marsupials using the comparatively well studied *S. crassicaudata* as a model species. In order to successfully achieve this goal it is important to review the literature describing the biological attributes of dasyurid gametes, in relation to the gametes of eutherians and other marsupials, whilst identifying the current gaps in knowledge and making an assessment of the potential pitfalls relevant to developing ART for the conservation of dasyurids.

1.2 GLOBAL EXTINCTIONS AND CONSERVATION TOOLS

Across the world one in five mammals are threatened with extinction due to processes such as habitat destruction, introduced predators, disease and unsustainable hunting (IUCN 2008). This loss of biodiversity is detrimental because it reduces the worth of a region through the loss of direct (agriculture, medicine) or indirect (pollination, tourism) value (Daily 2000; Daily *et al.* 2000). Furthermore, in addition to being valuable to the cultural identities of local communities, biodiversity has significant intrinsic value (Soulé 1985).

Conservation actions can be divided into two major divisions- *in situ* or *ex situ* conservation. *In situ* efforts conserve the species within their natural habitat; they are normally the first conservation action proposed and involve mitigation of threats such as land clearing or introduced predators. For example, the orang-utan (*Pongo pygmaeus*) is threatened by deforestation and land cultivation in Borneo but *in situ* conservation efforts aim to deter deforestation by suggesting the use of alternative lands and economic incentives based on carbon trading (Swarna Nantha and Tisdell 2009).

In some cases the threatening processes cannot be mitigated, or population sizes are unsustainable, and that is when *ex situ* conservation involving the removal of the species from the wild is employed. *Ex situ* conservation efforts create insurance colonies in the form of captive breeding populations and have been used in the conservation of the Przewalski's horse (*Equus ferus przewalskii*) (Boyd *et al.* 2008) and Californian condor (*Gymnogyps californianus*) (Harvey *et al.* 2004). However *ex situ* conservation can also take a more modern approach and involve genome resource banking where the gametes of individuals are preserved and can be reintroduced to the population at a later date (reviewed in Holt 2001), as is discussed in detail in section 1.4.

A recent assessment of threatened species across the globe determined that Australia had 57 mammalian species identified as being threatened (IUCN 2008). The only two countries with greater numbers of threatened mammals were Indonesia (183 species) and Mexico (100 species). One family of marsupial which is unfortunately well represented in this list is the Dasyuridae which has ten Australian species listed as vulnerable or endangered (IUCN 2008).

1.3 DASYURID MARSUPIALS AND CONSERVATION

Members of the family Dasyuridae are carnivorous or insectivorous marsupials from Australia or New Guinea (Figure 1-1). This is the largest marsupial family and, in Australia, contains 51 species ranging in size from the 2 g Pilbara ningaui (*Ningaui timealeyi*) to Australia's largest surviving native carnivore the 9 kg Tasmanian devil (*S. harrisii*) and the now extinct Tasmanian tiger, or thylacine (*Thylacinus cynocephalus*) (Strahan 1995). Dasyurids are, in general, short lived with a large reproductive output. The extreme of the dasyurid life history strategy is typified by several dasyurids in the genus Antechinus which experience male die-off (Lee *et al.* 1982). After breeding and before their offspring are born the male dies, thus putting all their energy into one reproductive event and freeing resources for the lactating females (Bradley *et al.* 1980; McDonald *et al.* 1981). Male die-off is a consequence of high concentrations of corticosteroids which occur as a result of seasonal increases in testosterone and a subsequent drop in corticosteroid binding protein, this causes immune suppression and makes the individual vulnerable to parasitic and pathogenic infection (Barker *et al.* 1978; Bradley *et al.* 1980; McDonald *et al.* 1981; Poskitt *et al.* 1984). The largest species to demonstrate a tempered version of male die-off is the 1 kg northern quoll (*Dasyurus hallucatus*) where only a portion of males die after breeding (Schmitt *et al.* 1989; Oakwood *et al.* 2001).

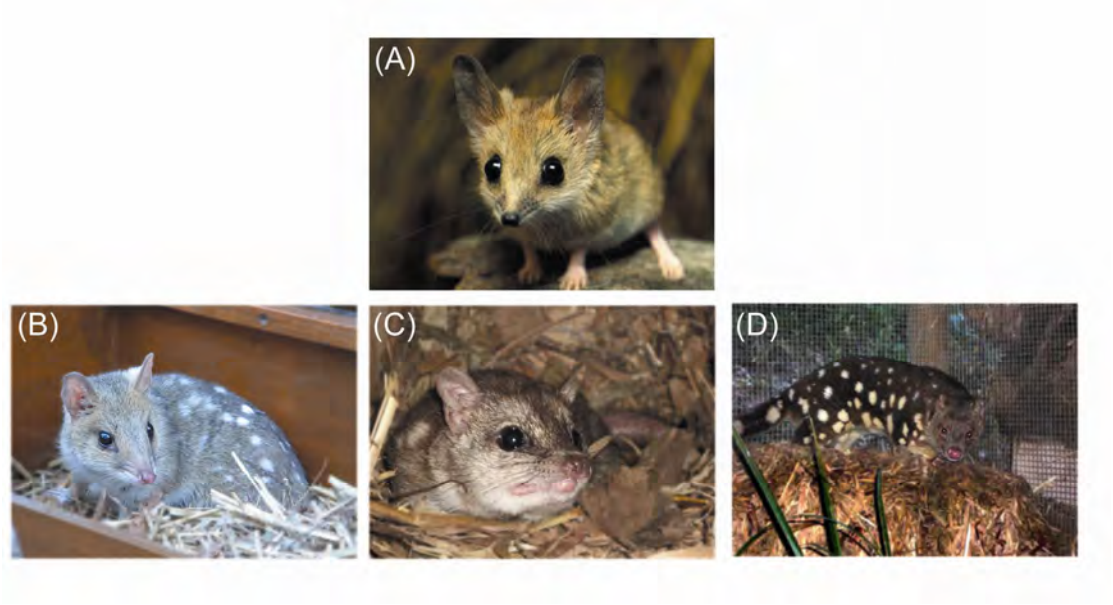

Figure 1-1: Some of the dasyurid marsupials which will be examined in this thesis (A) the fat tailed dunnart (*Sminthopsis crassicaudata*) image courtesy of Museum Victoria (B) the eastern quoll (*Dasyurus viverrinus*) (C) the northern quoll (*Dasyurus hallucatus*) (D) the spotted tailed quoll (*Dasyurus maculatus*). Images are not to scale.

Because many dasyurids put all their resources into one or two breeding events their population size can rapidly increase, but this life history strategy can also be detrimental in the event of an ecological disaster such as drought or fire which reduces, or significantly changes, the available food resources (Jones *et al.* 2003; Dawson *et al.* 2007). The failure of just one breeding season can have dramatic effects on the size of the breeding population, especially in those species which experience male die-off (Oakwood 2000; Jones *et al.* 2003). As a result, nine Australian dasyurids are endangered and another nine are considered vulnerable by the Australian Federal Government (Department of the Environment and Heritage 1999). As this thesis will focus on developing ART for the larger dasyurids, whose conservation status is described in Table 1-1, the threatening processes and conservation efforts pertinent to these species will now be described.

1.3.1 Conservation Issues in the Eastern Quoll, *Dasyurus viverrinus*

The eastern quoll (*Dasyurus viverrinus*) is a medium sized 1 to 2 kg quoll (Figure 1-1B) which was once common throughout south east Australia as indicated by the large number of reproductive studies carried out on this species during the early 1900s (Sandes 1903; Hill 1910; O'Donoghue 1911; O'Donoghue 1912; Hill and O'Donoghue 1913). However, the species is now presumed extinct on the mainland only surviving in Tasmania (Mansergh and Seebeck 1992). Their decline has been linked with predation by, and competition for food resources with, introduced carnivores and a toxoplasmosis-like disease epidemic (reviewed in Jones and Rose 2001). *D. viverrinus* also suffered from human induced mortality because of road kills and were hunted due to their appetite for domestic poultry- Jones *et al.* (2003) describes *D. viverrinus* killing species as large as a turkey and Nathan (1966) describes a history of hunting the poultry-yard raiding *D. viverrinus* in suburban Sydney during the 1930s.

An immediate threat to *D. viverrinus* in Tasmania is the presence of foxes illegally released onto the island during 2001 (Dennis 2002; Jones *et al.* 2003). Predation of juvenile *D. viverrinus* by foxes is proposed to be a major reason for the decline of the mainland population (Jones and Rose 2001). Furthermore the density of *D. viverrinus* in Tasmania has been significantly impacted by the decline of *S. harrisii* which suffer Tasmanian Devil Facial Tumour Disease (see section 1.3.6) (CE Hawkins personal communication 2008). The decline in *S. harrisii* has allowed populations of introduced carnivores to expand via increased food availability and reduced predation leaving juvenile *D. viverrinus* susceptible to increased competition and predation (Jones *et al.* 2007; Mooney 2007).

Table 1-1: The conservation status of large Australian dasyurids as determined by the Australian Federal Government (Department of the Environment and Heritage 1999) and the State Government of New South Wales (NSW) (National Parks and Wildlife Services 1995), Northern Territory (NT) (Parks and Wildlife Commission 2000), Queensland (QLD) (Environmental Protection Agency 1992), South Australia (SA) (Department of Environment and Heritage 1972), Tasmania (TAS) (Department of Primary Industries, Water and Environment 1995), Victoria (VIC) (Department of Sustainability and Environment 1988) and Western Australia (WA) (Department of Conservation and Land Management 1950).

Species	State	Status
Eastern quoll *Dasyurus viverrinus*	Federal	Not listed
	NSW	Endangered[1]
	SA	Extinct
	TAS	Common (not listed)
	VIC	Threatened[1]
Northern quoll *Dasyurus hallucatus*	Federal	Endangered
	NT	Critically Endangered
	QLD	Near Threatened
	WA	Common (not listed)
Spotted tailed quoll *Dasyurus maculatus*	Federal	Endangered
	NSW	Endangered[2]
	QLD	Vulnerable
		Vulnerable
		Endangered[2]
	TAS	Rare
	VIC	Threatened
Western quoll or chuditch *Dasyurus geoffroii*	Federal	Vulnerable
	WA	Threatened
Tasmanian devil *Sarcophilus harrisii*	Federal	Endangered
	TAS	Endangered

[1] *Dasyurus viverrinus* has been presumed extinct on the mainland since 1964 (see section 1.3.1)

[2] *Dasyurus maculatus gracilis* is only found in Queensland and is distinct from *Dasyurus maculatus maculatus* (see section 1.3.3).

1.3.2 Conservation Issues in the Northern Quoll, *Dasyurus hallucatus*

D. hallucatus is a smaller quoll weighing up to 1 kg (Figure 1-1C) with a patchy distribution throughout Northern Australia. It is one of the most threatened species because it consumes the introduced poisonous cane toad (*Bufo marinus*) which has recently arrived into its habitat (Covacevich and Archer 1965; Burnett 1997). Oakwood (2004) describes the decimation of a healthy *D. hallucatus* population to three animals within ten months of the cane toad's arrival. This species is also sensitive to decline because of increased predation by native and introduced species as a consequence of shelter loss because of fire, road kills biased towards dispersing males and hunting by poultry farmers (Oakwood 2000; Oakwood *et al.* 2001; Jones *et al.* 2003).

1.3.3 Conservation issues in the Spotted Tailed Quoll, *Dasyurus maculatus*

D. maculatus is the largest quoll species and males can grow to 7 kg (Edgar 1983)(Figure 1-1D). There are two subspecies, *D. maculatus maculatus* which occurs in Tasmania and south eastern Australia and *D. maculatus gracilis* which is found only in fragmented rainforest patches in far north Queensland (Jones *et al.* 2003). *D. maculatus* are affected by habitat loss and limited den availability as they require tree hollows in old growth forest (Settle 1978; Belcher and Darrant 2006). The destruction of these forests, by logging or fire, also limits the diversity and abundance of prey species (Belcher *et al.* 2007). As described for the other quolls, *D. maculatus* are known to raid poultry yards and hence suffer from hunting by farmers (Troughton 1954; Settle 1978), although community programs are in place to teach farmers effective methods to exclude *D. maculatus* from poultry pens (Tree Kangaroo and Mammal Group 2001). This species also suffers from competition and predation from introduced carnivores (Mansergh and Belcher 1992) and *D. maculatus gracilis* is susceptible to poisoning after consuming the introduced cane toad (Burnett 1997).

1.3.4 Conservation Issues in the Western Quoll, *Dasyurus geoffroii*

The western quoll, or chuditch (*Dasyurus geoffroii*), is a medium sized quoll weighing up to 2 kg (Serena and Soderquist 1995). It was listed as endangered, existing in only 2% of its former range in south-west Western Australia (Jones *et al.* 2003). However due to a successful captive breeding and reintroduction program (described in section 1.3.7) its classification has recently been downgraded to threatened (Department of Conservation and Land Management 1950; Morris *et al.* 2003). The main causes for species decline were predation and competition from introduced carnivores combined with habitat and den reduction, fire and introduced cane toads (Covacevich and Archer 1965; Orell and Morris 1994; Burnett 1997) but *D. geoffroii* are also threatened by road mortality and hunting by farmers (Soderquist and Serena 1989; Smith *et al.* 2004).

1.3.5 Conservation issues in New Guinea Quolls

The New Guinea quoll (*Dasyurus albopunctatus*) and the Bronze quoll, (*Dasyurus spartacus*) are both endemic to New Guinea and are threatened by predation and competition from cats and dogs, habitat destruction and hunting (Jones *et al.* 2003; Leary *et al.* 2008; Woolley *et al.* 2008). One factor limiting their survival may be hunting by farmers as *D. spartacus* has been reported to raid poultry pens and *D. albopunctatus* is locally referred to as "stilman" which translates to "thief" (Van Dyck 1987; Flannery 1990; Jones *et al.* 2003). *D. albopunctatus* is also reported to have declined in areas where feral cats exist (Dwyer 1983; Flannery 1990) and road kills are recorded for *D. spartacus* (Van Dyck 1987).

1.3.6 Conservation Issues in the Tasmanian Devil, *Sarcophilus harrisii*

S. harrisii is Australia's largest surviving native carnivore growing up to 9 kg, and it is only found on the island of Tasmania (Jones 1995). Since 1996 *S. harrisii* has been seriously threatened by the outbreak of Tasmanian Devil Facial Tumour Disease (Young 2003; Jones *et al.* 2007), an ailment which causes large tumours on the face and eventually prohibits normal feeding causing starvation (Pearse and Swift 2006). These tumours occur because of a cancerous cell line which is transferred between highly inbred animals following physical contact. Inbreeding in *S. harrisii* means that when the cancerous cells are transferred to another individual they are not recognised as foreign and no immune reaction occurs (Pearse and Swift 2006; Siddle *et al.* 2007). The seriousness of this disease is expressed by McCallum *et al.* (2007) who predict the likely extinction of *S. harrisii* in the wild within 25 to 35 years.

1.3.7 Conservation Tools for Dasyurids

As described above, several major threats to larger dasyurid marsupials involve introduced species or disease. As there are no immediate solutions to these issues (Burnett 1997; Hawkins *et al.* 2006), *in situ* conservation is not likely to be an effective conservation initiative for species such as *D. hallucatus* and *S. harrisii*. In contrast *ex situ* programs have been successful, especially in *D. geoffroii* where animals were removed from the wild and bred in captivity at Perth Zoo (Western Australia, Australia) (Orell and Morris 1994; Bradley *et al.* 1999). Populations have now been reintroduced to field sites that were cleared of introduced carnivores by baiting with a locally derived toxin which does not affect native species but is poisonous to introduced pests, such as the fox and feral cats (King *et al.* 1989). More recently long term captive Tasmanian Devil Facial Tumour Disease free *S. harrisii* colonies have been established (Jones *et al.* 2007) and in *D. hallucatus* several free ranging colonies have been translocated onto offshore islands which are cane toad free (Rankmore 2005).

Although these programs are having a positive conservation outcome there are drawbacks of long term captive breeding colonies. Housing carnivores in captivity is extremely costly and evolutionary processes continue within the captive environment, potentially introducing traits which are not advantageous in the wild (Snyder *et al.* 1996). These issues are avoided when using ART, such as genome resource banking, which preserves genetic diversity through storing the gametes of deceased individuals for reintroduction back into the population at a later date. Frozen gametes do not require the spatial and economic resources that live animals do, and they are not subject to the adaptive changes which can occur over several generations in captivity.

1.4 ASSISTED REPRODUCTIVE TECHNIQUES FOR CONSERVATION

Assisted reproductive techniques involve technologies such as synchronisation of oestrus, semen collection, gamete cryopreservation, *in vitro* fertilization (IVF) and nuclear transfer or cloning. Some of the earliest ART began in the late 1700 and 1800s and involved artificial insemination (AI) in dogs and horses (reviewed in Foote 2002). However the development of cryopreservation was not studied intensively until 1949 when experiments on fowl spermatozoa demonstrated that freezing with cryoprotectants resulted in post-thaw sperm motility (Polge *et al.* 1949). Subsequently sperm preservation and artificial insemination was investigated in agricultural species (Polge and Rowson 1952) and techniques such as embryo transfer were routinely used to greatly increase the productivity of beef and dairy herds by the 1970s (reviewed in Mapletoft and Hasler 2005). Once established in agricultural species similar techniques could begin to be applied to, and modified to suit, closely related endangered species. For example, Wildt *et al.* (1986) describes superovulation protocols and embryo collection techniques developed in a model species, the domestic sheep, being successfully used in another even–toed ungulate, the scimitar-horned oryx (*Oryx tao*).

ART are important as they can be used to overcome the restrictions of natural breeding. For example, ART can overcome mate incompatibility and in giant pandas (*Ailuropoda melanoleuca*) as the insemination of females with semen collected by electroejaculation removes the threat of aggressive male-female interactions (Moore *et al.* 1984; Zhang *et al.* 2004). ART is also of benefit when animals need to be kept separate because of transmissible diseases. The koala (*Phascolarctos cinereus*) suffers from sexually transmitted chlamydial infection but disease free populations containing the offspring of genetically important chlamydia infected males could be established with the development of sperm screening and AI (Johnston and Holt 2001). In some cases ART is used when geographic boundaries prevent natural breeding- the international transportation of genetically valuable individuals is costly and stressful to

the animal but cryopreserved spermatozoa can be easily shipped (Pukazhenthi and Wildt 2004). Temporal restriction can also be overcome with ART allowing increased production of offspring. For example, seasonally anoestrus female southern hairy-nosed wombats (*Lasiorhinus latifrons*) can be induced to ovulate with endocrine stimulation (Druery *et al.* 2007). Embryo transfer is another example of an ART which can increase the genetic contribution that a single female can make to a population and this has been used to increase the productivity of genetically valuable Przewalski's horses (*E. przewalskii*) (Summers *et al.* 1987). Furthermore males who are highly inbred can have low quality spermatozoa and limited potential for fertilisation (Gage *et al.* 2006), but ART which either assists (subzonal insemination- SUZI) or injects (intracytoplasmic sperm injection- ICSI) a spermatozoon into the oocyte can result in live births in felids (Pope *et al.* 1995; Pope *et al.* 1998).

One of the most valuable forms of ART is the long term preservation of gametes through genome resource banking. This concept collects and stores genetic diversity and allows individuals which are no longer alive, or who have been castrated or ovariectomised, to contribute to the gene pool many years later (reviewed in Holt 2001). Recently the importance of the long term storage of spermatozoa was demonstrated by the successful production of genetically valuable pups from a female black footed ferret (*Mustela nigripes*) inseminated with spermatozoa from a genetically valuable male which had been cryopreserved nine years prior (Howard and Wildt 2009). Genome resource banking is a fundamentally important method for preserving biological diversity while it exists, even if techniques for using frozen gametes are not yet established. In light of the conservation issues facing dasyurids (see section 1.3) it is timely to now establish methods for genome resource banking for these species.

1.4.1 The Value of a Model Species in ART

To date successful ART has been carried out on those species which are well studied or which have well studied domestic models. For example, embryo transfer in Przewalski's horse (*E. przewalskii*) (Summers *et al.* 1987), IVF in the tiger (*Panthera tigris)* (Donoghue *et al.* 1990), nuclear cloning in the gaur (*Bos gaurus*) (Lanza *et al.* 2000) and artificial insemination in the *M. eugenii* (Paris *et al.* 2005)- the most well studied marsupial. However, for species without such a model, there has been minimal success and this can be largely attributed to the limited understanding of their reproductive physiology (Rodger 1990; Holt *et al.* 2004; Pukazhenthi and Wildt 2004), a paucity of material to study (Johnston *et al.* 2003; Andrabi and Maxwell 2007) and absence of funding for the research or conservation of species with little perceived economic importance (Paris *et al.* 2007).

S. crassicaudata is an appropriate model species for examining ART directed towards the conservation of dasyurids as it is easy to house and breed, with large litters and a short gestation, and has equivalent reproductive attributes to the larger threatened dasyurids (Figure 1-1A) (Martin 1965; Morton 1983; Bennett *et al.* 1990; Chesson and Hope 1995). *S. crassicaudata* has a characteristic broad tail, in which 25% of their body fat is stored, and this is suggested to limit body size allowing increased mobility for hunting and predator avoidance (Morton 1983; Hope *et al.* 1997). In the wild, female *S. crassicaudata* are seasonal and polyoestrus with two peaks in births during August and October (southern hemisphere Winter and Spring) (Morton 1978). But in captivity, photoperiod manipulation allows year round breeding (Smith *et al.* 1978; Bennett *et al.* 1990). Females have eight to ten teats and give birth to supernumerary young (up to fifteen offspring) however, the average litter size surviving to weaning at 65 to 68 days is only five or six (Ewer 1968; Godfrey and Crowcroft 1971; Morton 1978; Bennett *et al.* 1990; Chesson and Hope 1995). Females are sexually mature at four months and, although rare, they are capable of breeding until 30 months of age in captivity (Smith *et al.* 1978; Bennett *et al.* 1990). Males show evidence of sperm production at five months but are seven months of age before they are capable of breeding in captivity (Godfrey and Crowcroft 1971; Chesson and Hope 1995). On average animals live for three years in captivity (Ewer 1968; Aslin 1979; Bennett *et al.* 1990). In *S. crassicaudata* there is no evidence of the male die-off (see section 1.3) which is observed in several other small dasyurids (Lee *et al.* 1982).

The captive research population of *S. crassicaudata* was established at the University of Adelaide (South Australia, Australia) in 1965 (Bennett *et al.* 1990) and in 2002 a colony of these animals were moved to the University of Newcastle (New South Wales, Australia). This species has previously been used as a model for genetics and medical issues (reviewed in Greaves *et al.* 2001; McAllan 2006) but it has most commonly been used for fundamental studies examining marsupial fertilisation (Breed and Leigh 1988). However there are also limited studies into *S. crassicaudata* ART such as ovarian stimulation, xenotransplantation of ovarian tissue, sperm cryopreservation and embryo transfer which will be covered in further detail in subsequent sections of this chapter (Rodger *et al.* 1992a; Breed and Leigh 1996; Shaw *et al.* 1996; Taggart *et al.* 1996). The understanding of *S. crassicaudata* reproductive biology is also complimented by the studies carried out on the closely related *S. macroura* and *A. stuartii* which provide helpful information regarding dasyurid embryo development, endocrine profiles and induced ovulation (Selwood 1980; Hinds and Selwood 1990; Selwood and Woolley 1991; Menkhorst *et al.* 2007).

The biology of male and female reproduction in the model species, *S. crassicaudata*, will be reviewed in the following sections. This will be complimented by information regarding the larger threatened dasyurids, where available, because of their importance as conservation targets and to highlight the relevancy of the model species.

1.5 MALE GAMETE BIOLOGY

A good understanding of fundamental gamete biology is essential for underpinning all ART. This section will describe the production and maturation of spermatozoa, in both eutherians and marsupials. The unique features which occur in dasyurid spermatozoa will be highlighted with special reference to how these issues may affect ART.

1.5.1 Sperm Production

Eutherian spermatozoa are produced in the testes which are comprised of 10 to 20 long convoluted seminiferous tubules containing non-germinal Sertoli cells in which spermatozoa develop (reviewed in de Kretser and Kerr 1994; reviewed in Setchell *et al.* 1994). The space between the seminiferous tubules is called the interstitial tissue and contains immune cells, vessels for blood and lymphatic transport, nerves and Leydig cells which play a significant role in the endocrine milieu (Fawcett 1973; reviewed in de Kretser and Kerr 1994).

The process of sperm production is represented diagrammatically in Figure 1-2. The stem cells of the male gamete are diploid spermatogonia which are connected by intercellular bridges. Type A dark spermatogonia are found on the basement membrane of the seminiferous tubules and as they mature to type B spermatogonia they progress towards the lumen (reviewed in de Kretser and Kerr 1994; reviewed in Setchell *et al.* 1994). These cells undergo mitosis to produce two primary spermatocytes in response to increased endocrine activity at sexual maturity (reviewed in Byskov and Hoyer 1994), however not all spermatogonia develop and some remain undifferentiated to allow continuous sperm production. The diploid primary spermatocytes undergo meiosis to produce secondary spermatocytes which undergo a second meiotic division to produce the haploid round spermatid (Fawcett *et al.* 1971). The round spermatid loses contact with the Sertoli cells via spermiation and is freed into the lumen of the seminiferous tubules (reviewed in de Kretser and Kerr 1994; reviewed in Setchell *et al.* 1994). During spermiogenesis the spermatid develops to a spermatozoa following reduction of the cytoplasm, condensation of the nucleus, development of the acrosome and elongation of the flagellum as described in the following sections (Fawcett *et al.* 1971; reviewed in de Kretser and Kerr 1994; reviewed in Setchell *et al.* 1994).

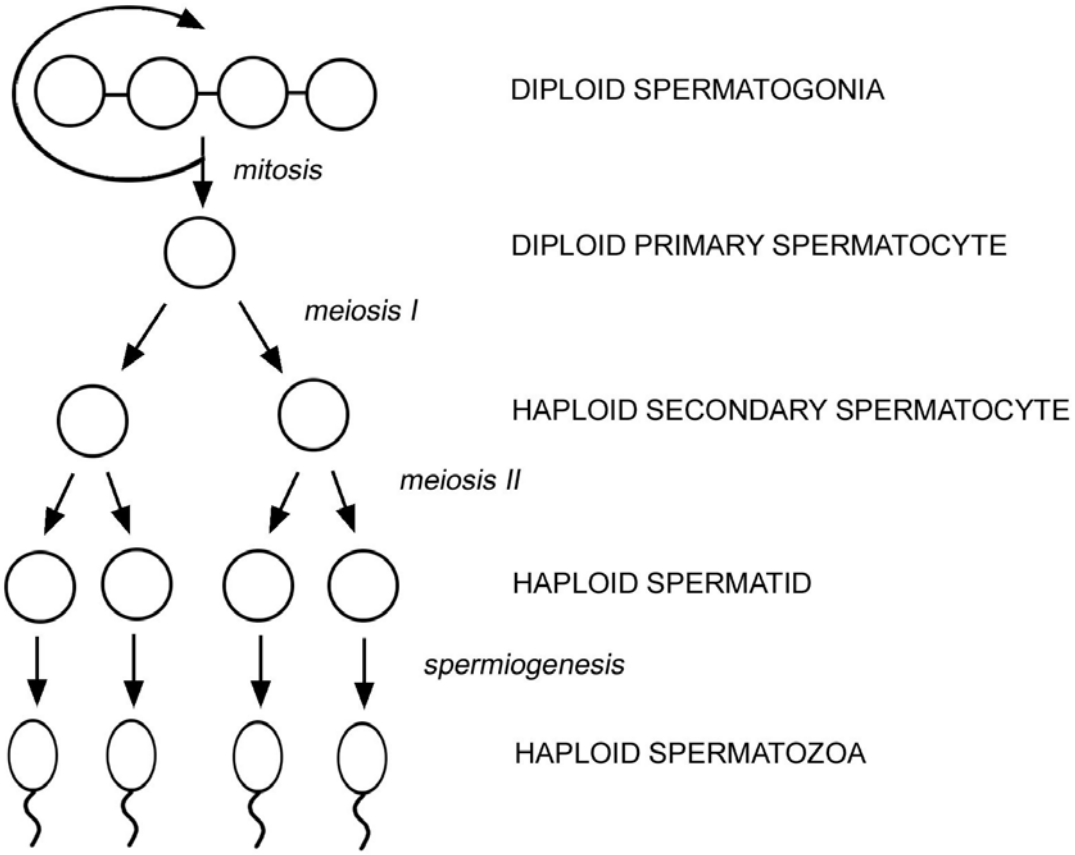

Figure 1-2: A schematic representation of spermatogenesis, describing the development of the male gamete from spermatogonia to spermatozoa.

This process is similar in the marsupial however the testes contain comparatively more Leydig cells in the interstitium than eutherians (Fawcett *et al.* 1973). Dasyurids differ from the other marsupials and eutherians because the spermatozoa develop in the lumen of very few seminiferous tubules- in *S. crassicaudata* only one tube is present and in the kowari (*Dasyuroides byrnei*) there are four seminiferous tubules (Woolley 1975). Another major difference is observed in the spermatogonia of *A. stuartii*, which undergoes male die-off (see section 1.3). In eutherians some spermatogonia remain for continuous sperm production but in *A. stuartii* all spermatogonia develop into mature spermatozoa and at the end of the breeding season the seminiferous tubules are atrophic (Kerr and Hedger 1983).

1.5.1.1 Nuclear condensation

During eutherian spermiogenesis the DNA condenses and the nucleus and sperm head elongates dorso-ventrally. Nuclear DNA is initially wrapped around simple nuclear proteins called histones but condenses as the histones are replaced, first by transition proteins then by the smaller protamines (Fawcett *et al.* 1971; Eddy and O'Brien 1994; Balhorn 2007). Protamines contain cystine residues which allow significant three dimensional disulphide bonding to crosslink and stabilise adjacent protamines and protect the DNA (Bedford and Calvin 1974; Balhorn 1982; Eddy and O'Brien 1994). This stabilisation occurs in all eutherian mammals but there is variation in the number and type of protamines (Balhorn 1982; Balhorn 2007).

Morphological maturation of the nucleus also occurs at this stage in marsupials and has a similar duration (Setchell and Carrick 1973). But the process differs in the marsupial as the nucleus undergoes dorso-ventral flattening producing a T-shaped sperm head at right angles to the tail which creates the marsupial sperm thumbtack morphology (Hughes 1965; Temple-Smith and Bedford 1976). The other major difference is that the nucleus of marsupial spermatozoa is more fragile than that of eutherians and is prone to decondensation when air dried or treated with detergents (Temple-Smith and Bedford 1976; Cummins 1980). This occurs because marsupial nuclear protamines lack cystine residues and cannot form stabilising disulphide bonds (Fifis *et al.* 1990; Mate *et al.* 1994; Lin *et al.* 1995; Retief *et al.* 1995b). The exception to this is a dasyurid, the long tailed planigale (*Planigale ingrami*), whose P1 protamine molecule contains five cystine residues (Retief *et al.* 1995a).

The nuclei of mature *S. crassicaudata* spermatozoa is comprised of two regions of condensed DNA, the C1 electron dense region and the later developing and less stable C2 region containing flocculent DNA (Breed *et al.* 1994a; Soon and Breed 1996). In mature *S. crassicaudata* sperm nuclei, histones are present and treatments which damage histones result in sperm decondensation (Soon *et al.* 1997). Protamine-like proteins are also present in *S. crassicaudata* sperm nuclei, but due to the absence of cystine residues the condensed DNA would still be relatively unstable (Retief *et al.* 1995a; Soon *et al.* 1997) as demonstrated by decondensation following exposure to detergents (Breed *et al.* 1994a). The lack of cystine residues in protamines has also been described in several larger dasyurids including *D. viverrinus*, *D. hallucatus* and *S. harrisii* (Retief *et al.* 1995a).

This lack of nuclear stability is of concern to ART that involves the preservation of spermatozoa. Eutherian spermatozoa which have undergone treatment to reduce nuclear disulphide bonds are unable to fertilise oocytes after freeze drying (described in further detail in section 1.6.5) (Kaneko *et al.* 2003). The absence of nuclear stability in dasyurid spermatozoa may suggest vulnerability to nuclear decondensation following freeze drying and this will be a consideration in the development of freeze drying protocols for the preservation of dasyurid spermatozoa.

1.5.1.2 Acrosomal development

The acrosome is a specialised vesicular compartment on the head of spermatozoa which develops during spermiogenesis and contains hydrolytic enzymes such as acrosin, acid phosphatase, hyaluronidase and phospholipase which help penetrate the zona pellucida of a mature ovum (Ackerman 1970; reviewed in Eddy and O'Brien 1994; reviewed in Yanagimachi 1994). In eutherians the acrosome begins as pro-acrosomal granules formed by the Golgi apparatus. These coalesce to become a single large granule that forms a symmetrical cap, delineated by a continuous inner and outer acrosomal membrane, which covers the apical surface of the elongated sperm head (Figure 1-3A)(reviewed in de Kretser and Kerr 1994; reviewed in Eddy and O'Brien 1994).

In marsupials the acrosome develops from Golgi derived vesicles which coalesce to form an empty acrosomal vesicle that fills with granular material (Sapsford *et al.* 1967; Lin *et al.* 1997). The marsupial acrosome does not take the form of a cap but instead covers the dorsal side of the flattened sperm head (Figure 1-3B) (Temple-Smith 1994). In gliding possums, dasyurids and the American marsupials the acrosome is flat over the horizontal surface of the T-shaped spermatozoa but in *T. vulpecula* and *M. eugenii* the acrosome is defined as “scoop-like” until epididymal maturational occurs (see section 1.5.2) (Temple-Smith 1994; Lin and Rodger 1999).

In *S. crassicaudata* the acrosome extends along four fifths of the dorsal side of the nucleus, but it is thicker at the apical tip (Hughes 1965; Harding *et al.* 1982). Although there are no additional studies on the acrosome of other dasyurids, Harding *et al.* (1982) describe remarkable similarity in the morphology of spermatozoa from thirteen species of dasyurids suggesting that acrosomal morphology will not show considerable variation.

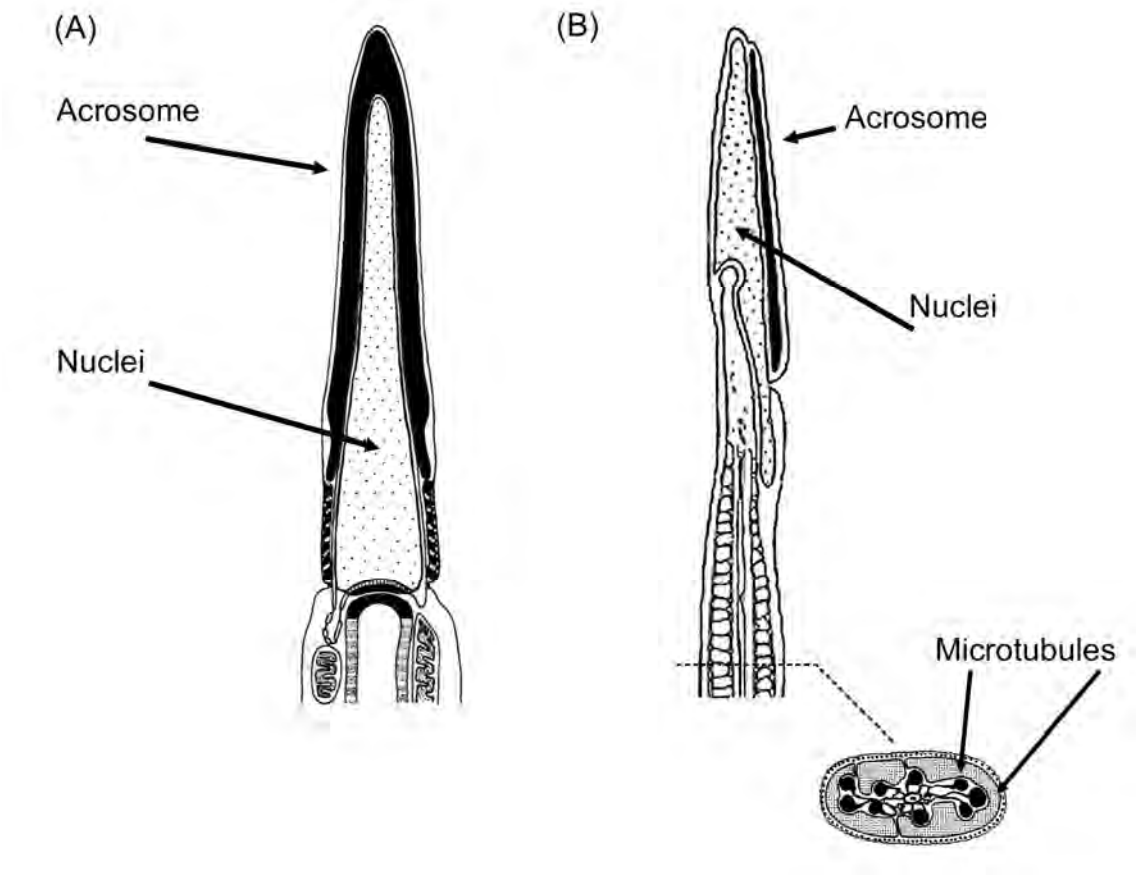

Figure 1-3: The morphology of the spermatozoa from (A) a eutherian, note the dorso-ventral elongation of the sperm nuclei (stippled) and the cap-like acrosome (filled) (B) a dasyurid marsupial, note the insertion of the connecting piece deep in the dorso-ventrally flattened sperm nuclei (stippled) which can reorient 90 degrees to be T-shaped, the acrosome (filled) is also present on one side of the sperm head and the ovoid midpiece contains asymmetrically placed microtubules. This figure has been modified from Temple-Smith (1994) and the eutherian and marsupial spermatozoa are not to scale.

1.5.1.2.1 The acrosome reaction *in vivo*

In vivo the eutherian acrosome reaction takes place following capacitation of spermatozoa inside the female tract in response to zona binding which causes an increase in calcium (reviewed in Eddy and O'Brien 1994; reviewed in Yanagimachi 1994). A true acrosome reaction is typified by fusion of the overlying plasma membrane with the outer acrosomal membrane at multiple locations creating a fenestrated membrane which allows the release of acrosomal contents into the extracellular space to aid zona penetration (Barros *et al.* 1967; Jones 1973; reviewed in Yanagimachi 1994).

The acrosome reaction in marsupial spermatozoa also occurs due to a fenestrated membrane (Sistina *et al.* 1993b; Taggart *et al.* 1993). However zona binding and the proposed role of the acrosome varies within marsupials. In the Virginian opossum (*Didelphis virginiana)* and the grey short tailed opossum (*Monodelphis domestica*) a large penetration hole is found suggesting sperm entry occurs by enzymatic, and not physical, forces (Rodger and Bedford 1982a; Taggart *et al.* 1993). In *S. crassicaudata* the T-shaped sperm head lies against the zona but instead of making a large hole in the zona, only a thin slit occurs (Breed and Leigh 1988). This arises from the localised action of acrosomal enzymes originating from the curved rostral tip of the sperm head and may suggest that in dasyurids both physical and enzymatic forces are involved (Breed and Leigh 1988; Bedford 1998). The actual importance of the acrosome reaction in *S. crassicaudata* is still unclear as transmission electron microscopy (TEM) studies show that spermatozoa contacting the zona surface have either undergone the acrosome reaction (Breed and Leigh 1988; Breed 1994b) or are still partially or fully intact (Breed and Leigh 1992; Breed 1994b). Further uncertainty is introduced by the finding that spermatozoa which are actually penetrating the zona still have a partially intact acrosome (Breed 1994b).

1.5.1.2.2 The acrosome reaction *in vitro*

In eutherians the acrosome reaction can be artificially induced *in vitro* with calcium ionophore- a hydrophobic molecule which binds to calcium and transports it across the plasma membrane (Reed and Lardy 1972; Talbot *et al.* 1976; Shams-Borhan and Harrison 1981). But a false acrosome reaction, occurring from degradation, can also be induced in most eutherian spermatozoa from physical challenge such as freezing without cryoprotection or chemical challenge such as detergent treatment (Yanagimachi 1975; Oettlé 1986; Simpson *et al.* 1987). As a result of its fragility, acrosome loss is a useful tool for assessing morphological damage in studies examining eutherian sperm cryopreservation (Molinia *et al.* 1994; O'Brien and Roth 2000; Isachenko *et al.* 2008).

The marsupial acrosome was reported to be more stable than that of eutherian mammals, maintaining normal morphology following *in vitro* chemical or physical treatment when investigated in *T. vulpecula*, *M. eugenii* and *M. domestica* (Cummins 1980; Mate and Rodger 1991; Mate 1992; Sistina *et al.* 1993a; Sistina *et al.* 1993b). TEM indicated that stabilising disulphide bonds were present in the acrosomal membranes and acrosomal matrix of *M. eugenii* and it is likely that these resulted in a robust acrosome (Mate *et al.* 1994; Lin *et al.* 1995). In these species examination of acrosomal morphology would not be indicative of morphological damage following

sperm preservation. There have been no studies on the acrosomal stability of any dasyurid species and experimental investigations regarding this could not only contribute to the knowledge of fundamental reproductive biology in dasyurids, but could also provide a tool for morphological assessment of spermatozoa following preservation treatments.

1.5.1.3 Development of the flagellum

Another major morphological transformation during spermiogenesis is the development of a flagellum which is comprised of a connecting piece, middle piece, principle piece and end piece. In eutherians the flagellum is connected to the nucleus via a connecting piece and capitulum which gives rise to segmented columns that anchor the axoneme- a microtubule complex which continues into the distal proximal piece (reviewed in Eddy and O'Brien 1994). The eutherian axoneme consists of an inner microtubule doublet surrounded by nine symmetrically placed outer doublets, all surrounded by outer dense fibers (reviewed in Eddy and O'Brien 1994). The midpiece is further surrounded by a mitochondrial sheath and in the principle piece the outer dense fibers are surrounded by a fibrous sheath (reviewed in Eddy and O'Brien 1994).

The flagellum in marsupial spermatozoa also has the same four regions. But they also have an additional mid-piece fiber network which develops during epididymal maturation and a morphologically different connecting piece which resembles a ball and socket articulation (Temple-Smith 1994; Lin *et al.* 1997). This allows reorientation of the sperm head, from T-shaped to streamlined, and is relatively unstable when compared with the eutherian connecting piece (Cleland and Rothschild 1959; Temple-Smith 1994; Lin *et al.* 1998). Within marsupials the most significant differences between species are found in the symmetry of the fibers and dimensions of the four regions of the flagellum (Temple-Smith 1987).

Studies of dasyurid sperm morphology show that the connecting piece is rod-like and joins the implantation fossa at a ball-and-socket articulation deep in the nuclear space (Hughes 1965; Harding *et al.* 1982; Temple-Smith 1994). In addition the midpiece is ovoid in dasyurids, resulting in asymmetric microtubule placement, and this gives the flagellum a flattened paddle-like surface providing a greater surface area to enable efficient sperm motility (Harding *et al.* 1982; Hughes 1982; Taggart and Temple-Smith 1990b).

1.5.2 Maturation of Spermatozoa in the Epididymis

Spermatozoa which are released into seminiferous tubules drain into the rete testes via the tubus rectus which combines several seminiferous tubules (reviewed in Setchell *et al.* 1994). The efferent ducts export spermatozoa from the testes to the epididymis which is a highly convoluted structure found along the anterior-posterior surface of the testes. In eutherians it is divided into three regions: the caput for fluid resorption, corpus for maturation and the cauda for storage (reviewed in Moore and Smith 1988; Setchell *et al.* 1994). As the spermatozoa passively pass through these regions they become capable of fertilising an oocyte due to sperm surface changes, acrosomal remodeling and the acquisition of the capacity to become motile (reviewed in Moore and Smith 1988; Yanagimachi 1994). During epididymal maturation in the boar the cytoplasmic droplet migrates from the neck to the midpiece and can be observed in highly concentrated caudal spermatozoa (Lasley and Bogart 1944) but in the bull the cytoplasmic droplet has been removed by the time spermatozoa reach the caudal epididymidis (Branton and Salisbury 1947).

Marsupial spermatozoa from the caput epididymidis also lack the capacity for motility and during epididymal maturation they undergo similar developmental changes with a few notable exceptions (Hughes 1965; Temple-Smith and Bedford 1976; Temple-Smith and Bedford 1980). As marsupial spermatozoa pass from the corpus to the caudal epididymidis their heads become reoriented and pivot 90 degrees, from their T-shaped morphology, to become streamlined (Hughes 1965; Temple-Smith and Bedford 1976; Temple-Smith and Bedford 1980). However there is a well studied exception to this pattern of marsupial epididymal sperm maturation; in the long nosed bandicoot (*Perameles nasuta*) the sperm head becomes reoriented in the testis (Sapsford *et al.* 1969). Vast morphological changes also occur to the acrosome of possums and macropods during epididymal maturation, with the immature acrosomal scoop condensing and folding to create a compact button-like mature acrosome (Lin and Rodger 1999). In didelphid marsupials, sperm pairing occurs during epididymal maturation: two spermatozoa become coupled at the plasma membrane overlying the acrosome and they do not separate until capacitation (Temple-Smith and Bedford 1980; Temple-Smith 1987; Moore and Taggart 1995). The cytoplasmic droplet is lost from marsupial spermatozoa prior to storage in the caudal epididymidis and in *T. vulpecula* the droplets are actually phagocytosed by specialised luminal cells (Temple-Smith and Bedford 1976; Temple-Smith and Bedford 1980; Temple-Smith 1984).

In dasyurids, spermatozoa are transported from the seminiferous tubules to the centrally placed rete testis which leads to the epididymis (Woolley 1975). Epididymal maturation is similar to that of other marsupials and there is no evidence of phagocytosis of cytoplasmic droplets (Hughes 1965; Taggart and Temple-Smith 1989). But dasyurids do differ in that they have minimal sperm storage- the number of spermatozoa actually decreases between the distal corpus and caudal epididymidis (Harding *et al.* 1982; Taggart and Temple-Smith 1989). This may be a tactic to reduce the concentration of spermatozoa per ejaculate and support a polygamous lifestyle (Taggart and Temple-Smith 1989; Taggart and Temple-Smith 1990a). Macropods produce 1000×10^6 spermatozoa per epididymis but in *A. stuartii* each epididymis contains just 3.5×10^6 spermatozoa, with one tenth released per ejaculate (Bedford *et al.* 1984; Taggart and Temple-Smith 1990a). In *S. crassicaudata* both caudal epididymides contain 0.69×10^6 spermatozoa and this low sperm count is also observed in *S. macroura* and *D. byrnei* (Bedford *et al.* 1984; Breed *et al.* 1989). Despite the low number of spermatozoa, dasyurids have efficient sperm transport to the site of fertilisation in the upper oviduct (Taggart and Temple-Smith 1991; Taggart 1994).

1.5.3 Conclusion to Male Reproductive Biology in Dasyurids

Studies examining the fundamental biology of dasyurid spermatozoa suggest that spermiogenesis does not vary greatly from that described for eutherians (Soon and Breed 1996). There is also little morphological variation in the mature spermatozoon within the dasyurid family (Harding *et al.* 1982). The literature has shown that dasyurid spermatozoa are comparatively large and streamlined. *S. crassicaudata* spermatozoa have a 10 x 2 µm head and a flagellum which is 270 µm long; this is five to ten times longer than eutherian spermatozoa (Harding *et al.* 1982; Breed *et al.* 1989). Dasyurid spermatozoa also lack nuclear stabilisation and are produced in low numbers with limited caudal storage (Harding *et al.* 1982; Breed *et al.* 1989; Taggart and Temple-Smith 1989) and this will need to be taken into consideration when examining sperm preservation techniques. The spermatozoa of marsupials have robust acrosomes and although ultrastructural studies have examined the acrosome reaction *in vivo* (Breed and Leigh 1988; Breed and Leigh 1992; Breed 1994b) the stability of the dasyurid acrosome has not been examined. The fragility of the acrosome is a commonly used indicator of post-freezing morphological damage in eutherians, but this would not be appropriate in those marsupials with acrosomal stabilisation. As such it will be important to determine if the acrosome of dasyurids is stable, as observed in other marsupials, or if it is unstable, allowing it to be used as an indicator of morphological damage post-freezing.

1.6 SPERM PRESERVATION

Preservation of spermatozoa allows the gametes to be stored as part of a genome resource bank for reintroduction into the population in the future (reviewed in Holt 2001). This has great benefits for genetic management of endangered populations though the selective reintroduction of genetic diversity as reported in the black footed ferret (*M. nigripes*) where cryopreserved spermatozoa were used to inseminate a female almost a decade after its collection and cryopreservation (Howard and Wildt 2009). The first report of sperm cryopreservation occurred in amphibians (Luyet and Hodapp 1938) and by 1949 glycerol was successfully being used as a sperm cryoprotectant (Polge *et al.* 1949). Subsequently sperm cryopreservation has been used to preserve the male gamete in the health sciences (Sherman 1973), medical research institutions (Noiles *et al.* 1995; Kusakabe *et al.* 2001), agricultural industry (Hammerstedt and Graham 1992; Woelders *et al.* 1997), veterinary practices (Cardoso *et al.* 2003) and wildlife conservation (Gist *et al.* 2000). However sperm preservation does not need to be restricted to cryopreserved material as more recent developments have examined freeze drying (Hoshi *et al.* 1994; Wakayama and Yanagimachi 1998; Kusakabe *et al.* 2001; Kwon *et al.* 2004) and heat treating spermatozoa (Yanagida *et al.* 1991; Cozzi *et al.* 2001; Lee and Niwa 2006). These two latter techniques produce spermatozoa which are non-viable in a traditional sense with damaged membranes and no motility. However the advent of ICSI has provided technical methods to overcome these problems and as a result freeze drying techniques have become increasingly popular (Wakayama *et al.* 1998; Wakayama and Yanagimachi 1998).

1.6.1 The Theory of Cryopreservation

Successful cryopreservation involves the removal of intracellular water from cells to avoid ice crystal formation which can damage cell membranes. As the temperature decreases, ice crystals form in the extracellular fluid thus removing water from the solution and increasing its ionic strength (Mazur 1965; Watson 1979; Watson and Fuller 2001). To maintain an equilibrium, cells lose water and dehydrate (Mazur 1965; Watson 1979; Watson and Fuller 2001). The absence of intracellular water avoids the formation of intracellular ice crystals but the dehydration is detrimental to lipid bilayers and the three dimensional structure of proteins, and results in lethally high intracellular ionic concentrations (Mazur 1965; Watson 1979; Crowe *et al.* 1990). The two-factor hypothesis presented by Mazur (1965) suggests that an equilibrium must be found between the rate of cooling and water loss. If the rate of cooling is too fast there will not be sufficient time for water to leave the cell and ice formation will occur, piercing the plasma membrane. If the rate of cooling is too slow the cell is exposed to the high ionic concentrations for too long and it is susceptible to dehydration.

1.6.2 Cryoprotectants and Sperm Extenders

The detrimental effects of dehydration can be overcome by the addition of cryoprotective substances. Penetrating cryoprotectants, such as glycerol and dimethyl sulphoxide (DMSO), have a low molecular weight and form non-specific hydrogen bonds with polar protein residues. They act as a water replacement by allowing the maintenance of the three dimensional structure of proteins, lowering the intracellular ionic concentrations and avoiding detrimental cytoplasmic leakage caused by fusion between lipid bilayers (Ackerman 1970; Crowe *et al.* 1987; Crowe *et al.* 1990; Fabbri *et al.* 2000).

Despite their positive attributes, penetrating cryoprotectants such as glycerol, are toxic in some species if metabolised (Polge *et al.* 1949; Crowe *et al.* 1990; Hammerstedt and Graham 1992). Glycerol is generally used at concentrations of 4 to 10% in the cryopreservation of eutherian spermatozoa (Watson and Fuller 2001) but in the boar it has toxic effect when used at greater than 3% (reviewed in Watson 1979) and it can have a contraceptive effect in avian species (Hammerstedt and Graham 1992). Marsupial spermatozoa are in general tolerant of glycerol, maintaining viability in concentrations as high as 17.5% (Rodger *et al.* 1991; Taggart *et al.* 1996; MacCallum and Johnston 2005), but the exception to this is macropod spermatozoa in which glycerol is toxic (McClean *et al.* 2006). Cryoprotectant toxicity can be overcome by lowering the working temperature to reduce cellular metabolism (McClean and Johnston 2003; Fowler and Toner 2005), stepwise addition of the cryoprotectant (Gao *et al.* 1995) or by combining two or more cryoprotectants at a lower concentration (De Leeuw *et al.* 1993).

The other class of cryoprotectants are high molecular weight non-penetrating molecules which establish an osmotic gradient to dehydrate the cell and increase the viscosity of the extracellular solution to slow the rate of water molecules joining ice crystals (Watson 1979; De Leeuw *et al.* 1993). Some disaccharide sugars, such as trehalose, are commonly used non-penetrating molecules which increase the osmotic gradient. But if these sugars are internalised they can also interact with the phospholipid heads of lipid bilayers increasing their stabilisation (Crowe *et al.* 1987).

In addition to cryoprotectants, spermatozoa are diluted with extenders which were initially designed to protect bull semen over several hours and dilute samples to allow insemination in multiple cows (Watson 1979). This was advantageous to the production-focused livestock industry but is less essential for wildlife studies where spermatozoa may have a low concentration and be of reduced quality, as can be observed in highly inbred populations (Wildt *et al.* 1983; Gage *et al.* 2006). Extenders can contain additives which protect the spermatozoa from the effects of preservation by using different mechanisms to cryoprotectants. These can include egg yolk, or low density lipoprotein, which stabilise plasma membranes (Watson and Martin 1973; Watson and Martin 1975; Watson 1976), cytochalasin D which increases the flexibility of the cytoskeleton (Ledda *et al.* 2007) and antioxidants to reduce oxidative damage (Holt 2000).

1.6.3 Cooling and Thawing Protocols

Spermatozoa may be frozen in a variety of containers including narrow 250 µL straws, large 5 mL straws, ampoules or cryotubes- each with different thermal conductivity (Eriksson and Rodriguez-Martinez 2000; Watson and Fuller 2001). Slow cooling protocols have a cooling rate of between 0.3 and 1 °C minute^{-1} and once the spermatozoa are frozen to approximately -40 ºC samples are plunged into liquid nitrogen (LN) for storage at -196 °C (Phillips *et al.* 2008). The slow cooling ensures that no intracellular ice crystals grow but the cells are exposed to the toxic effects of the cryoprotectants for longer and this technique requires the use of a temperature controlled cooling system (Fowler and Toner 2005). Fast cooling protocols require that cells are capable of surviving cold shock (see section 1.6.3.1) and is achieved by suspending samples 5 cm above LN in the vapour phase (~160 °C) or on dry ice (-80 °C) as pellets for a set period followed by plunging into LN for storage (Molinia and Rodger 1996; Taggart *et al.* 1996). Making pellets on dry ice requires very little equipment thus is highly applicable to field studies and the rate of cooling can be manipulated for interspecies differences by adjusting the pellet size, with larger pellets cooling more slowly (MacCallum and Johnston 2005). The most significant danger with fast cooling protocols is that ice crystals will form inside the cell due to incomplete dehydration (Holt *et al.* 1992).

During thawing the same osmotic and toxicity issues which were discussed for freezing arise (see section 1.6.1) and this is the time when membrane damage often occurs (Holt *et al.* 1992; Watson 1995; Holt *et al.* 1999). Hence most protocols recommend a rapid thaw which melts transient ice crystals that form inside the cell before they have the opportunity to grow (Mazur 1965). Also important is the removal of the cryoprotectant using stepwise dilution to stop water entering the cell too quickly and resulting in membrane rupture (Gao *et al.* 1995). Interestingly thawed macropod spermatozoa maintain normal motility following thawing up to 20 °C but at higher temperatures the motility decreases and spermatozoa become immotile- potentially due to the inability of macropod sperm lipid membranes to pass through multiple phase transitions (Holt *et al.* 1999).

1.6.3.1 Cold shock

Spermatozoa from several eutherian mammals including cattle, pigs, sheep and horses are susceptible to cold shock in response to rapid exposure to low temperatures (Mazur 1965; Darin-Bennett *et al.* 1973; Watson *et al.* 1987; White 1993). Spermatozoa become immotile and cannot recover when warmed as a result of physical changes including structural membrane modification, increases in membrane permeability, swelling and alterations to the cytoskeleton (reviewed in White 1993; Watson 1995).

One of the paradigms of marsupial sperm cryobiology has suggested that spermatozoa are not susceptible to cold shock. True cold shock is described by Mann (1964) as occurring following a rapid exposure to approximately 0 ºC in ice water. Studies on *T. vulpecula* indicated that phalangerid spermatozoa were not susceptible to cold shock (Rodger and White 1978). Other marsupial studies which followed this finding suggested cold shock tolerance but used inconsistent methods, sometimes only examining refrigerated temperatures, which are 4 to 5 ºC, and at other times not exposing the spermatozoa to rapidly temperature declines. The literature reports that macropod spermatozoa are cold shock tolerant because they survive rapid chilling to 4 ºC (Miller *et al.* 2004) and cooling to -7 ºC without ice crystal formation at the moderately fast rate of 10 ºC minute^{-1} (Holt *et al.* 1999). Taggart *et al.* (1996) suggests cold shock does not occur in *S. crassicaudata*, the long footed potoroo (*Potorous longipes*) and the ringtail possum (*Pseudocheirus peregrinus*) as spermatozoa survived several days in the refrigerator at 4 ºC within the scrotal tissue. An assessment of *P. cinereus* spermatozoa was the first to hint at cold induced damage as a small, but significant, 12% decrease in motility was observed following chilling in a refrigerator at 5 ºC (Johnston *et al.* 2000).

Although it is quite possible that several marsupial species are not susceptible to cold shock, it is important to recognise that since Rodger and White (1978) there have been no systematic studies in marsupial spermatozoa which examine true cold shock- that is, rapid exposure to approximately 0 ºC (Mann 1964). This could have significant impact on the outcomes of sperm freezing which is difficult in some marsupials, as described below.

1.6.4 Progress in Marsupial Sperm Cryopreservation

The spermatozoa of vombatid, peramelid and phalangerid marsupials withstand cryopreservation well (Rodger *et al.* 2009). In both the common wombat (*Vombatus ursinus*) and *T. vulpecula* post-thaw motility is almost 80% (Taggart *et al.* 1996; MacCallum and Johnston 2005) with no evidence of glycerol toxicity (Rodger *et al.* 1991; Molinia and Rodger 1996). Sperm cryopreservation in these studies was successful with rapid freezing protocols including pellet freezing and freezing in LN vapour.

Cryopreservation is less successful in *P. cinereus* with post-thaw motility limited to approximately 50% and this was achieved using 14% glycerol and a moderate cooling protocol at -6 ºC minute^{-1} (Johnston *et al.* 2006). Post-thaw recovery of viable macropod spermatozoa is very poor with only 10% retaining motility (Molinia and Rodger 1996; Holt *et al.* 1999; McClean *et al.* 2006). This may be in part due to the sensitivity of macropod spermatozoa to glycerol (McClean *et al.* 2006). However freezing spermatozoa with dimethylacetamide instead of glycerol resulted in only a small improvement, with post-thaw motility reaching approximately 13% (McClean *et al.* 2008b).

There is currently only one published study regarding the cryopreservation of spermatozoa from dasyurid marsupials. This preliminary investigation examined the spermatozoa of one individual *S. crassicaudata* and reports low motility (3%) using 8% glycerol and 7.5% egg yolk when spermatozoa were frozen rapidly in liquid nitrogen vapour (Taggart *et al.* 1996). Clearly, in order to establish sperm cryopreservation protocols for genome resource banking as a tool to conserve dasyurid marsupials, a systematic examination of the tolerance of dasyurid spermatozoa to cryoprotectants and varied freezing protocols is warranted.

1.6.5 Freeze Drying Spermatozoa

An alternative to sperm cryopreservation is freeze drying. Storage of freeze dried spermatozoa has the advantage that samples can be maintained at room temperature and do not need to be stored in a large tank containing liquid nitrogen which is difficult to transport, requires continual maintenance and can be dangerous (Hoshi *et al.* 1994; Kawase *et al.* 2005; Dinnyes *et al.* 2007). However freeze dried sperm nuclei and lipid bilayers may potentially be harmed by free radicals formed following exposure to solar radiation, and spermatozoa which contain a high proportion of unsaturated fatty acids are particularly susceptible to such damage (White 1993; Potts 1994; Puhlev *et al.* 2001; Kawase *et al.* 2005; Lee and Niwa 2006).

Freeze drying desiccates cells in a vacuum environment after rapid freezing by the process of sublimation (Mellor 1978; Hoshi *et al.* 1994) and subsequently samples are stored in an oxygen free environment to avoid oxidative damage (Hoshi *et al.* 1994; Wakayama and Yanagimachi 1998). The total removal of intercellular water allows cells to tolerate temperature changes and preserves protein function (Keilin 1959; Holt 1997). This does not occur in cryopreserved cells as a small amount of water remains associated with membranes and proteins (Crowe *et al.* 1990). Desiccated spermatozoa are not motile and have permeabilised membranes, hence are not traditionally viable or useful for AI or IVF. However oocytes can still be fertilised by ICSI (Wakayama and Yanagimachi 1998; Kwon *et al.* 2004; Liu *et al.* 2004) and to date, live young from mice, rats and rabbits have been born following fertilisation with freeze dried spermatozoa (Wakayama and Yanagimachi 1998; Liu *et al.* 2004; Hirabayashi *et al.* 2005).

There have not been any studies into freeze drying marsupial spermatozoa and reports of freeze drying in non-domestic species are limited to a study on fish spermatozoa (Poleo *et al.* 2005). In marsupials the likelihood of success may be limited by the lack of nuclear stabilisation (see section 1.5.1.1) because the ability of eutherian spermatozoa to fertilise an oocyte after freeze drying is related to the presence of nuclear disulphide bonds. Kaneko *et al.* (2003) demonstrated that immature murine spermatozoa which are not disulphide stabilised are only capable of fertilizing an oocyte if treated with a substance that creates disulphide bonds prior to freeze drying. The protection from light and oxygen is also highly important in marsupials as the lipid membranes in spermatozoa from the eastern grey kangaroo (*Macropus giganteus*), *P. cinereus* and *V. ursinus* contain a comparatively high proportion of unsaturated fatty acids which are hypothesised to be highly vulnerable to oxidative damage via lipid peroxidation (White 1993; Miller *et al.* 2004).

1.6.6 Conclusion to Sperm Preservation in Dasyurids

Sperm cryopreservation is a complex process with two major areas for manipulation- the choice and concentration of cryoprotectants and the rate of freezing. Although the spermatozoa of some marsupials tolerate almost 20% glycerol (Rodger *et al.* 1991), Taggart *et al.* (1996) suggests that *S. crassicaudata* is intolerant of glycerol concentrations above 8%. The potential for cold shock is dismissed in *S. crassicaudata* as the spermatozoa remain viable at 4 ºC when still inside the scrotal sack (Taggart *et al.* 1996) however there is yet to be any systematic assessment of cold shock in dasyurids which is consistent with the methodology described by Mann (1964). Despite the wide discussion of fundamental sperm biology in several dasyurids (Harding *et al.* 1982; Retief *et al.* 1995a), sperm cryopreservation has been almost exclusively limited to the single study on *S. crassicaudata* with the exception of a current project examining sperm cooling in *S. harrisii* (Taggart *et al.* 1996; Keeley 2008). Clearly a systematic assessment of cold shock, cryoprotectants, freezing protocols and alternative preservation techniques such as freeze drying are required to make progress in the field of dasyurid sperm preservation and enable the establishment of genome resource banking for the conservation of threatened dasyurid marsupials.

1.7 FEMALE GAMETE BIOLOGY

The ability to understand the female reproductive system is also fundamental to the success of ART. For example, understanding the timing of ovulation allows AI to be carried out during the post-ovulatory period (Holt *et al.* 1996; Paris *et al.* 2005; Molinia *et al.* 2007) and the collection of a large cohort of mature oocytes is essential to the progress of ICSI (West *et al.* 2007) and IVF, which is possible in American didelphids (Rodger and Bedford 1982b; Taggart *et al.* 1993) but has had little success in the Australian marsupials- with the exception of *T. vulpecula* (Mate *et al.* 2000b; Sidhu *et al.* 2003). It is also important to include the female gamete in genome resource banks which are of little value if they exclusively contain spermatozoa. An understanding of ovarian biology is essential to underpin the development of oocyte preservation techniques. This section will review the development and maturation of female gametes, examining both eutherians and marsupials. Special reference will be made to the literature describing dasyurids, and how the unique features of dasyurid oocytes may impact ART. Although the majority of dasyurid studies will describe reproduction in the small dasyurids such as *A. stuartii*, *S. crassicaudata* and *S. macroura,* there are also several large and extremely relevant studies from the early 1900s describing fundamental ovarian biology in the eastern quoll, *D. viverrinus* (Sandes 1903; Hill 1910; O'Donoghue 1911; O'Donoghue 1912; Hill and O'Donoghue 1913; Hall 1994).

1.7.1 Oocyte and Follicle Development

Mammalian oocytes begin as primordial germ cells which migrate from the hindgut to populate the gonad where they undergo several rounds of mitosis before becoming primordial oocytes capable of meiosis (reviewed in Byskov and Hoyer 1994). In the developing oocyte, meiosis is disrupted by two periods of arrest and the initial period of arrest occurs in dictyate of the first meiotic prophase during follicle growth when the oocyte has a prominent germinal vesicle and is diploid. Just prior to ovulation the germinal vesicle breaks down and one set of chromosomes is extruded in the first polar body (Brambell 1928; reviewed in Wassarman and Albertini 1994). The oocyte arrests again during metaphase II until sperm binding and activation after which the oocyte ejects the second polar body (Brambell 1928; Motlík and Kubelka 1990).

Figure 1-4 shows that eutherian primordial oocytes are surrounded by flat pre-granulosa cells to form a primordial follicle (Brambell 1928). As the follicle develops it becomes surrounded by a single layer of cuboidal granulosa cells creating a primary follicle (Brambell 1928; reviewed in Greenwald and Roy 1994). At this stage many follicles are not recruited by hormonal cues (see section 1.7.3) and become atretic. In those which are recruited the zona pellucida is secreted and thickens upon development into a secondary follicle which is defined by two or more layers of granulosa cells (Léveillé *et al.* 1987; reviewed in Greenwald and Roy 1994). When fluid filled follicular vesicles coalesce and form an antrum, a tertiary follicle has developed (Greenwald and Roy 1994). Eutherian oocytes inside tertiary follicles no longer increase in size, and are fully grown with a diameter of 70 µm in mice and 160 µm in humans, but the follicle itself continues to grow larger and this developmental pattern is termed biphasic growth (Brambell 1928; Bertrand *et al.* 1995).

Oocyte and follicle development in marsupials is fundamentally similar to that described for eutherians (Sandes 1903; Hinds *et al.* 1996), but the timetable of events differs because sexual differentiation of the gonads does not occur until after birth (Ullmann 1981; Eckery *et al.* 1996; Kress 1996). However if emergence from the marsupial pouch is aligned with birth in eutherians then the timetable of oogenesis is comparable (Alcorn and Robinson 1983). Initial studies suggested marsupial follicles followed the biphasic growth pattern, as observed in eutherians (Lintern-Moore *et al.* 1976). However, subsequent studies in phalangerid, macropodid, didelphid and dasyurid marsupials have shown that the oocyte continues to grow, albeit slowly, until the periovulatory period (Giles 1990; Falconnier and Kress 1992; Rodger *et al.* 1992b; Moritz *et al.* 1998; Kress *et al.* 2001). This developmental pattern has been termed monophasic growth (Rodger *et al.* 1992b).

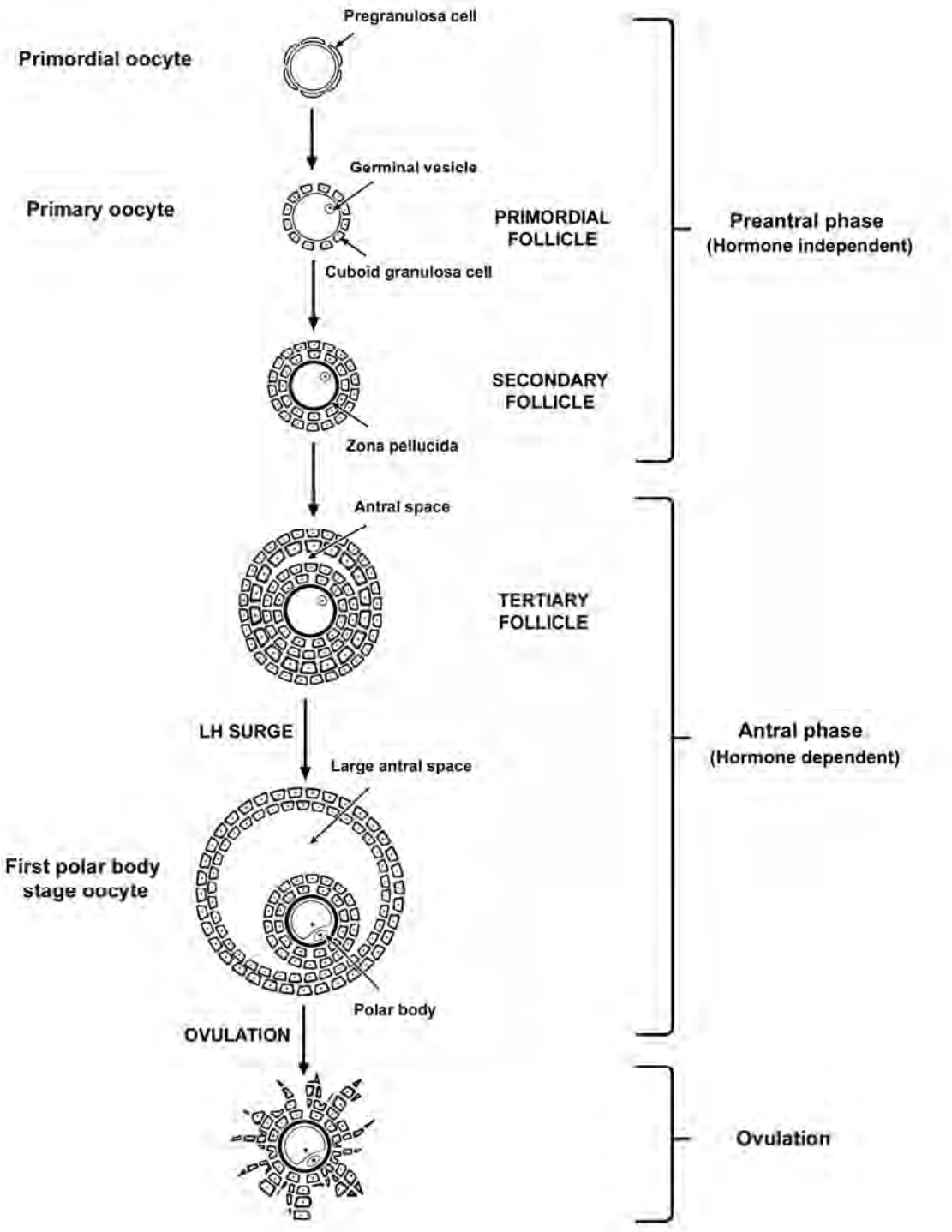

Figure 1-4: Schematic representation of the development of an oocyte and follicle in a eutherian mammal.

1.7.2 Oocyte Maturation and Ovulation

Just prior to ovulation, a eutherian oocyte contained within a large antral follicle resumes meiosis to progress from prophase I to metaphase II, undergoing germinal vesicle breakdown and extruding the first polar body (Motlík and Kubelka 1990; Wassarman and Albertini 1994). However maturation events also occur within the cytoplasm and involve changes to the physical properties of the oocyte surface and the development and pre-ovulatory migration of cortical granules to the cortical cytoplasm underlying the oolemma (Hyttel *et al.* 1986; Wassarman and Albertini 1994). Ovulated oocytes have a thick zona pellucida of up to 17.5 µm in humans and they are surrounded by an expanded cumulus cell layer (Talbot and DiCarlantonio 1984; Bertrand *et al.* 1995).

Although the timing of germinal vesicle break down is similar in marsupial oocytes there are several major differences between eutherian and marsupial oocytes at this stage of development. In *T. vulpecula*, *M. eugenii* and *M. domestica* but not *S. crassicaudata*, cytoplasmic maturation may be a post-ovulatory process as the peripherally situated cortical granules do not become obvious until after ovulation (Breed and Leigh 1990; Falconnier and Kress 1992; Mate *et al.* 1992; Mate 1996). Marsupial oocytes are also considerably larger and in *D. viverrinus* mature oocytes have a diameter of 240 µm (Hill 1910). The zona pellucida is also thinner in marsupial oocytes, having a width of 8 µm in *M. eugenii* and 2 µm in *D. viverrinus* (Hill 1910; Breed and Leigh 1988; Mate 1998). Furthermore the expanded cumulus cell layer is never established around oocytes which are instead ovulated naked (Phillips and Fadem 1987; Breed and Leigh 1988). The ovulated marsupial oocyte is rapidly surrounded by the mucoid coat and shell layer secreted by the oviduct and uterotubal junction, respectively. In *D. virginiana, D. viverrinus* and *M. domestica* a thick mucoid coat surrounds the oocyte even before the spermatozoon is fully incorporated into the egg (Hill 1910; Rodger and Bedford 1982a; Phillips and Fadem 1987) and these layers are suggested to provide a block to polyspermy (Hartman 1916; Breed and Leigh 1988; Jungnickel 1999).

1.7.3 Endocrinology

Oocyte growth and development is largely controlled by cyclic changes regulated by the endocrine system. The main hormones of the reproductive cycle are follicle stimulating hormone (FSH), luteinising hormone (LH) and oestradiol (reviewed in Greenwald and Roy 1994). In both eutherians and marsupials FSH and LH are released from the anterior pituitary gland in response to pulsatile hypothalamic secretion of gonadotrophin releasing hormone (GnRH). For the majority of the cycle these hormones act on ovarian tissue, and are regulated by a negative feedback system based on oestradiol secretion from the target tissue (reviewed in Short 1972).

The development of preantral follicles is asynchronous and gonadotrophin independent (Panyaniti *et al.* 1985; McNatty *et al.* 1990). Thecal cells, which surround the follicle, possess LH receptors but none are present on the granulosa cells which instead have FSH receptors. When recruitment occurs FSH concentrations increase and the granulosa cells respond by developing LH receptors. At this stage follicle development becomes hormone dependent (Ireland 1987).

During the follicular phase of the reproductive cycle, under the influence of LH, thecal cells convert cholesterol to progesterone which is transported to the granulosa cells which, under the influence of FSH, aromatise it into oestradiol (Adashi 1994). Those oocytes with a lower threshold requirement for FSH are able to produce oestrogens much more efficiently than those with a higher threshold requirement for FSH. This allows a limited number of follicles to become dominant because the oestradiol negatively feeds back to the pituitary and limits the secretion of gonadotrophins, thus causing the less mature follicles to become atretic (Ireland 1987; Adashi 1994)

When the concentration of oestradiol from the developing follicles is sufficiently high, the feedback system changes to positive feedback resulting in a GnRH, and subsequent LH, surge. This initiates ovulation, the progression from prophase I to metaphase II and stimulates the granulosa cells to form a corpus luteum (CL) containing luteal cells which secrete progesterone to dominate the luteal phase (Short 1972; Gemmell 1995). In eutherians the CL, or placenta, is required for the maintenance of pregnancy (Gemmell 1995). The eutherian CL requires hormonal support and its action can be halted with progesterone-based treatments or prostaglandin $F_{2\alpha}$ which is advantageous for herd synchronisation or superovulation (described in further detail in section 1.8.1.1) (Schiewe *et al.* 1991; Gemmell 1995; Bó *et al.* 2002).

1.7.4 Female Marsupial Reproductive Patterns

Reproductive cycles in female marsupials follow two possible patterns, differing in the timing of resumption of follicle growth (Tyndale-Biscoe and Renfree 1987). In dasyurids, phalangerids and didelphids gestation occupies the luteal phase and follicles do not grow until the CL undergoes luteolysis in the non-pregnant female, or lactation ends after pregnancy. The other strategy occurs in most macropods, gestation occupies the luteal phase but oocyte growth resumes towards the end of pregnancy allowing a post-partum oestrus. The embryo formed during post-partum mating enters diapause until the sucking stimulus from the newly born joey is removed. In marsupials the secretory life of the CL is not gonadotrophin dependent hence drugs which end the luteal phase in eutherians are not applicable to marsupials, an understanding of which has been important in the development of ovarian stimulation protocols for marsupials (see section 1.8.1.1) (Hearn 1974; Tyndale-Biscoe *et al.* 1974; Short *et al.* 1985).

1.7.4.1 Female dasyurid reproductive patterns

The female reproductive cycle in dasyurids has been well studied using plasma and faecal hormones, and similar endocrine patters exist in *A. stuartii*, *D. viverrinus*, *D. maculatus*, *D. hallucatus*, *S. harrisii*, *D. byrnei* and *D. geoffroii* (Fletcher 1989; Hinds 1989; Hinds and Selwood 1990; Vanderlely 1991; Stead-Richardson *et al.* 2001; Czarny 2004; Hesterman *et al.* 2008c; Hesterman *et al.* 2008b). The cycle comprises of an intermediate phase with low activity except for a short pro-oestrus increase in progesterone, potentially arising from the atresia of non-ovulated follicles (Sandes 1903). The follicular phase, encompassing oocyte growth, begins when transient peaks of oestradiol occur and ends with ovulation as a result of the oestradiol peak.

The morphology and development of the progesterone secreting CL in *D. viverrinus* is described by Sandes (1903) who reports atrophy of growing follicles in the presence of a CL and no difference in the CL of pregnant and non-pregnant *D. viverrinus*. From an endocrine perspective the luteal phase is progesterone dominated although transient peaks in oestradiol are detected in *D. geoffroii*, *D. maculatus* and *S. harrisii* (Stead-Richardson *et al.* 2001; Hesterman *et al.* 2008b; Hesterman *et al.* 2008c). The gestation period, ranges from 9.8 days in *S. macroura* to 32.5 days in *D. byrnei*, *and* is contained within the luteal phase (Fletcher 1989; Au *et al.* 2008). In *S. macroura* progesterone concentrations peak half way through gestation when embryos implant (Menkhorst *et al.* 2009) and the CL reaches a maximum size of 600 µm during the mid to late luteal phase, stopping secreting progesterone prior to its decrease in size (Woolley 1966; Hinds and Selwood 1990; Selwood and Woolley 1991).

Until recently there were no reports of an endocrine difference between the pregnant and non-pregnant luteal phase in female dasyurids (Fletcher 1989; Hinds 1989). However studies in *S. harrisii* demonstrate the presence of different faecal progesterone metabolites in pregnant females and studies in *S. macroura* suggest progesterone concentrations are correlated with embryo number and that there is a difference in gestation length, hence the luteal phase, and endometrial mitotic index of pregnant and non-pregnant females (Menkhorst and Selwood 2005; Hesterman *et al.* 2008b; Menkhorst *et al.* 2009).

These cyclic endocrine changes are also indicated morphologically by changes in pouch morphology, body weight fluctuations and the pattern of vaginal cornified epithelial cells (described in further detail in section 1.8.1.1.1). These morphological changes are well established for several dasyurids including *D. viverrinus*, *D. byrnei*, *S. macroura* and *S. harrisii* (O'Donoghue 1911; Fletcher 1989; Woolley 1990; Hesterman *et al.* 2008a). As morphological changes represent more immediate signs of the reproductive state they can be used as indicators of oestrus for captive breeding or ovarian stimulation (Woolley 1990; Hickford *et al.* 2001; Hesterman *et al.* 2008a). However their precise association with endocrine events is often not established. For example, although pouch changes indicate periods of sexual receptivity in *S. harrisii* (Hesterman and Jones 2009), this type of monitoring is less useful for ART as it does not indicate the timing of ovulation which is essential for establishing AI or harvesting naturally grown oocytes.

1.7.5 Conclusion to Female Reproductive Biology in Dasyurids

Female dasyurids have been the focus of several studies regarding fundamental reproductive biology and the literature contains valuable anatomical studies regarding *D. viverrinus*. Although oogenesis in dasyurids is similar to eutherians, striking differences exist during the periovulatory period as dasyurids have monophasic oocyte growth and ovulate supernumerary naked oocytes with a thin zona. In *S. crassicaudata* up to fifteen first polar body stage oocytes, which are 210 µm in diameter and surrounded by a thin 3.5 µm zona pellucida, are ovulated and become surrounded by a mucoid layer in the oviduct followed by a shell layer secreted by the uterotubal junction (Breed and Leigh 1988; Roberts and Breed 1996). Although not specifically investigated in *S. crassicaudata*, studies on other dasyurids demonstrate a consistent endocrine profile typified by a pro-oestrus progesterone rise, an oestradiol dominated follicular phase and a CL derived progesterone dominated luteal phase which contains the gestation period. Morphological changes follow these endocrine cycles and can be used as non-invasive indicators of behavioural oestrus.

Knowledge of the fundamental reproductive biology of both model dasyurids and *D. viverrinus*, a species now considered to be threatened, will be essential to the success of programs establishing ART for threatened dasyurids. It is now important to take the findings of these fundamental studies and transform them into practical methods to improve captive breeding or develop dasyurid specific ART.

1.8 OVARIAN ASSISTED REPRODUCTIVE TECHNIQUES

In order to utilise spermatozoa which has been preserved, female ART such as *in vitro* oocyte maturation are required (Hodges 2003). Female ART also includes the synchronisation of females for AI (Bó *et al.* 2002), ovarian stimulation to encourage reproductive cyclicity in anoestrus females (Druery *et al.* 2007), the harvest and culture of immature follicles for developmental research (Eppig and Telfer 1993), *in vivo* oocyte growth through xenotransplantation (reviewed in Paris *et al.* 2004) and maturation of large numbers of oocytes for embryo transfer, IVF or ICSI (Donoghue *et al.* 1990; Loskutoff *et al.* 1990). The majority of ovarian ART have been developed in eutherian agricultural and laboratory species and adapted to closely related wildlife before being used on more diverse species, including marsupials (Loskutoff *et al.* 1995; Molinia *et al.* 1998a; Bó *et al.* 2002).

1.8.1 Ovarian Stimulation in Marsupials

Ovarian stimulation can be achieved with equine serum gonadotrophin (eSG), also called pregnant mare serum gonadotrophin (PMSG), a glycoprotein comprised of two inactive subunits (α and β) which, when combined, have the effect of both FSH and LH (Papkoff 1974; Greenwald and Roy 1994). This compound has a long half life necessitating only a single dose which is of benefit when working with stress prone species, but a limitation is that it can cause ovarian overstimulation (McIntosh *et al.* 1975; Rodger and Mate 1988; Greenwald and Roy 1994). Ovarian stimulation can also be achieved with several doses of FSH to support follicle development followed by LH to induce ovulation (Schiewe *et al.* 1991). These protocols are less likely to result in overstimulation and may provide more control over stimulation, but a limitation is that they also require regular drug administration and as a result are less practical (Molinia *et al.* 1998b; Magarey and Mate 2003).

Ovarian stimulation is a well studied field in monovular marsupials because it stops the selection of one dominant follicle, increasing oocyte yield. In polyovular marsupials ovarian stimulation allows cycle manipulation and the collection of larger numbers of oocytes. However ovarian stimulation is also important in marsupials because it allows precisely timed ovulation (Hinds *et al.* 1996). This is essential to marsupial AI studies as oocytes become surrounded by a mucoid and shell coat, stopping sperm penetration, soon after ovulation (see section 1.7.2) (Hill 1910; Hartman 1916). Thus there is only a small window of opportunity in which AI can be achieved, and to date AI has only been successful in those marsupial species where the precise timing of ovulation was known (Johnston *et al.* 2003; Paris *et al.* 2005; Molinia *et al.* 2007; Allen *et al.* 2008; Rodger *et al.* 2009). One exception to this general rule was a creative study describing ICSI in post-ovulatory, shell coated, *M. eugenii* oocytes where a small section of the shell was microdissected to allow passage of the microinjection pipette (Richings 2004; Richings *et al.* 2004).

In the polyovular *M. domestica* eSG successfully initiated superovulation when followed three days later by repetitive doses of gonadotrophin releasing hormone (GnRH or LHRH) (Mate 1992). In dasyurids eSG has also been used with varied success for ovarian stimulation and this is described in detail in section 1.8.1.1. In the monovular *L. latifrons* and *T. vulpecula* ovarian stimulation has been achieved with eSG and LH during the breeding season (Glazier 1999; McDonald *et al.* 2006; Molinia *et al.* 2007). However eSG does not provide adequate ovarian stimulation to *M. eugenii*, nor *T. vulpecula* or *L. latifrons*, during the non-breeding season (Rodger *et al.* 1993; Glazier 1998; Molinia *et al.* 1998b; Glazier *et al.* 2002). Instead repeated doses of the short acting FSH provide superior results. In *M. eugenii* ovarian stimulation was achieved after eight doses of FSH every twelve hours followed by a single dose of LH (Molinia *et al.* 1998b). This resulted in increased oocyte yield, allowing further ART studies, and overcame the issue of seasonal anoestrus (Molinia *et al.* 1998b; Magarey and Mate 2003). The same protocol was also used to induce ovulation in anoestrus *T. vulpecula* with a significant improvement in the number of females responding and the number of oocytes harvested (Glazier and Molinia 2002). Nevertheless this protocol did not successfully stimulate the ovaries of immature or anoestrus *V. ursinus* (West *et al.* 2007) although a fourteen dose FSH protocol was successful in producing an increased number of growing follicles and oocytes with greater maturity in the closely related *L. latifrons* (Druery *et al.* 2007).

1.8.1.1 Ovarian stimulation in dasyurids

Very little work has been done on ovarian stimulation in dasyurids outside of the Sminthopsis genus. In one study *D. byrnei* were given up to 20 IU eSG daily for ten days followed by human chorionic gonadotrophin (hCG) on day 11. This resulted in the development of follicles in anoestrus females and subsequent ovulation, but only one female gave birth (Fletcher 1983). In *D. hallucatus* seven daily doses of LH releasing hormone (LHRH) with eSG on day five resulted in increased plasma progesterone, enlarged uteri and follicular development, but no mating occurred (Vanderlely 1991).

Ovarian stimulation has been the focus of several studies in *S. crassicaudata*. An early study examined the effect of 20 IU eSG on the 15 g *S. crassicaudata* and report ovulation and mating behavior, however the high eSG dose resulted in overstimulation and most oocytes did not progress beyond the oviduct (Smith and Godfrey 1970). When doses of 2.5 to 20 IU eSG were followed two days later with 2.5 to 5 IU hCG no improvement was seen (Smith and Godfrey 1970). Subsequent studies examined 5 IU and 1.5 IU eSG and still observed overstimulation (M Smola and JC Rodger, unpublished observations) but when the dose was reduced to 1 IU and hCG was excluded *S. crassicaudata* females ovulated 5 to 6 days later and one litter was born (Rodger *et al.* 1992a). This suggested that the increasing hormone concentrations were sufficient to generate an endogenous LH surge (Papkoff 1974). Although this study reports uterine embryos and the birth of a litter, the reliability of the protocol was limited and not all females responded (Rodger *et al.* 1992a). Despite this variability, *S. crassicaudata* embryos have been produced at an acceptable rate for carrying out morphological studies, however this variability is unlikely to be appropriate for ART particularly in threatened species (Anderson and Breed 1993; Breed and Leigh 1996).

To date dasyurid ovarian stimulation has been best studied in *S. macroura*. In this species follicle growth and ovulation can be stimulated in cycling and non-cycling females when treated with either a single dose of 1.3 to 2 IU of eSG, or two doses five days apart showing again that an endogenous LH surge was generated (Hickford *et al.* 2001). However the ovarian response and timing of ovulation was improved if LH was administered four days following the initial eSG stimulation (Maleszewski and Selwood 2004). This protocol was made more accurate by Menkhorst *et al.* (2007) who used 20 IU eSG at day 0 and day 4 to successfully induce ovulation. Despite this success there was a high proportion of atretic and luteinised follicles, which was perhaps not surprising given that a previous study had shown that doses as low as 2 IU caused negative effects akin to nausea in the 25 g *S. macroura* (Hickford *et al.* 2001; Menkhorst *et al.* 2007).

Fundamental to the success of ovarian stimulation in *S. macroura* has been the avoidance of CL which persist though the majority of the luteal phase and in marsupials do not regress when the female is treated with exogenous hormones (Tyndale-Biscoe *et al.* 1974). Hickford *et al.* (2001) demonstrated that the *S. macroura* ovarian stimulation protocols did not work during the mid-luteal phase of the cycle when the CL is fully functional. Thus it is likely that the variation in the response to stimulation in *S. crassicaudata* has been due to CL effects.

1.8.1.1.1 Cornified epithelial cell (CEC) monitoring

In order to avoid the CL the patterns of vaginal cornified epithelial cells (CEC) can be examined to establish the day of oestrus and hence estimate the duration of the luteal phase. The increased circulating oestradiol during the late follicular phase results in changes to the epithelial cells in the vagina causing them to mature, lose their nuclei and slough off into the vaginal lumen (de Brux 1958; Junqueira *et al.* 1989). Following an increased presence of CEC in urine there is an influx of leukocytes which signal ovulation in several dasyurids (Selwood and Woolley 1991). CEC assessment is used in eutherians by swabbing the vagina but in marsupials it can be achieved in a non-invasive manner as female marsupials have a combined urogenital tract (Pearson and De Bavay 1951). This anatomy means that during oestrus, non-invasively collected urine samples contain the shed CEC which can be used to monitor the reproductive status of females (Godfrey 1969b). This technique has been used in successfully in *S. macroura* ovarian stimulation protocols to avoid the CL (Hickford *et al.* 2001; Menkhorst *et al.* 2007).

In dasyurids, monitoring the pattern of CEC and leukocytes is a well established tool, but the relationship between CEC and ovulation or mating differs slightly in each species. For example, *S. macroura* mates when urine contains high CEC with ovulation occurring just prior to the influx of leukocytes (Godfrey 1969b; Woolley 1990; Selwood and Woolley 1991). In *A. stuartii* mating also occurs when CEC scores are high, but ovulation occurs when urinary CEC decrease (Selwood 1980). Although *S. crassicaudata* mates when urine contains high CEC and ovulation occurs once CEC are declining the precise timing of ovulation in relation to CEC patterns is yet to be established (Godfrey and Crowcroft 1971; Bennett *et al.* 1979; Selwood 1987). Understanding this relationship will be an important step in developing *S. crassicaudata* as a model for ART in more threatened larger dasyurids.

1.8.2 *In Vitro* Oocyte Maturation

Marsupial oocytes collected for IVF or ICSI need to be harvested from pre-ovulatory antral follicles because those collected following ovulation will be surrounded by mucoid and shell layers which stop sperm binding and make sperm injection difficult (Hill 1910; Hartman 1916; Richings 2004). Following ovarian stimulation with exogenous hormones, oocytes within antral follicles may require *in vitro* maturation to reach the first polar body stage before they are able to be used for IVF or ICSI.

In eutherians, if antral follicles have already been exposed to the LH surge *in vivo* they resume meiosis and mature upon collection without additional hormonal support (Pincus and Enzmann 1935; Ayalon *et al.* 1972; Motlík and Kubelka 1990; Eppig *et al.* 1996). However follicles harvested prior to the LH surge require endocrine support in order to mature. Techniques to achieve this are well developed in laboratory species (Tsafriri *et al.* 1972) and some wildlife (Johnston *et al.* 1991). However in other species such as canids *in vitro* maturation remains difficult (Songsasen and Wildt 2007).

Oocytes from LH exposed follicles will spontaneously mature *in vitro* in *M. domestica* (Selwood and VandeBerg 1992) but in other marsupials the ovarian stimulation protocol can affect the potential for maturation. Oocytes collected from *M. eugenii* stimulated with FSH required exogenous LH and the presence of granulosa cells to mature (Mate and Buist 1999). However those collected from eSG stimulated *T. vulpecula* and *M. eugenii* were capable of maturation in the absence of exogenous hormones (Mate and Rodger 1993a; Glazier *et al.* 2002). In *V. ursinus* stimulated with FSH and LH, oocytes from antral follicles were cultured with FSH and LH supplements, but only 15% had matured within 24 hours (West *et al.* 2007).

Despite the large amount of literature regarding *in vivo* oocyte maturation (Breed and Leigh 1988; Kress *et al.* 2001) and several ovarian stimulation studies (Rodger *et al.* 1992a; Hickford *et al.* 2001; Menkhorst *et al.* 2007) there has been little examination of *in vitro* oocyte maturation in dasyurids. In *S. macroura* half of the oocytes collected from eSG stimulated females lost their granulosa cells *in vitro*, only 8% reached germinal vesicle breakdown and only half of those extruded the first polar body (Merry *et al.* 1995). Subsequently eSG stimulated *S. macroura* oocytes were cultured for 24 hours with LH supplements and 60% matured to the polar body stage (Maleszewski and Selwood 2004). There have been no studies regarding the *in vitro* maturation of oocytes from *S. crassicaudata*, nor any of the larger dasyurid marsupials. Establishing rigorous *in vitro* maturation protocols will be essential to the establishment of assisted fertilisation techniques which are especially important given the potential difficulties faced in the preservation of dasyurid spermatozoa (described in section 1.6.6.).

1.8.3 Oocyte Preservation

In addition to ovarian stimulation and oocyte maturation, preservation of oocytes can effectively make use of opportunistically available gametes from female animals which are deceased. Although preservation of large eutherian oocytes is possible at the germinal vesicle stage (Otoi *et al.* 1995), and after extrusion of the first polar body (Eroglu *et al.* 1998; Lucena *et al.* 2006), a large proportion of oocytes are non-viable or suffer the effects of meiotic spindle depolymerisation. Alternatively the oocytes from small immature preantral follicles can be preserved. This is advantageous because preantral follicles are numerous in the ovary and present throughout the reproductive cycle (Shaw *et al.* 2000). Younger oocytes are also capable of surviving an ischemic environment better than more mature oocytes because they have a lower metabolic rate, hence they are likely to be of better quality when collected post-mortem (Gosden *et al.* 1994; Nugent *et al.* 1997; Cleary *et al.* 2001). The low surface area to volume ratio of smaller oocytes is also advantageous for rapid diffusion of cryoprotectants (Shaw *et al.* 2000), and this is even more poignant when examining the large yolky oocytes of dasyurid marsupials (Hill 1910; Breed and Leigh 1988). The concurrent lack of a zona pellucida and absence of a temperature sensitive meiotic spindle also makes immature oocytes more likely to survive freezing (Vincent *et al.* 1990; Eroglu *et al.* 1998). These factors encourage the preservation of preantral follicles and indicate that it is an essential component of the efforts to establish ART for dasyurid marsupials. But before genome resource banking of preantral follicles becomes an accepted conservation tool, methods for the collection, preservation and post-preservation culture of dasyurid ovarian follicles need to be developed.

1.8.3.1 The harvesting of preantral ovarian follicles

Ovarian follicles can be isolated by either collagenase based enzymatic disruption (Eppig 1976) or mechanical methods (Nayudu and Osborn 1992; Spears *et al.* 1994). Enzymatic dissociation was established in hamsters and early studies demonstrated only 14% immediate post-dissociation atresia (Roy and Greenwald 1985). The method is rapid with a large yield and enables the collection of very small primordial follicles (Telfer 1996) in the human (Martinez-Madrid *et al.* 2004), mouse (Eppig and Telfer 1993), rat (Daniel *et al.* 1989) and dog (Bolamba *et al.* 2002). It is important to understand that enzymatically harvested follicles are considered cumulus cell-oocyte complexes instead of intact follicles as they lack the basement membrane and thecal cells of a normal follicle (Eppig 1994; Eppig *et al.* 1996).

Despite the ease of enzymatic dissociation, mechanical dissection is used in cats (Jewgenow and Göritz 1995), sheep (Cecconi *et al.* 1999), rabbits (Nicosia *et al.* 1975), cows and pigs (Telfer 1996) as these species have a high proportion of fibrous interstitial tissue within their ovaries or are sensitive to the toxic effects of the enzymatic treatment (Nicosia *et al.* 1975; Telfer 1996). However mechanical dissection of follicles gives a lower yield (Figueiredo *et al.* 1993; Telfer 1996) and the follicles can suffer physical stress from mechanical handling (Figueiredo *et al.* 1994). In cats this results in approximately 50% non-viable oocytes (Jewgenow and Göritz 1995). It is also virtually impossible to mechanically isolate primordial follicles or primary follicles smaller than 100 µm (Nayudu and Osborn 1992; Telfer 1996).

Mechanical isolation of secondary follicles had been achieved in *M. eugenii* (Richings *et al.* 2006) and preantral follicles were mechanically dissected in *S. macroura* (Nation and Selwood 2005) and *M. domestica* (Butcher and Ullmann 1996). There are yet to be any studies regarding enzymatic dissociation in marsupials. However the description of limited collagen based connective tissues in the ovaries of the red tailed phascogale (*Phascogale calura*) indicates that enzymatic dissociation is likely to be appropriate for dasyurid marsupials (Thomas 2006).

1.8.3.2 The preservation of immature oocytes

The concepts of cryopreservation in reference to spermatozoa have already been described in section 1.6.1. Oocytes and ovarian tissue slices from laboratory species, humans and even marsupials can be frozen using standard cryopreservation protocols involving the use of a programmable freezer or a passive cooling device which cools at 1 ºC minute^{-1} when placed in a -80 ºC environment (Parrott 1960; Carroll *et al.* 1990; Cleary *et al.* 2003; Donnez *et al.* 2004). Initially success was limited, possibly due to the long equilibration times in cryoprotectant and glycerol toxicity (Deanesly 1954; Green *et al.* 1956; Parrott 1960; Candy *et al.* 1995). However current protocols which use DMSO as a cryoprotectant demonstrate high success, resulting in only small differences between oocyte morphology and the potential for growth and development in fresh and freeze thawed ovarian slices (Candy *et al.* 1995; Liu *et al.* 2002).

Oocytes and embryos from humans, laboratory and agricultural species can also be preserved more rapidly using vitrification protocols (Vajta *et al.* 1998; Vajta 2000; Lucena *et al.* 2006; Yamada *et al.* 2007). Vitrification is an alternative freezing method which involves the use of highly concentrated cryoprotective solutions and an extremely fast cooling rate achieved by plunging samples directly into LN (Nawroth *et al.* 2002; Fowler and Toner 2005). Vitrification creates a highly viscous cytoplasm, limiting molecular mobility thus halting diffusion across lipid bilayers and metabolic processes (Isachenko *et al.* 2003; Fowler and Toner 2005). The high viscosity also means that no ice crystals form and instead a glass-like solid phase is reached upon freezing. Vitrification is rapid and does not necessarily require expensive equipment. But disadvantages of the technique include the toxicity of highly concentrated cryoprotectants, the creation of an extremely large osmotic gradient and the potential for semi-thawing to occur during storage which could allow the formation of ice crystals (Nawroth *et al.* 2002; Isachenko *et al.* 2003; Fowler and Toner 2005).

In marsupials the ovarian tissue of several species has been cryopreserved using slow cooling protocols and subsequent growth has required the use of xenotransplantation. Acceptable survival of oocytes cryopreserved in a passive cooling device was reported in *V. ursinus* (Cleary *et al.* 2003; Cleary *et al.* 2004) and *L. latifrons* and *M. eugenii* oocytes have been cryopreserved with a programmable slow cooling device (Wolvekamp *et al.* 2001; Mattiske *et al.* 2002). Dasyurid ovarian slices have also been cryopreserved, using a programmable slow cooling device in *S. crassicaudata* (Shaw *et al.* 1996) or a passive cooling device in *P. calura* (Thomas 2006). These studies, working with slim slices of ovarian tissue, suggest that a portion of dasyurid oocytes survive cryopreservation. However the relatively new technique of vitrification of individual follicles has not yet been examined in any marsupial. Establishment of a robust protocol for the preservation of individual oocytes which can then be cultured *in vitro*, instead of requiring xenotransplantation, is a priority for efforts to develop economical and broadly available genome resource banking for dasyurids.

1.8.3.3 The growth of preantral follicles

Following preservation, immature oocytes must grow and undergo maturation before they can be used for IVF or ICSI. Growth can occur *in vivo* if tissue pieces are auto- or xenotransplanted and this technology is progressing well in wildlife although it is costly and highly technical (reviewed in Paris *et al.* 2004). In the marmoset (*Callithrix jacchus*) and domestic cat large antral follicles have been grown from tissues transplanted into mice (Candy *et al.* 1995; Bosch *et al.* 2004) and ovarian tissue from the African elephant (*Loxodonta africana*) can produce large antral follicles which secrete sufficient hormones to initiate normal vaginal cytology in mice- however these oocytes have abnormal morphology (Gunasena *et al.* 1998). There has also been significant research into xenografting marsupial tissue. In *V. ursinus* ovarian tissue was transplanted into mice and oocytes were harvested from antral follicles, however *in vitro* maturation protocols failed to stimulate nuclear maturation (Cleary *et al.* 2003). Studies in *M. eugenii* have also demonstrated the development of antral follicles from ovarian tissue transplanted to mice (Mattiske *et al.* 2002). Preliminary investigations have been reported for *S. crassicaudata*, where grafted ovarian slices become established in mice and show normal ovarian development (Shaw *et al.* 1996), and *P. calura* where secondary follicles were observed in freeze-thawed ovarian slices transplanted into mice (Thomas 2006).

Immature oocytes can also be cultured *in vitro* under carefully determined conditions (Eppig and Telfer 1993). When culturing enzymatically harvested preantral follicles it is important to maintain contact between the oocyte and surrounding granulosa cells to prevent the formation of a granulosa cell monolayer on the culture dish. The oocyte-granulosa cell connection is essential as granulosa cell cytoplasmic processes communicate with the oocyte via gap junctions (Anderson and Albertini 1976; Motta *et al.* 1994). This connection is essential for continued growth and maturation. If naked primordial oocytes are grown on a granulosa cell layer they do not grow normally (Eppig 1979). However the presence of granulosa cells, but not fibroblastic monolayers, in the culture system does confer partial benefit (Bachvarova *et al.* 1980; Herlands and Schultz 1984).

In order to restrict the migration of granulosa cells away from the oocyte during *in vitro* culture, follicles must be grown on, or within, a substrate such as agar or collagen (Eppig *et al.* 1996; Telfer 1996) or on native or collagen impregnated tissue culture inserts or pre-prepared granulosa cell monolayers (Eppig and Schroeder 1989; Jewgenow and Göritz 1995; Bolamba *et al.* 2002; Adam *et al.* 2004). Such tools are not required in the culture of mechanically isolated follicles which maintain the basal lamina and do not lose the connection between the granulosa cells and the oocyte when attachment to the culture dish occurs (Figueiredo *et al.* 1994; Cortvrindt *et al.* 1996).

Successful growth following by development and fertilisation of *in vitro* grown preantral follicles has been achieved in mice, rats and cows (Daniel *et al.* 1989; Eppig and Schroeder 1989; Hirao *et al.* 2004). But similar success has not been achieved in marsupials. Mechanically harvested preantral follicles from *M. domestica* increase in size but do not develop an antrum when cultured with FSH (Butcher and Ullmann 1996). In *M. eugenii* mechanically harvested secondary follicles increase in size but do not form an antrum and degenerate after four days in culture (Richings *et al.* 2006). In dasyurids, mechanically harvested *S. macroura* follicles which were grown using a roller culture technique had limited degradation but again no antrum formation occurred (Nation and Selwood 2005).

1.8.4 Conclusion to Female ART in Dasyurids

Despite the large body of literature describing dasyurid ovarian biology there has been limited progress in dasyurid ART. The best studied area is ovarian stimulation where protocols for *S. crassicaudata* have had partial success (Rodger *et al.* 1992a). However a series of studies in *S. macroura*, which avoid stimulating females during the luteal phase, provide an insight into methods to improve *S. crassicaudata* ovarian stimulation (Hickford *et al.* 2001; Menkhorst *et al.* 2007). There have been only a few investigations into dasyurid oocyte *in vitro* maturation but the literature regarding other species suggests that eSG stimulated oocytes will mature spontaneously if exposed to the LH surge (Mate and Rodger 1993a; Glazier *et al.* 2002). To make use of opportunistically available gametes from deceased dasyurids, ovarian preservation can be carried out, and cryopreservation of ovarian slices has been described briefly in *S. crassicaudata* (Shaw *et al.* 1996). But there have been no studies describing the collection of preantral follicles using enzymatic dissociation, preservation of individual oocytes using vitrification or the *in vitro* culture of enzymatically isolated marsupial follicles. The establishment of oocyte preservation protocols and associated ART including ovarian stimulation are important steps which would significantly contribute to the efforts towards developing ART for the conservation of dasyurid marsupials.

1.9 SCOPE OF THIS THESIS

There are at present no effective controls for the threatening processes facing dasyurids. The migration of the introduced the cane toad is likely to continue (Sutherst *et al.* 1996; Burnett 1997) and the only current method being investigated for mitigating Tasmanian Devil Facial Tumour Disease is a suppression trial where diseased individuals are euthanised to stop the spread of the disease (Jones *et al.* 2007; McCallum 2008). In light of the limitations of *in situ* conservation, *ex situ* captive breeding has been initiated. But captive breeding is costly, especially when housing large numbers of carnivores, and individuals are exposed to selection pressures exerted by the captive environment which may not be valuable when they are eventually returned to the wild (Snyder *et al.* 1996).

ART, especially genome resource banking, provides an additional form of insurance for the conservation of dasyurid marsupials. This chapter has highlighted the solid background of fundamental reproductive biology in dasyurids but a paucity of studies focused on reliable techniques that can be used for ART. To date the tools available for dasyurid ART include: a sperm cryopreservation protocol in *S. crassicaudata* which results in 3% motile spermatozoa (Taggart *et al.* 1996); ovarian stimulation protocols in which are effective but highly variable in *S. crassicaudata* (Rodger *et al.* 1992a) or induce overstimulation in *S. macroura* (Menkhorst *et al.* 2007); a brief explanation of ovarian freezing and xenotransplantation in *S. crassicaudata* (Shaw *et al.* 1996); and morphological studies on embryo culture in *A. stuartii*, *S. crassicaudata* and *S. macroura* (Selwood 1980; Selwood 1987). In male dasyurids are no studies examining acrosomal stability and no systematic investigations of the tolerance of dasyurid spermatozoa to cryoprotectants, low temperatures or alternative freezing protocols. In female dasyurids inconsistent ovarian stimulation protocols are followed by *in vitro* maturation protocols which have been largely unsuccessful and there have been only extremely limited studies into protocols which would allow long term gamete banking, and the subsequent use, of dasyurid oocytes.

This thesis proposes to focus and extend the knowledge from the literature describing fundamental reproductive biology in dasyurids and advance dasyurid in ART for the purpose of conservation of threatened dasyurid marsupials. This will be achieved by concentrating on male and female gamete preservation for the purpose of genome resource banking and the development of a reliable protocol for ovarian stimulation to facilitate future ART such as AI, IVF and ICSI.

These advances will be achieved by examining both fundamental reproductive mechanisms and ART and will systematically investigate:

- If the dasyurid acrosome is stabilised or fragile, to determine if it can be used as an indicator of post-preservation damage (*Chapter 2*).
- Optimal protocols for dasyurid sperm preservation using conventional sperm freezing and the alternative strategy of freeze drying, to establish a reliable method of long term gamete banking for the preservation of biodiversity in threatened male dasyurids (*Chapter 3*).
- The potential for cold shock in dasyurid spermatozoa to help understand the problems encountered with rapid cooling protocols and contribute to current understanding of low temperature marsupial sperm biology (*Chapter 4*).
- The development of reliable high yield ovarian stimulation and *in vitro* maturation protocols to underpin the development of post-preservation techniques such as AI, IVF and ICSI (*Chapter 5*).
- The potential for cryostorage and *in vitro* culture of individual ovarian follicles to establish a reliable method of long term gamete banking, and post-preservation techniques, for the preservation of biodiversity in threatened female dasyurids (*Chapter 6*).

In addition to these major aims, four addendum studies were conducted and the preliminary findings from these describe:

- Acrosomal stability in an additional dasyurid (*Addendum to Chapter 2*).
- A pilot study describing preliminary development of ICSI (*Addendum to Chapter 3*).
- The use of ovarian dissociation in an additional dasyurid and a macropod (*Addendum 1 to Chapter 6*).
- A pilot study to determine the most effective system for *in vitro* culture of enzymatically dissociated follicles (*Addendum 2 to Chapter 6*).

CHAPTER 2

Acrosome stability in the spermatozoa of dasyurid marsupials

PUBLISHED ARTICLE

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We K. E. Mate and J. C. Rodger attest that the Research Higher Degree candidate Natasha Czarny had an integral role in the experimental design, carried out the research, analysed the data and prepared the publication entitled “Acrosome stability in the spermatozoa of dasyurid marsupials”. We consulted and advised on the experimental design, interpretation of results and preparation of the manuscript.

Name……..

Signature……... Date……

Name……..

Signature……... Date……

Candidate signature…….(Natasha Czarny) Date……

CHAPTER 2: Acrosome stability in the spermatozoa of dasyurid marsupials

2.1 INTRODUCTION

Dasyurids are carnivorous, or insectivorous, marsupials found in Australian and New Guinea. There are currently 18 species of dasyurid marsupials listed as Threatened (Department of the Environment and Heritage 1999) and this study forms part of a program seeking to explore the potential for assisted reproductive techniques (ART) as a conservation tool for threatened dasyurids using the fat tailed dunnart (*Sminthopsis crassicaudata*) as an experimental model.

To achieve assisted reproduction a sound understanding of specific reproductive events is necessary. The acrosome is a critical sperm organelle fundamental to successful fertilisation in eutherians (Eddy and O'Brien 1994; Yanagimachi 1994) but the role of the marsupial acrosome in fertilisation is less well understood (reviewed in Mate *et al.* 2000a). Dasyurid spermatozoa observed on the zona surface maintain intact acrosomal membranes and those seen penetrating the layer have undergone partial acrosomal vesiculation (Breed 1994a). Furthermore during marsupial fertilisation, sperm entry into the oocyte leaves a large hole in the zona pellucida- presumably caused by enzymatic activity resulting from the acrosome reaction (Breed and Leigh 1990; Taggart *et al.* 1993; Jungnickel *et al.* 2000; Sidhu *et al.* 2003)

In eutherians the acrosome is a fragile structure suffering breakdown following sperm cooling (Simpson *et al.* 1987), freezing (Oettlé 1986) and detergent treatment (Yanagimachi 1975). Acrosomal breakdown also causes leakage of hydrolytic enzymes and this can be used as a subtle marker for sperm damage in post-thaw viability assays (Froman *et al.* 1987; Schenk and Amann 1987). In contrast, the acrosome of several marsupials studied to date is unusually stable, withstanding cryopreservation without cryoprotectant, dehydration and detergent treatment (Cummins 1980; Mate and Rodger 1991; Sistina *et al.* 1993a). Furthermore the marsupial acrosome reaction is not induced artificially by calcium ionophore as in eutherians (Green 1978; Shams-Borhan and Harrison 1981; Mate and Rodger 1991). But acrosome loss, with the ultrastructural features of an acrosome reaction (Barros *et al.* 1967), can be induced in marsupials by treatment with the phosphoinositol pathway intermediate, diacylglycerol (DiC_8)- although this requires a higher concentration than that used to cause the acrosome reaction in eutherian species (Sistina *et al.* 1993b).

The apparent strength of the marsupial acrosome has been attributed to the presence of stabilising disulphide bonds in the acrosomal matrix and membranes at the light (fluorescent thiol staining- (Mate *et al.* 1994)) and electron (direct monomaleimido-nanogold staining (Lin *et al.* 1995)) microscope level in the Tammar wallaby (*Macropus eugenii*) and the brush tailed possum (*Trichosurus vulpecula*). The presence of these stabilising bonds may be correlated with progress towards successful cryopreservation in these species (Molinia and Rodger 1996; Holt *et al.* 1999; McClean *et al.* 2007) and this is currently being assessed by our laboratory. Cryopreservation is not yet possible in *S. crassicaudata* (Taggart *et al.* 1996) despite preliminary studies which demonstrate that dasyurid spermatozoa have a surprisingly high tolerance to cryoprotectants (Czarny *et al.* 2007)

The current study is the first to investigate acrosomal stability of dasyurid spermatozoa by examining the response to physical (air drying, freeze thawing or detergent treatment) and chemical (exposure to calcium ionophore or DiC_8) challenges.

2.2 METHODS

2.2.1 Husbandry

S. crassicaudata were sourced from the small marsupial facility at the University of Newcastle (Australia) and originated from the long established colony at the University of Adelaide (Australia). Animals were housed in bachelor groups of up to five males in opaque polypropylene boxes (420 mm x 280 mm x 160 mm) with a sawdust substrate, plastic and metal shelters and paper to build nests. They had *ad. libitium* access to food (IAMS chicken adult cat food, Dayton, OH, USA) and water via dripper bottles and plastic bowls. The animals were exposed to a 16 hours light, 8 hours dark light cycle to promote constant breeding (Smith *et al.* 1978; Bennett *et al.* 1990).

Eastern quolls (*Dasyurus viverrinus*) were housed at the University of Newcastle within indoor pens (1.8 x 4.6 m) on a cement floor covered with tee-tree mulch, gum leaves, hay and logs. Animals were housed individually or in male female pairs and fed daily raw meat, bones, eggs or whole mice. There was a constant supply of dry cat food (Whiskas, Mars Incorporated, McLean, VA, USA) and water was provided *ad. libitium*. Animals were exposed to 10 hour light: 14 hour dark light cycles and were examined during the southern hemisphere winter breeding season.

The use of protected native species was licensed by New South Wales National Parks and Wildlife Service (Australia) and all experiments were approved by Newcastle University Animal Care and Ethics Committee.

2.2.2 Semen Collection

Adult male *S. crassicaudata* were euthanised by CO_2 inhalation and testes were immediately dissected and wrapped in saline dampened gauze. As marsupial spermatozoa do not suffer cold shock (Rodger and White 1978; Taggart *et al.* 1996) testes were stored on ice for up 30 to 60 min before processing. The epididymis was removed and approximately 4 mm of the caudal epididymidis was placed in 400 µL of dasyurid sperm medium comprised of phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA) supplemented with 4% (v/v) fetal calf serum (FCS) (Trace Biosciences, Castle Hill, NSW, Australia) at 35 °C. Preliminary studies determined this to be an optimal medium as spermatozoa incubated an alternative base medium (HEPES buffered DMEM- Sigma-Aldrich, St Louis, MO, USA) showed a reduction in motility and appeared attached to the base of the culture dish after three hours. The addition of 4% FCS to PBS resulted in an increased motility of some samples and was used in subsequent experiments.

Spermatozoa from each individual epididymis were "swum out" for 30 min before initial motility was assessed by eye on a heated stage using an Axiovert 35 inverted microscope (Zeiss, Jena, Germany). Sperm concentration was determined for samples fixed in 4% (w/v) paraformaldehyde (PFA) (Probing and Structure, Thuringowa, QLD, Australia) and counted using a haemocytometer (Improved Neubauer, Bad Mergentheim, Germany). Triton-X experiments used ~70 x 10^3 spermatozoa mL^{-1} and the remainder of the study used ~12 x 10^4 spermatozoa mL^{-1}.

Dasyurid spermatozoa are not highly motile when compared with the spermatozoa of eutherians or other marsupials (Taggart and Temple-Smith 1990b). The present study only included samples with greater than 40% motility- this is the average motility of spermatozoa collected from *S. crassicaudata*. In dasyurids, unlike other marsupial species, the motility and viability of spermatozoa is highly variable both between, and within, individuals. The mechanisms affecting this, and indicators predicting sperm quality, are currently being investigated by our laboratory.

D. viverrinus were euthanised by intra-cardiac injection of sodium pentobarbitone (Lethabarb, Laser Animal Health, Salisbury, SA, Australia) whilst anesthetised with 4% isolfuorane (Virbac Animal Health, Peakhurst, NSW, Australia). Testes were removed and spermatozoa were processed as described above with the volume of sperm medium taking into consideration the increased size of the animal. Triton-X experiments used ~90 x 10^3 spermatozoa mL^{-1} and the remainder of the study used ~14 x 10^4 spermatozoa mL^{-1}.

Opportunistically collected spermatozoa were available from ten reproductively active northern quolls (*Dasyurus hallucatus*) supplied by Territory Wildlife Park (Berry Springs, NT, Australia). Testicular tissue was transported wrapped in saline dampened gauze on ice and spermatozoa were collected as described above with the volume of sperm medium taking into consideration the increased size of the animal. Triton-X experiments used 75×10^3 spermatozoa mL^{-1} and the remainder of the study used 16×10^4 spermatozoa mL^{-1}.

Electroejaculated or epididymal spermatozoa were collected from adult *M. eugenii* as a positive control. Electroejaculations and swim-up of epididymal spermatozoa into PBS were performed as described by Mate and Rodger (1991). Spermatozoa were centrifuged to pellet (Hettich Zentrifugen EBA12, Tuttlingen, Germany) (500 *g*; 5 min) then resuspended in 35 °C PBS + 4% FCS. On some occasions spermatozoa were mixed with a protease inhibitor cocktail (Sigma-Aldrich) for another study (Harris and Rodger 2005). Neither the collection method nor the addition of protease inhibitors affected the outcome of experiments. Triton-X experiments used ~15×10^5 spermatozoa mL^{-1} and the remainder of the study used ~38×10^6 spermatozoa mL^{-1}.

2.2.3 Air Drying

The sperm sample was split into two aliquots; one was immediately fixed in PFA and concentrated onto slides by "cytospin" centrifugation (Mate and Rodger 1991) (Shandon Inc., Pittsburgh, PA, USA) (200 *g*, 3 min). The other was loaded into cytospin vessels without fixation. The slides were air dried before staining with Bryan's stain (Bryan 1970)- eosin Y, flavanic acid, fast acid green (all 0.1% w/v, Sigma-Aldrich) in 1% (v/v) acetic acid (Fronine, Riverstone, NSW, Australia) for 3 to 6 min then mounted in DePex (Gurr, Poole, UK).

2.2.4 Freezing without Cryoprotection

The sperm sample was split into two 50 µL aliquots. One was immediately fixed in PFA and the other was immersed in liquid nitrogen for 3 min then thawed by continuous agitation in a 35 °C water bath and subsequently fixed. Spermatozoa were concentrated and processed with Bryan's stain as described above.

2.2.5 Triton-X

Spermatozoa were mixed 1:1 with 35 °C 0.4 to 0.001% (v/v) Triton-X (Sigma-Aldrich) (final concentration) in sperm medium (control). Immediately after the addition of Triton-X and after 30 min motility was noted and an aliquot of each concentration was fixed in PFA. Samples were concentrated and processed with Bryan's stain as described above.

2.2.6 Calcium Ionophore

Spermatozoa were mixed 1:1 with 35° C 10 to 0.01 µM calcium ionophore (Sigma-Aldrich) initially dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich) and diluted in sperm medium. The control contained 2% (v/v) DMSO in sperm medium- equivalent to the highest concentration treatment. Immediately after the treatments and at 60 and 120 min motility was noted and an aliquot of each concentration was fixed in PFA. Samples were concentrated and processed with Bryan's stain as described above.

2.2.7 DiC_8

Spermatozoa were mixed 1:1 with 100 to 10 µM 35 °C 1,2-dioctanoyl-*sn* glycerol (DiC_8) (Sigma-Aldrich) dissolved in DMSO and diluted in sperm medium. The control contained 2% (v/v) DMSO in sperm medium- equivalent to the highest concentration treatment. Immediately after the treatments and at 60 and 120 min motility was noted and an aliquot of each concentration was fixed in PFA. Samples were concentrated and processed with Bryan's stain as described above.

2.2.8 Bromobimane

Spermatozoa from all species were fixed in PFA and concentrated using the "cytospin" method as described above. Slides were then treated with 0, 1 or 5 mM of the reducing agent, dithiothreitol (DTT) (Sigma-Aldrich) for 20 min. After washing with PBS slides were stained for 15 min with the fluorescent thiol labelling agents, monobromobimane (mBBr- penetrating) (Calbiochem, La Jolla, Ca, USA) or monobromotrimethylammoniobimane (qBBr- non-penetrating) (Calbiochem). Slides were washed and mounted in Mowiol (Calbiochem) and viewed with a Zeiss Axioplan 2 with fluorescence filter set 2 (exciter filter 365 nm, emission filter 420 nm). Images were captured using an Olympus D970 digital camera (Tokyo, Japan).

2.2.9 Statistical Analysis of Acrosome Loss

For all dasyurid experiments duplicate counts of 50 spermatozoa were made. *M. eugenii* experiments contained sufficient spermatozoa to perform duplicate counts of 100. Spermatozoa were classified as acrosome present if they had a dark acrosomal region or acrosome absent if the sperm head was unstained. Significant differences between the control and treatment were determined by using student's T-tests on square-root transformed data. All experiments were repeated at least three times. Values are presented as mean ± standard error of the mean (SEM).

2.3 RESULTS

2.3.1 Bryan's Staining in Dasyurid Marsupials

Bryan's staining enabled the visualisation of the acrosomal region in all dasyurid species examined. Figure 2-1 shows the pattern of staining in *S. crassicaudata*.

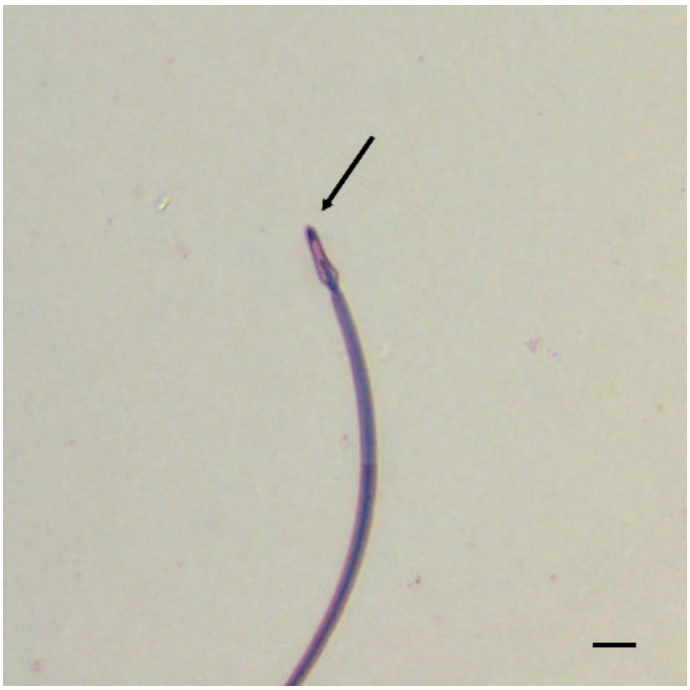

Figure 2-1: Fat tailed dunnart (*Sminthopsis crassicaudata*) spermatozoa stained with Bryan's stain viewed with light microscopy. Note the presence of a stained region at the proximal tip of the streamlined sperm head (arrow). Bar=10 µm.

2.3.2 Effect of Air Drying

Air drying resulted in 63% of *S. crassicaudata* spermatozoa demonstrating acrosome loss- and this is greater than pre-treatment values (Figure 2-2, P<0.01). Post-treatment acrosomal loss was also seen in *D. viverrinus* (P<0.05) and *D. hallucatus* (P<0.01) but there was no significant difference between pre- and post-air dry samples in *M. eugenii* (Figure 2-2). In dasyurid spermatozoa, head decondensation was apparent but decapitation did not occur. Decapitation was common in *M. eugenii* samples.

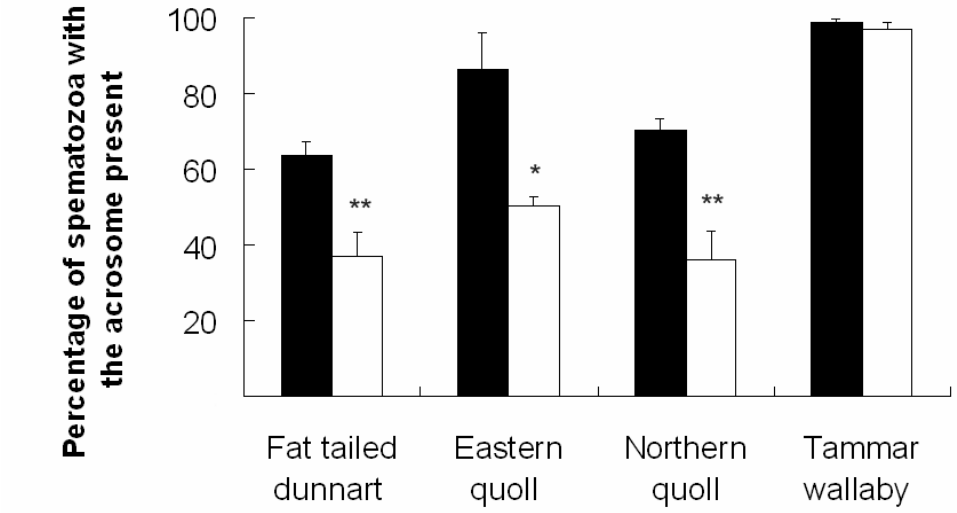

Figure 2-2: Mean percentage (± SEM) of fat tailed dunnart (*Sminthopsis crassicaudata,* n=4), eastern quoll (*Dasyurus viverrinus,* n=3), northern quoll (*Dasyurus hallucatus,* n=3) and Tammar wallaby (*Macropus eugenii,* n=3) spermatozoa with intact acrosomes before (control, filled) and after (unfilled) air drying. * P<0.05 and ** P<0.01.

2.3.3 Effect of Freezing without Cryoprotection

Freeze-thawing resulted in 25% of *S. crassicaudata* spermatozoa loosing their acrosome, and this is greater than pre-treatment values (Figure 2-3, P<0.001). Post-treatment acrosomal loss was also seen in *D. viverrinus* (P<0.05) and the pattern repeated in *D. hallucatus* (P<0.01) but there was no significant difference between control and post-freeze samples in *M. eugenii* (Figure 2-3).

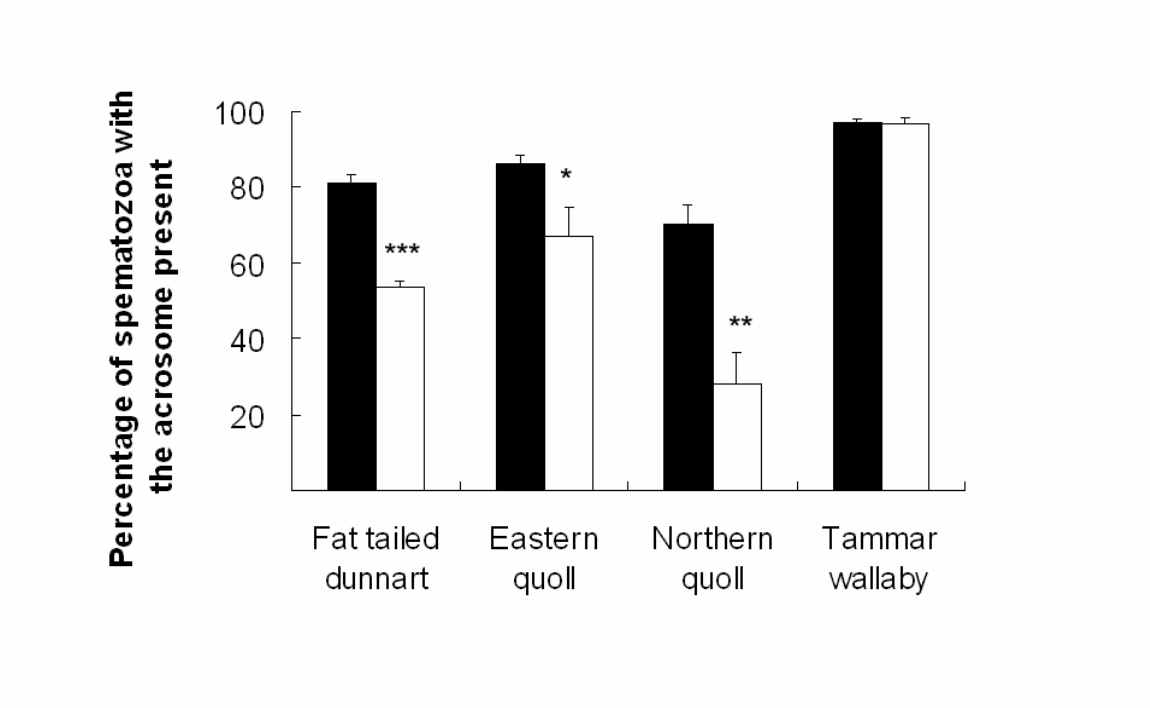

Figure 2-3: Mean percentage (± SEM) of fat tailed dunnart (*Sminthopsis crassicaudata,* n=4), eastern quoll (*Dasyurus viverrinus,* n=4), northern quoll (*Dasyurus hallucatus*, n=3) and Tammar wallaby (*Macropus eugenii,* n=3) spermatozoa with acrosomes intact prior to (control, filled) and following (unfilled) freeze-thawing in the absence of cryoprotectant. * P<0.05, ** P<0.01 and *** P<0.001.

2.3.4 Effect of Triton-X Treatment

In *S. crassicaudata* high concentrations of Triton-X (0.04, 0.02 and 0.01%) reduced the percentage of acrosomes present by up to 40% when assessed immediately (P<0.05) (Figure 2-4). After 30 min reductions (P<0.05) in the percentage of acrosomes present were seen in all but the lowest (0.001% Triton-X) concentration (Figure 2-4). Addition of ≥0.01% Triton-X caused spermatozoa to become immotile immediately but lower concentrations showed no change from control values at either T0 or T30.

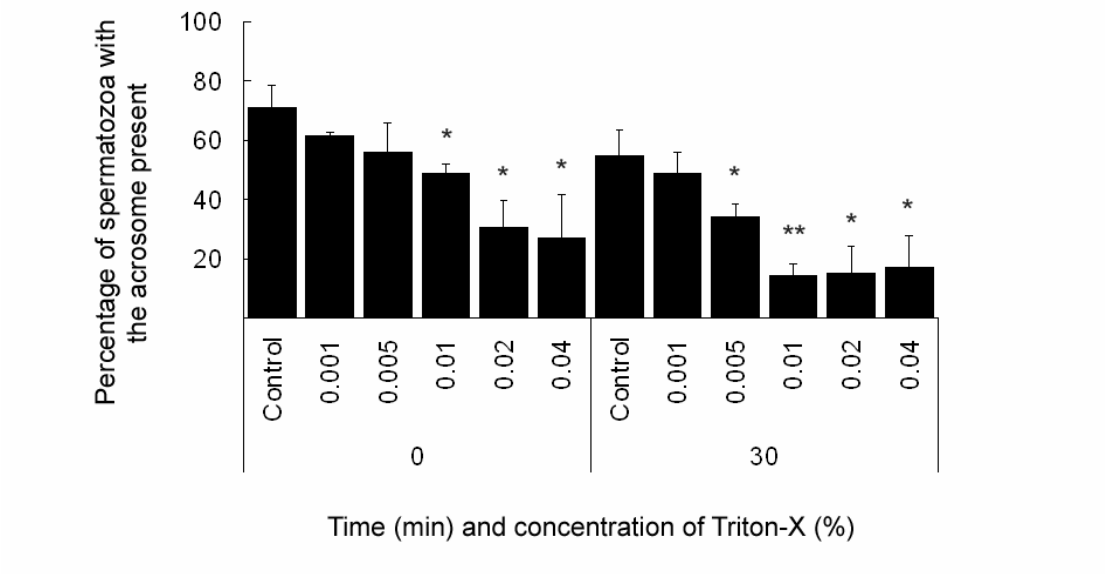

Figure 2-4: Mean percentage (± SEM) of acrosomes present in the spermatozoa of the fat tailed dunnart (*Sminthopsis crassicaudata*) following treatment with 0 (control) to 0.04% Triton-X immediately and at thirty minutes (n=3). * P<0.05 and ** P<0.01.

A similar pattern of sensitivity to Triton-X was seen in both *D. viverrinus* and *D. hallucatus* where an immediate decrease (P<0.05) in the proportion of spermatozoa with the acrosome present was detected at higher concentrations (≥0.01% for *D. viverrinus* and ≥0.02% for *D. hallucatus*) (Figure 2-5). Following the 30 min incubation period mixtures containing all but the lowest concentration of Triton-X (0.001%) resulted in acrosomal loss in both species (P<0.05, Figure 2-5). Spermatozoa from *D. viverrinus* were immediately immotile following treatment with 0.04% Triton-X but concentrations between 0.02 and 0.005% maintained motility, albeit at a reduced rate. In *D. hallucatus* spermatozoa treated with 0.04% Triton-X were again immotile and spermatozoa treated with 0.02% Triton-X showed reduced motility- all remaining treatments demonstrated no variation from control values. Wallaby spermatozoa treated with 0.04% or 0.02% Triton-X immediately demonstrated a reduction in the presence of acrosomes compared to untreated samples (P<0.05). After 30 min acrosomes were absent at 0.04%, 0.02% and 0.01% Triton-X (P<0.001).

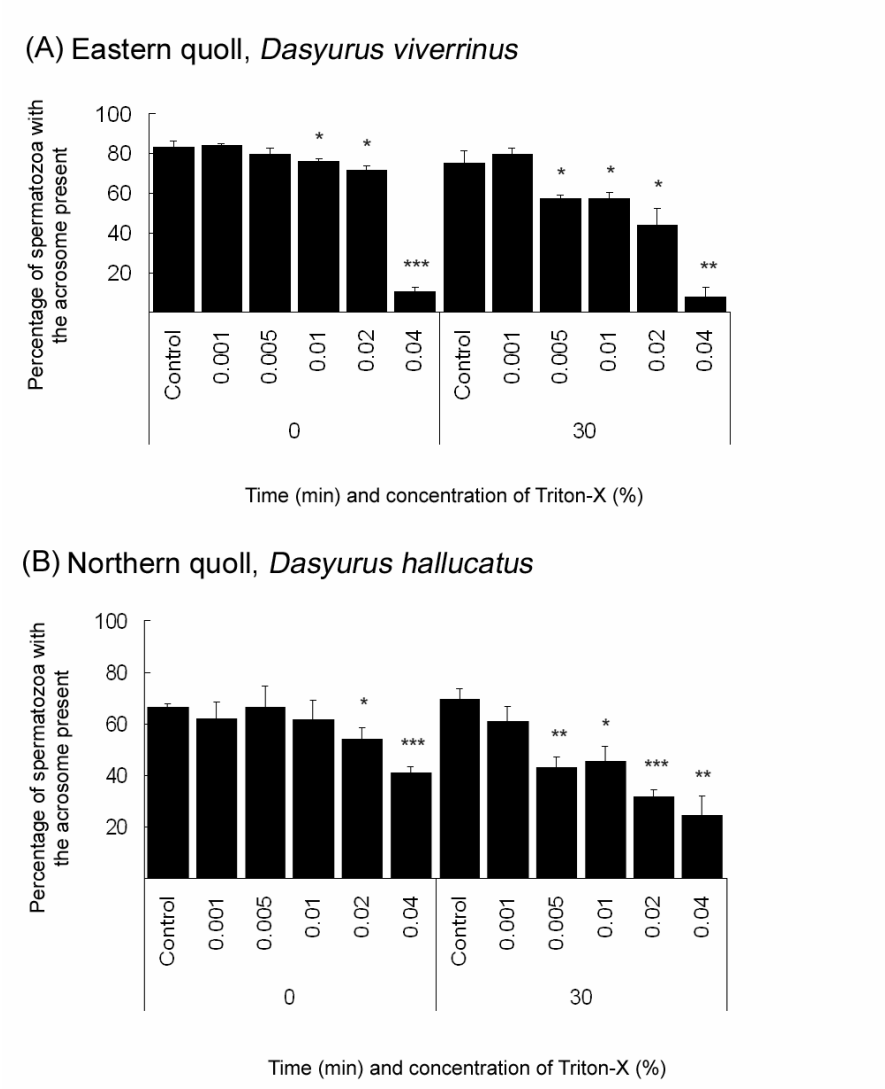

Figure 2-5: Mean percentage (± SEM) of acrosomes present in the spermatozoa of the (A) eastern quoll (*Dasyurus viverrinus*) and the (B) northern quoll (*Dasyurus hallucatus*) following treatment with 0 (control) to 0.04% Triton-X immediately and at thirty minutes (n=3). * P<0.05, ** P<0.01 and *** P<0.001.

2.3.5 Effect of Calcium Ionophore

Treatment of spermatozoa from *S. crassicaudata*, *D. viverrinus*, *D. hallucatus* and *M. eugenii* with concentrations of calcium ionophore up to 10 µM demonstrated no significant declines in the percentage of acrosomes present compared with spermatozoa treated with the control- dilution medium alone (data not shown). The treatment did however affect the motility of spermatozoa of *S. crassicaudata* and *D. viverrinus* which became immotile within 60 min when treated with ≥10 µM calcium ionophore. This pattern was not reflected in *D. hallucatus* where motility was maintained throughout the experiment.

2.3.6 Effect of DiC_8

Treatment of *S. crassicaudata* spermatozoa with 100 µM DiC_8 decreased the percentage of acrosomes present by up to 75% within 60 min (P<0.001) (Figure 2-6). There was no significant difference between control values and 10 µM DiC_8 at T0, T60 or T120. Treatment with 100 µM DiC_8 caused *S. crassicaudata* spermatozoa to become immotile within 60 min on all occasions but lower concentrations had no effect.

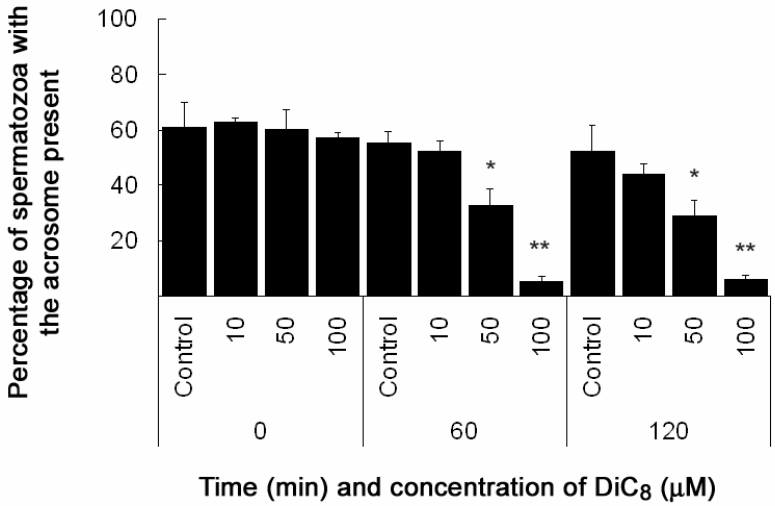

Figure 2-6: Mean percentage (± SEM) of acrosomes present in the spermatozoa of the fat tailed dunnart (*Sminthopsis crassicaudata*) following treatment with 0 (control) to 100 µM 1,2-dioctanoyl-*sn* glycerol (DiC_8) assessed upon addition, 60 and 120 minutes (n=3). * P<0.05 and ** P<0.001.

A decrease in the percentage spermatozoa with the acrosome present was observed one hour following the addition of 100 (P<0.01) and 50 µM (P<0.05) DiC_8 in *D. viverrinus* (Figure 2-7). In *D. hallucatus* the effect of 100 µM DiC_8 is seen earlier with an immediate 60% decrease (P<0.01) in the percentage of spermatozoa with the acrosome present, however the 60 min incubation is required for 50 µM DiC_8 to cause a decrease (P<0.05) in the percentage of spermatozoa with the acrosome present (Figure 2-7). *D. viverrinus* spermatozoa were immotile immediately following treatment with 100 µM DiC_8 and in some cases it was also immotile following 60 min incubation with 50 µM DiC_8. In *D. hallucatus* treatment with 100 µM DiC_8 caused spermatozoa to become immotile within 60 min but lower concentrations had no effect. *M. eugenii* spermatozoa demonstrated similar results with reductions (P<0.001) in the proportion of acrosomes present at 60 min with 100 µM but not 50 µM DiC_8.

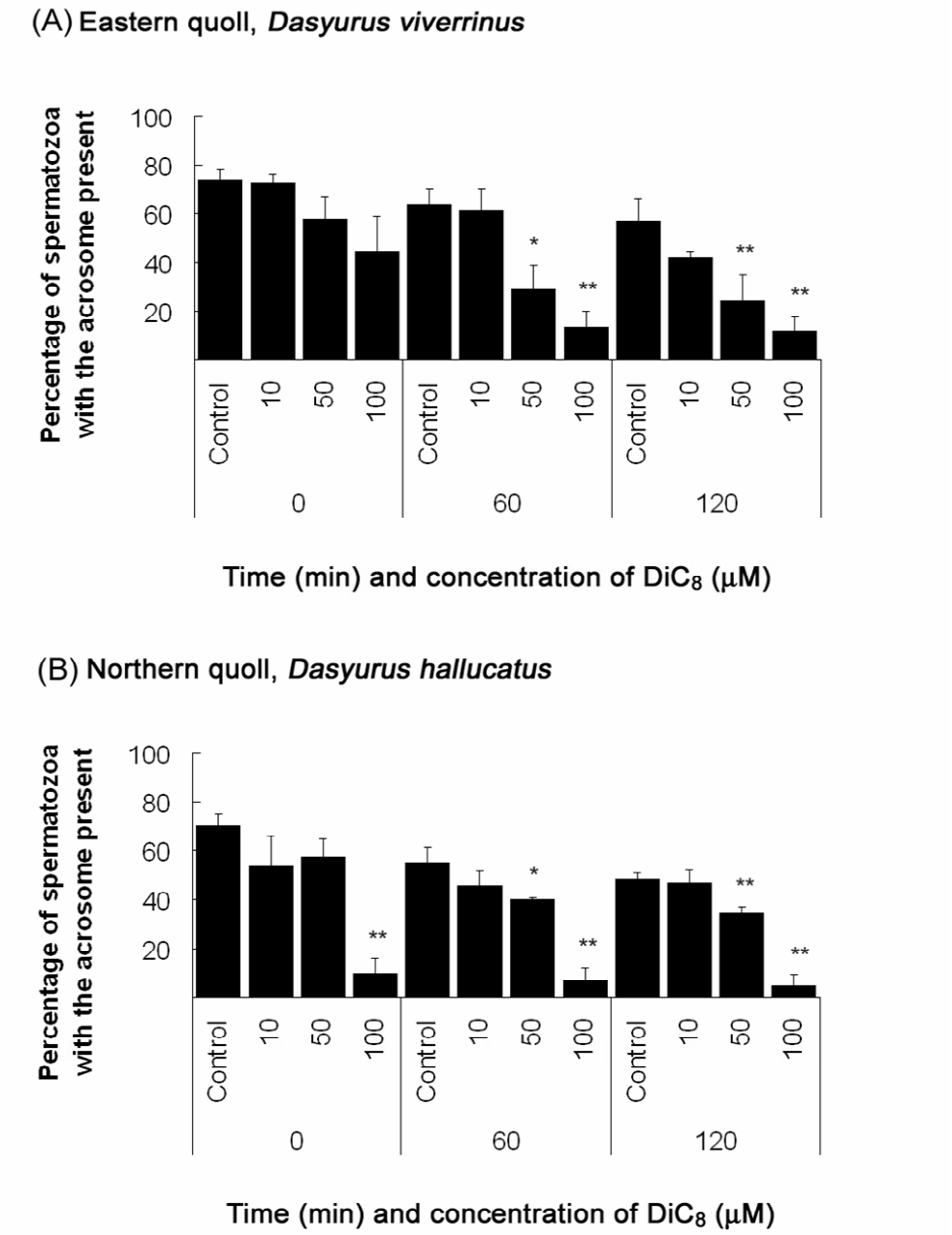

Figure 2-7: Mean percentage (± SEM) of acrosomes present in the spermatozoa of the (A) eastern quoll (*Dasyurus viverrinus*) and the (B) northern quoll (*Dasyurus hallucatus*) following treatment with 0 (control) to 100 μM 1,2-dioctanoyl-*sn* glycerol (DiC_8) assessed upon addition, 60 and 120 minutes (n=3). * $P<0.05$ and ** $P<0.01$.

2.3.7 Bromobimane Staining

No localised acrosomal or nuclear fluorescent staining was observed in the spermatozoa of *S. crassicaudata* (n=5), *D. viverrinus* (n=3) or *D. hallucatus* (n=3) following treatment with either concentration of reducing agent followed by mBBr or qBBr. Diffuse staining of the sperm head was observed, presumably from the overlying plasma membrane (Figure 2-8A to C). Staining patterns did not differ between mBBr and qBBr except that the former stained more brightly. Fluorescent staining on tail region, rich in disulphide bonds, was observed in all samples and the intensity of staining rose with increasing concentrations of DTT. The strong staining tail region extends to the implantation fossa and can be seen through the unstained areas on the sperm head in spermatozoa showing the streamlined morphology (see Figure 2-8B). Observation of *M. eugenii* spermatozoa (n=5) demonstrated localised staining surrounding the acrosomal region; fluorescence was also observed in the tail region and diffusely over the sperm head, as in dasyurids (Figure 2-8D).

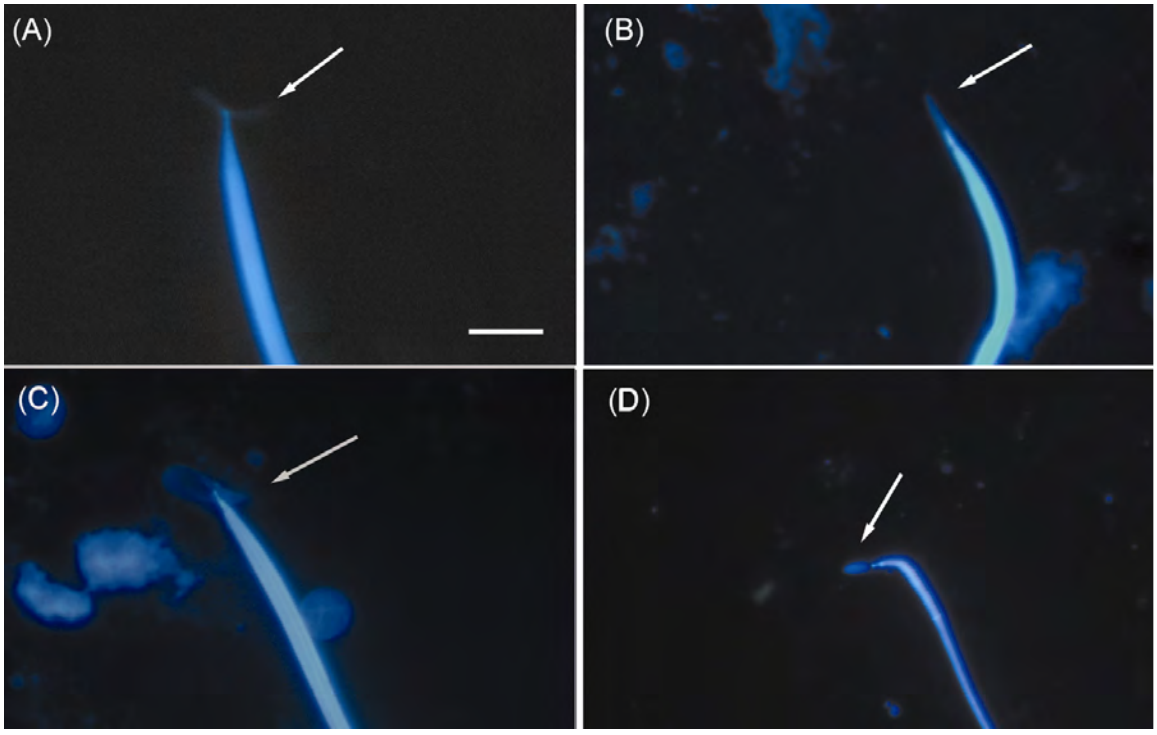

Figure 2-8: Images of spermatozoa treated with dithiothreitol to reduce disulphides to thiols followed by the fluorescent thiol labelling agent monobromobimane for spermatozoa from the (A) fat tailed dunnart (*Sminthopsis crassicaudata*) (B) eastern quoll (*Dasyurus viverrinus*), (C) northern quoll (*Dasyurus hallucatus*) and (D) Tammar wallaby (*Macropus eugenii*). Bar=10 um. In A to C note the absence of localised acrosomal staining in the area indicated by the arrow and the presence of thiol staining along the tail area, in D the arrow indicates localised acrosomal staining.

2.4 DISCUSSION

The acrosome of *S. crassicaudata*, *D. viverrinus* and *D. hallucatus* has been shown to be less stable following physical and chemical challenge than the spermatozoa of Australian macropodid and phalangerid families and the American didelphids. This study demonstrated that air drying and freeze-thawing in the absence of cryoprotectant resulted in incomplete but significant acrosome losses from dasyurid spermatozoa. This differs from the previous results published for macropodid, phalangerid and didelphid marsupials (Cummins 1980; Sistina *et al.* 1993a) and is more consistent with the reaction of eutherian spermatozoa to such treatments (Watson 1979; Oettlé 1986; Simpson *et al.* 1987).

The relative sensitivity of dasyurid spermatozoa is also reflected in the significant loss of motility and acrosomal loss at concentrations ≥0.005% Triton-X after 30 min. Although acrosomal loss was not reported to occur at 0.01% Triton-X in *M. eugenii* (Sistina *et al.* 1993a), the present study demonstrated significant decreases at this concentration as also reported for *T. vulpecula* and eutherian spermatozoa (Yanagimachi 1975; Sistina *et al.* 1993a). However, it is unlikely that these changes are a “true” acrosome reaction but rather cellular degradation in the form of acrosomal vesiculation as shown by Sistina *et al.* (1993a), this is consistent with the loss of motility. A “true” acrosome reaction features the maintenance of sperm motility and multiple point fusions between the outer acrosomal membrane and plasma membrane (Jones 1973; Didion *et al.* 1989; Sistina *et al.* 1993c).

The observed sensitivity of dasyurid spermatozoa seen in the previous experiments may be explained by the lack of stabilising disulphide bonds in the acrosomal matrix as detected by the lack of localised acrosomal fluorescent thiol staining. Most marsupials lack typical eutherian nuclear disulphide stabilisation (Balhorn 1982; Fifis *et al.* 1990; Soon *et al.* 1997) but the acrosomes of *M. eugenii* and *T. vulpecula* are stabilised by disulphide bonds in the matrix and membranes as shown by light (fluorescent thiol staining (Mate *et al.* 1994)) and electron (monomaleimido-nanogold staining (Lin *et al.* 1995)) microscopy. The fluorescent activity in the tail region of *S. crassicaudata* spermatozoa treated with the membrane impermeable qBBr is indicative of the presence of either disulphide bonds or thiols and is consistent with previous studies on *M. eugenii* and *T. vulpecula* (Mate *et al.* 1994). Although we are yet to confirm the lack of disulphide bonds in the acrosomal membranes of dasyurid spermatozoa at the electron level we postulate that the relative instability of dasyurid spermatozoa is due to the absence of both nuclear and acrosomal matrix disulphide bonds.

The present study indicated that dasyurids demonstrate resilience to acrosomal loss following treatment with calcium ionophore. This has also been observed in *M. eugenii*, *T. vulpecula* and they grey short tailed opossum (*Monodelphis domestica*) and conflicts with the well established eutherian model (Russell *et al.* 1979; Shams-Borhan and Harrison 1981; Mate and Rodger 1993b). Furthermore, as seen in previous marsupial studies (Mate and Rodger 1993b; Sistina *et al.* 1993b), when treated with the second messenger phosphoinositol pathway intermediate, diacylglycerol (DiC_8), the acrosome reaction was initiated at a similar range of treatment concentrations. As opposed to the changes seen following air drying, freeze-thawing and Triton-X treatment these changes are likely to represent those due to membrane fusion as described by Sistina *et al.* (1993b). This result supports the concept of Mate and Rodger (1993b) which describe the marsupial acrosome reaction being induced by a mechanism not dependent on extracellular calcium- potentially as a direct effect of DiC_8.

The percentage of acrosomes present in *M. eugenii* spermatozoa were consistent with those previously reported across all experiments (Cummins 1980; Mate and Rodger 1991; Mate and Rodger 1993b; Sistina *et al.* 1993a; Sistina *et al.* 1993b). But in general the dasyurid spermatozoa demonstrated a lower motility than that seen in *M. eugenii*, *T. vulpecula* and *M. domestica* however this is not surprising given past research (Taggart and Temple-Smith 1990b) and the low average motility of spermatozoa from animals within our colony (unpublished data).

This study has been the first to examine acrosomal stability in dasyurids. We demonstrated that *S. crassicaudata*, *D. viverrinus* and *D. hallucatus* spermatozoa lack the acrosomal disulphide stabilisation previously observed in other marsupials which results in greater fragility to physical challenge. We also demonstrated that in dasyurids, as in other marsupials studied, the phosphoinositol pathway intermediates but not calcium ionophore cause acrosomal loss. This lack of acrosomal stability in dasyurids may be associated with the with the poor freeze-thaw recovery (Taggart *et al.* 1996), however it is likely to only be a contributing factor as sperm cryopreservation is successful in a diverse array of eutherian species which also have a fragile acrosome (Penfold and Watson 2001). Another factor could be associated with the large size and asymmetric nature of the cell (Taggart *et al.* 1996).

An alternative strategy allowing the use of preserved dasyurid spermatozoa may be intracytoplasmic sperm injection (ICSI), as it removes the requirement for motile spermatozoa and acrosome reaction induced sperm binding factors which are presumably involved in marsupial fertilisation. Injection of heads from normal spermatozoa into oocytes has lead to fertilisation and early development in a macropod marsupial (*M. eugenii*) (Magarey and Mate 2004; Richings *et al.* 2004). Furthermore frozen spermatozoa are routinely used in human ICSI to treat infertility (Park *et al.* 2003; Meseguer *et al.* 2006) and the technique is also applied to fertilisation in livestock, domestic and laboratory species (Goto *et al.* 1991; Bogliolo *et al.* 2001; Ng *et al.* 2002). The widespread use of ICSI arguably makes this approach worthy of further examination for conservation applications, especially where post-thaw sperm motility is poor.

ADDENDUM TO CHAPTER 2: Acrosome stability and disulphide stabilisation in the red tailed phascogale (*Phascogale calura*)

1 INTRODUCTION

In this chapter the acrosomal biology of *S. crassicaudata*, *D. viverrinus* and *D. hallucatus* has been reported. Additional data regarding some aspects of the acrosomal biology of another species of dasyurid, the red tailed phascogale (*Phascogale calura*) has also been collected. This small study examined opportunistically available tissues from an arboreal dasyurid from the genus Phascogale, and further affirms that the acrosomal traits already described for several dasyurids were consistent in other members of the dasyurid family.

2 METHODS

Opportunistically collected spermatozoa were available from four adult *P. calura* supplied by Alice Springs Desert Park (Alice Springs, NT, Australia). Males were euthanised for population management at the end of their first breeding season, before the annual male die-off. Of the four animals examined, epididymal spermatozoa were present in three animals. This was not unexpected as sperm production is reported to continue throughout the breeding season and up until the period of die-off in the closely related brush tailed phascogale (*Phascogale tapoatafa*)(Millis *et al.* 1999).

Testicular tissue was transported wrapped in saline dampened gauze on ice and received within 24 hours of collection. Spermatozoa were collected as described in the body of this chapter (2.2.2) and used at a concentration of 120×10^3 spermatozoa mL^{-1}. *P. calura* spermatozoa were used in air drying, freezing without cryoprotection and bromobimane labelling experiments which were all carried out as described in the body of this chapter (2.2.3, 2.2.4 and 2.2.8), except that in the bromobimane labelling experiment the reducing agent (DTT) was only used at a concentration of 5 mM.

Duplicate counts of 50 spermatozoa were made and spermatozoa were classified as acrosome present if they had a dark acrosomal region or acrosome absent if the sperm head was unstained. Significant differences between the control and treatment were determined by using student's T-tests on square-root transformed data. Values are presented as mean ± standard error of the mean (SEM).

3 RESULTS

3.1 Bryan's Staining

Bryan's staining enabled the visualisation of the acrosomal region in *P. calura* as shown in Figure 1.

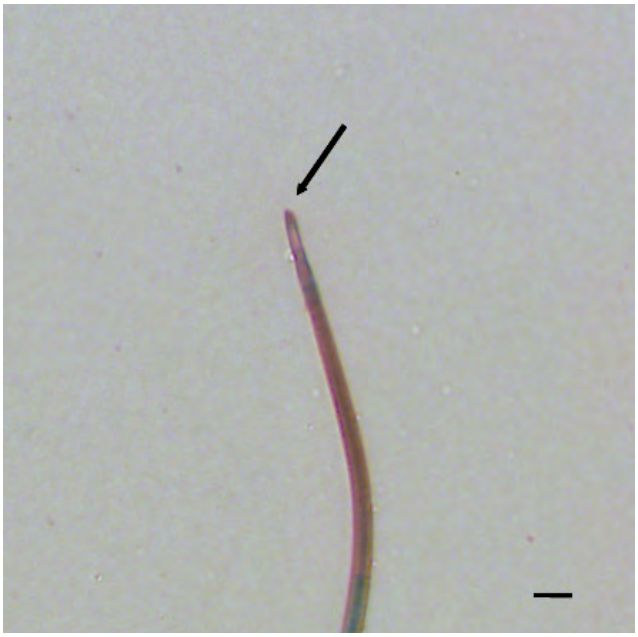

Figure 1: Spermatozoa from the red tailed phascogale (*Phascogale calura*) stained with Bryan's stain and viewed with light microscopy. Note the presence of a stained acrosomal region at the proximal tip of the streamlined sperm head (arrow). Bar=10 μm.

3.2 Effects of Air Drying and Freezing without Cryoprotection

Air drying the spermatozoa of *P. calura* resulted in a decline in the percentage of spermatozoa with the acrosome present ($P<0.01$, Figure 2). Following freeze-thawing without cryoprotection there was a 10% reduction in the proportion of spermatozoa indicating acrosomal loss but this was not significant (Figure 2). No sperm head decapitation was observed in any sample following either treatment.

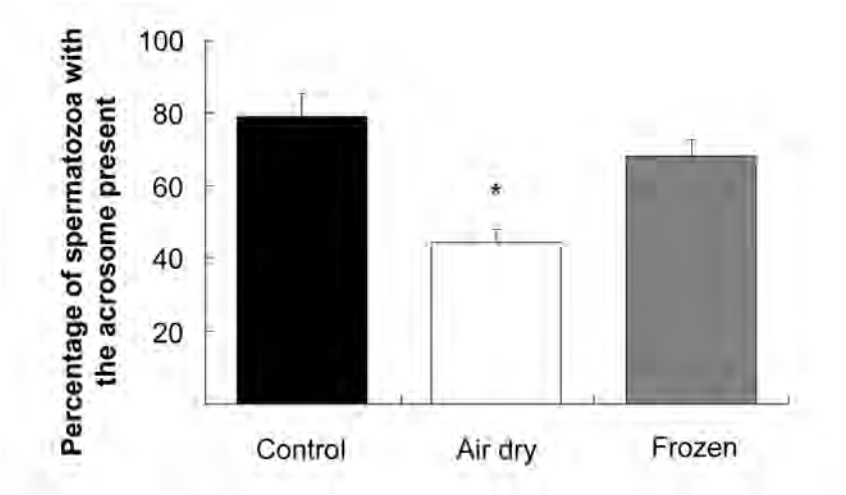

Figure 2: Mean percentage (± SEM) of spermatozoa from the red tailed phascogale (*Phascogale calura*) with intact acrosomes before (control, black) and after air drying (unfilled) or freezing without cryoprotection (grey). *P<0.01.

3.3 Bromobimane Staining

No localised acrosomal or nuclear fluorescent staining was observed in the spermatozoa of *P. calura* following treatment with the penetrating monobromobimane stain (Figure 3) or the non-penetrating monobromotrimethylammoniobimane stain, although the former stained the thiol groups on the sperm tail more brightly.

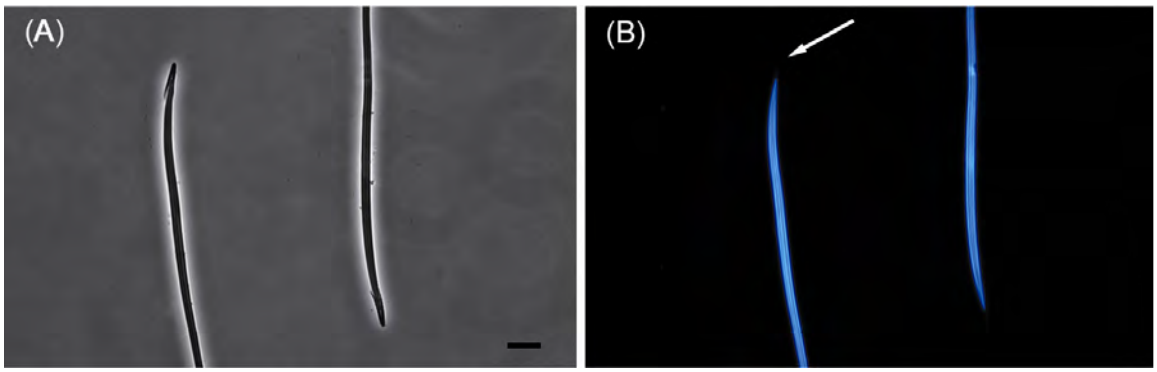

Figure 3: Spermatozoa from the red tailed phascogale (*Phascogale calura*) labeled with the fluorescent thiol labelling agent monobromobimane viewed with (A) light microscopy or (B) fluorescence. Note the absence of localised acrosomal staining in the area indicated by the arrow and the presence of thiol staining along the tail area. Bar=10 um.

4 DISCUSSION

This small study has demonstrated that spermatozoa from another dasyurid species, *P. calura*, has a similar lack of acrosomal stabilisation as that observed in *S. crassicaudata*, *D. viverrinus* and *D. hallucatus* in the body of this chapter. This has also been demonstrated to occur due to a lack of disulphide bonding in the acrosomal membranes, as had been observed in the other dasyurids. These findings differ from published reports regarding acrosomal stability in the spermatozoa of *T. vulpecula*, *M. eugenii* and *M. domestica* which are resilient to physical insult (Mate and Rodger 1991; Mate and Rodger 1993b; Sistina *et al.* 1993a; Sistina *et al.* 1993b; Sistina *et al.* 1993c) because of the presence of localised disulphide stabilisation in the acrosomal membranes and matrix (Mate *et al.* 1994; Lin *et al.* 1995).

This study did however show that *P. calura* spermatozoa were not as fragile as the spermatozoa from other dasyurids as the 10% acrosome loss following freeze-thawing without cryoprotection was not significant. This is a likely artifact of the small sample size of three and the variation between the samples. It is also important to note that the present finding only describes acrosome loss due to physical damage. Future studies could repeat the freeze-thawing experiment with more replicates and elaborate on the practical tests of acrosomal stability by examining the effect of detergent treatment and compounds which induce a true acrosome reaction, as has been described in the other dasyurid species.

The present small study is the first to report acrosomal stability using both practical and morphological tools in any phascogale. This is an important extension to the already described studies on dasyurids and demonstrates that an unstable acrosome is a feature common to dasyurids in the genus Sminthopsis, Dasyurus and Phascogale.

CHAPTER 3

Acrosomal integrity, viability, and DNA damage of sperm from dasyurid marsupials after freezing or freeze drying

PUBLISHED ARTICLE

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We M. S. Harris, G. N. De Iuliis and J. C. Rodger attest that the Research Higher Degree candidate Natasha Czarny had an integral role in the experimental design, carried out the research, analysed the data and prepared the publication entitled "Acrosomal integrity, viability, and DNA damage of sperm from dasyurid marsupials after freezing or freeze drying". We consulted and advised on the experimental design, interpretation of results and preparation of the manuscript.

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Candidate signature…….(Natasha Czarny) Date……

CHAPTER 3: Acrosomal integrity, viability, and DNA damage of sperm from dasyurid marsupials after freezing or freeze drying

3.1 INTRODUCTION

Dasyurids are carnivorous marsupials which play an important role as predators within Australian ecosystems. However many species are threatened by competition and predation from introduced carnivores, habitat fragmentation and disease (Jones *et al.* 2003). Habitat preservation, captive natural breeding and re-introduction into predator free environments are currently important and effective methods of managing some of these conservation problems (Bradley *et al.* 1999). However assisted reproductive techniques (ART), such as sperm preservation and gamete banking, have the additional advantage that they can be used to limit the loss of genetic diversity (Rodger *et al.* 2009).

Dasyurids have long, streamlined spermatozoa (approximately 300 μm in length) and in most species both the nucleus and acrosomal matrix lack disulphide stabilisation (Breed *et al.* 1989; Czarny *et al.* 2008). Lack of nuclear protamines is not unique to dasyurids and is observed in most marsupials (Cummins 1980). However the absence of disulphide stabilisation of the acrosomal matrix in a marsupial is unusual, as robust acrosomes were recorded in the brush tailed possum (*Trichosurus vulpecula*) and Tammar wallaby (*Macropus eugenii*) and had previously been thought to be a feature common to all marsupials (Mate and Rodger 1991; Sistina *et al.* 1993a; Czarny *et al.* 2008). This relative acrosomal instability in dasyurids is similar to that observed in eutherian species (Yanagimachi 1975) and it may have potential as an indicator of morphological disruption following potentially damaging treatments, such as cryopreservation (Oettlé 1986). Previous studies in our laboratory demonstrate this by reporting approximately 25% acrosome loss in spermatozoa from the fat tailed dunnart (*Sminthopsis crassicaudata*) following freezing in the absence of cryoprotectants (Czarny *et al.* 2008).

The spermatozoa of vombatid, peramelid and phalangerid marsupials withstand cryopreservation well (Rodger *et al.* 2009) and in both the common wombat (*Vombatus ursinus)* and *T. vulpecula* post-thaw motility is almost 80% (Taggart *et al.* 1996; MacCallum and Johnston 2005). These studies indicate a surprisingly high tolerance to penetrating cryoprotectants and *T. vulpecula* spermatozoa maintain viability in up to 17.5% glycerol (Molinia and Rodger 1996). Cryopreservation is less successful in the koala (*Phascolarctos cinereus*) with post-thaw motility limited to approximately 50% (Johnston *et al.* 2006). Post-thaw recovery in macropods is very poor with only 10% of

spermatozoa being motile (Holt *et al.* 1999; McClean *et al.* 2006). This may be in part due to the toxicity of glycerol in macropods (McClean *et al.* 2006) and recently post-thaw motility has been improved to approximately 13% using dimethylacetamide instead of glycerol (McClean *et al.* 2008b). Only one published study has examined sperm cryopreservation in a dasyurid marsupial and reports 3% motility following rapid freezing of spermatozoa from one *S. crassicaudata* with 8% glycerol and 7.5% egg yolk (Taggart *et al.* 1996).

Preservation of cells may also be achieved by desiccation which naturally occurs in some fungi, bacteria, insect and plants. Although desiccated spermatozoa are immotile, the advent of intracytoplasmic sperm injection (ICSI) has renewed interest in this technique for sperm preservation of laboratory species (Wakayama and Yanagimachi 1998) and more recently agricultural and companion species (Kwon *et al.* 2004; Liu *et al.* 2004). Freeze drying removes water from cells by the process of sublimation which occurs in a vacuum after freezing to at least -80 °C (Mellor 1978).

This study examined the preservation of spermatozoa from dasyurid marsupials using conventional sperm freezing and the alternative strategy of freeze drying. Our study aims to determine the treatment conditions for optimal sperm preservation by examining the viability, acrosome morphology, head presence and the integrity of the nuclear material upon thawing or rehydration.

3.2 MATERIALS AND METHODS

3.2.1 Husbandry

S. crassicaudata was the model dasyurid used in this study and was sourced from the small marsupial facility at the University of Newcastle (Australia). Animals were housed in male only bachelor groups containing up to five individuals in opaque polypropylene boxes (420 mm x 280 mm x 160 mm) with a recycled paper floor covering, shelters and nesting material. They had *ad. libitium* access to food (IAMS chicken adult cat food, Dayton, OH, USA) and water via dripper bottles and plastic bowls. The animals were exposed to a 16 hour light, 8 hour dark light cycle to promote continuous sperm production. For epididymal sperm collection adult male *S. crassicaudata* were euthanised by CO_2 inhalation and testes were immediately dissected and wrapped in saline dampened gauze. Testes were stored on ice for up to 1 hour before processing- this was shown to have no negative effect on sperm quality in previous studies (Czarny *et al.* 2008) as marsupial spermatozoa are not reported to suffer cold shock (Rodger and White 1978).

Eastern quolls (*Dasyurus viverrinus*) were donated to the study from Australian Ecosystems Foundation (Lithgow, New South Wales, Australia) and Remabi Park (Mount Barker, South Australia, Australia) and housed at The University of Newcastle (Australia) within indoor pens (1.8 x 4.6 m) on a cement floor covered with tee-tree mulch, gum leaves, hay and logs. Animals were housed individually or in male female pairs and fed daily raw meat, bones, eggs or whole mice. There was a constant supply of dry cat food (Whiskas, Mars Incorporated, McLean, VA, USA) and water was provided *ad. libitium*. Animals were exposed to a 10 hour light, 14 hour dark light cycle and examined during the southern hemisphere winter breeding season. For epididymal sperm collection adult *D. viverrinus* were euthanised by intra-cardiac injection of sodium pentobarbitone (Lethabarb, Laser Animal Health, Salisbury, SA, Australia) whilst anesthetised with 4% isolfuorane (Virbac Animal Health, Peakhurst, NSW, Australia). Testes were removed and transported as described above.

Spermatozoa were collected opportunistically from five reproductively active northern quolls (*Dasyurus hallucatus*) euthanised by Territory Wildlife Park (Berry Springs, Northern Territory, Australia) for population management. Males were fed a carnivore diet and housed individually in large (6 x 2 x 2 m) outdoor enclosures with a soil floor, climbing trees and a nest box. Animals were exposed to natural lighting and examined during the southern hemisphere winter breeding season. Males were euthanised by Lethabarb overdose under anesthetic as described for *D. viverrinus*. Testicular tissue was transported overnight wrapped in saline dampened gauze on ice. The spermatozoa from cold stored testes had been shown to remain motile for up to 48 hours in *D. hallucatus* (Czarny *et al.* 2008) and up to eight days in *S. crassicaudata* (Taggart *et al.* 1996).

The use of protected native species was licensed by New South Wales National Parks and Wildlife Service (Australia) and all experiments were approved by Newcastle University Animal Care and Ethics Committee.

3.2.2 Sperm Collection

An unusual feature of dasyurids is that the motility of spermatozoa from each caudal epididymidis within an individual can vary. We examined 132 males over several years and have observed that 44% had a bilateral difference of at least 20 percentage points difference in epididymal sperm motility and 10% of males had a bilateral difference of least 50 percentage points in sperm motility. To account for this experimentally, and avoid contamination with low quality samples, spermatozoa from each epididymis was handled separately.

Each caudal epididymidis was placed in a dasyurid sperm medium (pH 7.4, osmolarity 310 mOsm L^{-1}) comprised of Tris Buffered Saline (TBS- 150 mM NaCl, 2 mM KCl, 25 mM Tris, all components were purchased from Sigma-Aldrich, St Louis, MO, USA) supplemented with 4% (v/v) fetal calf serum (FCS) (Trace Biosciences, Castle Hill, NSW, Australia) and spermatozoa were extruded for 30 minutes (min) at 35 ºC. The dasyurid sperm medium was modified from Czarny *et al.* (2008) due to the necessity to exclude phosphate for use with the viability stain. Initial motility was assessed visually on a 35 ºC heated stage using an Axiovert 35 inverted microscope (Zeiss, Jena, Germany). Motility was assessed by the proportion of motile spermatozoa and only samples with greater than 40% forward progressive motility were used. This is the average motility of dasyurid spermatozoa, which is notably less motile than the spermatozoa of eutherians or other marsupials. Sperm concentration was determined following fixation with 4% (w/v) paraformaldehyde (PFA) (pH 7.5, Probing and Structure, Thuringowa, QLD, Australia) using a haemocytometer (Improved Neubauer, Bad Mergentheim, Germany). The large size of dasyurid spermatozoa required an assessment of five 1 mm^2 squares instead of the smaller 0.04 mm^2 squares used in many other species. Dasyurids have low sperm concentrations and the total yield from *S. crassicaudata* contains on average 6.9×10^5 spermatozoa (Breed *et al.* 1989). In this study *S. crassicaudata* samples were adjusted to between 50,000 to 100,000 spermatozoa mL^{-1}, *D. viverrinus* samples were adjusted to approximately 100,000 spermatozoa mL^{-1} and *D. hallucatus* samples were adjusted to approximately 150,000 spermatozoa mL^{-1}.

3.2.3 Experimental Design

3.2.3.1 Experiment 1: Freezing with egg yolk and 8% glycerol

Previous studies reporting sperm cryopreservation in *S. crassicaudata* had shown 3% motility when frozen in liquid nitrogen (LN) vapour with egg yolk and glycerol (Taggart *et al.* 1996). We replicated this experiment and examined the viability and morphology of *S. crassicaudata* spermatozoa (n=5) in 8% glycerol and 7.5% egg yolk after freezing in LN vapour. Spermatozoa were slowly diluted with an equal volume of room temperature dasyurid sperm medium (control) or the extender which contained Tris-Citrate Fructose buffer (TCF- 250 mM Tris, 88 mM citric acid, 7 mM fructose, all components were purchased from Sigma-Aldrich) and a final concentration of 8% (v/v) glycerol (Sigma-Aldrich) and 7.5% yolk from eggs which were recently laid by hens on organic feed (pH 6.5, osmolarity 1200 mOsm L^{-1}). Fresh spermatozoa in dasyurid sperm medium were fixed immediately as a negative control. Aliquots of spermatozoa (50 µL) were loaded into 0.25 mL straws (IMV, L'Aigle, France) and sealed with

dampened polyvinyl alcohol (PVA) (Sigma-Aldrich). Spermatozoa were equilibrated at room temperature for 5 min then frozen using the suspension method which involves suspending straws on a rack made from non-conductive material 3 cm above LN for 30 min after which the straws are immersed in LN. Samples were stored in LN for at least 4 weeks before assessment. Straws were thawed by immersion in a 35 ºC water bath for 10 seconds (sec) then expelled into a warmed culture dish. Viability was assessed with Fertilight and head presence was assessed with Bryan's stain as described in section 3.2.4.

3.2.3.2 Experiment 2: Tolerance to cryoprotectants

As experiment 1 did not produce viable spermatozoa we increased the concentration of glycerol and carried out a preliminary experiment to determine which concentrations of glycerol were detrimental to the viability of dasyurid spermatozoa. This toxicity trial was carried out by incubating *S. crassicaudata* spermatozoa (n=3) at room temperature in a final concentration of 8%, 20% and 40% glycerol and assessed their viability at 30, 60 and 180 min. Spermatozoa were slowly diluted with an equal volume of room temperature dasyurid sperm medium (control) or the extender which contained TCF and a final concentration of 8% (v/v) glycerol (TCF + 8G, pH 6.6, osmolarity 940 mOsm L^{-1}), 20% glycerol (TCF + 20G, pH 6.7, osmolarity 3368 mOsm L^{-1}) or 40% glycerol (TCF + 40G, pH 6.5, osmolarity ~7000 mOsm L^{-1}). Spermatozoa were incubated at room temperature and viability was assessed with Fertilight as described in section 3.2.4 immediately after the addition of the extender then at 30, 60 and 180 min. Loss of viability was suggested to be indicative of glycerol toxicity.

3.2.3.3 Experiment 3: Freezing with increased glycerol concentrations

On the basis that spermatozoa remained viable in up to 40% glycerol we examined the viability, morphology or DNA damage of frozen spermatozoa. *S. crassicaudata* spermatozoa (n=5) in TCF with a final concentration of 8% glycerol (TCF + 8G), 16% glycerol (TCF + 16G, pH 6.7, osmolarity 2743 mOsm L^{-1}), 20% glycerol (TCF + 20G) and 40% glycerol (TCF + 40G) were frozen using the suspension method and thawed by immersion in a 35 ºC water bath. Fresh spermatozoa in dasyurid sperm medium were fixed immediately as a negative control or loaded into straws and directly immersed in LN as a positive control. Viability was assessed with Fertilight, both acrosome and head presence was assessed with Bryan's stain. The damage to DNA in post-thaw positive control and TCF + 40G samples was examined using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) (n=4) as described in section 3.2.4.

An additional *S. crassicaudata* experiment examined the survival of spermatozoa frozen with TCF + 40G in a controlled rate freezer (CL 863 Freeze Control, Cryologic, Mulgrave, Australia) (n=6). All procedures were carried out as described above and spermatozoa were exposed to a 1.0 ºC min^{-1} or a 5.0 ºC min^{-1} protocol starting at 22 ºC. Straws were immersed in LN once they had reached -80 ºC, stored for at least 4 weeks and thawed as described above. For this experiment only viability with Fertilight was assessed, as described in section 3.2.4.

Spermatozoa from *D. viverrinus* (n=3) and *D. hallucatus* (n=4) were frozen using the suspension method with TCF + 20G and TCF + 40G. The control groups, suspension freezing and thawing was carried out as described above. Viability was assessed with Fertilight and both acrosome and head presence was assessed with Bryan's stain as described in section 3.2.4.

3.2.3.4 Experiment 4: Freeze drying

As an alternative preservation technique, *S. crassicaudata* (n=4) spermatozoa were freeze dried in either dasyurid sperm medium or trehalose solution. As freeze dried spermatozoa are not viable the morphology and DNA damage of spermatozoa was assessed upon rehydration. Freshly collected spermatozoa were diluted 1:1 with dasyurid sperm medium or trehalose solution comprised of 0.27 M trehalose (Sigma-Aldrich) in 1.5% (w/v) Diploma powdered skim milk (Fonterra Brands Pty Ltd, Mt Waverley, VIC, Australia) at room temperature. Aliquots of 100 µL were transferred to amber ampoules (Weaton, Millville, NJ, USA) and immersed in LN before being placed in chilled vessels attached to a freeze drier (Labconco, Kansas City, MO, USA) at 200 to 300 x 10^{-3} mbar for 4 hours (Liu *et al.* 2004). Ampoules were purged with nitrogen gas before being sealed and stored at room temperature for 3 months. Samples were rehydrated with 100 µL of Milli-Q water for 5 min then fixed. Controls comprised of fresh spermatozoa in dasyurid sperm medium which had been fixed. Controls and rehydrated spermatozoa were stained to determine both acrosomal and head presence using Bryan's stain and DNA damage was examined using TUNEL as described in section 3.2.4.

3.2.4 Assessment of Viability, Morphology and DNA Damage

Experiments 1 to 3 were assessed by sperm viability using the Fertilight live/dead staining kit (Molecular Probes, Carlsbad, CA, USA). Samples were stained with 10 nM SYBR14 and 240 nM propidium iodide (PI) for 10 min at 35 ºC then assessed at x200 on a Zeiss microscope with a heated stage using filter set 09 (excitation 450 to 490 nm, beam splitter 510 nm, emission 520 nm). Sperm heads which stained green (SYBR14) were viable and those which stained entirely or partially red (PI) were non-viable.

Sperm morphology (head loss and acrosome loss) was assessed in experiment 1, 3 and 4 using Bryan's stain (Bryan 1970). Spermatozoa were fixed with 4% PFA and concentrated onto slides by "cytospin" centrifugation (200 *g*, 3 min, Shandon Inc., Pittsburgh, PA, USA) (Mate and Rodger 1991). The slides were air dried before staining with Bryan's stain- eosin Y, flavanic acid, fast acid green (all 0.1% w/v, Sigma-Aldrich) in 1% (v/v) acetic acid (Fronine, Riverstone, NSW, Australia) for 3 to 6 min then mounted in DePex mounting medium (Gurr, Poole, UK). Duplicate counts of 100 spermatozoa were examined on a Reichert-Jung Microstar IV microscope (Buffalo, NY, USA) at x400 and the presence of a sperm head or acrosome was assessed.

S. crassicaudata spermatozoa from experiment 3 and 4 were also assessed for DNA damage using TUNEL. Spermatozoa (fresh and post-preservation) were fixed using 4% PFA and concentrated onto slides using the cytospin method. Slides were exposed to 4 ºC permeabilisation solution containing 0.2% sodium citrate (w/v, Sigma-Aldrich) and 0.2% Triton-X (v/v, Sigma-Aldrich) in phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) for 2 min then rinsed gently with PBS. The TUNEL protocol was performed according to the manufacturer's instructions with an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, USA) for 1 hour at 35 °C. Slides were then washed with PBS again, mounted in Mowiol mounting medium (Calbiochem, La Jolla, Ca, USA) and kept in the dark until analysis. Damage to DNA was identified by bright staining of the sperm head when viewed on a Zeiss Imager A1 (Jena, Germany) with narrow band Colibri light source set at 470 nm at x400. All experiments included stained and unstained fresh spermatozoa as negative controls and a DNA damage positive control which had been treated with 1 mg mL^{-1} DNase I (Roche Diagnostics) and 2.47 mg mL^{-1} magnesium sulphate (Sigma-Aldrich) for 1 hour at 35 °C prior to staining. Duplicate counts of 100 spermatozoa detected the percentage of stained sperm heads containing damaged DNA.

3.2.5 Statistical Analysis

All values of viability, acrosome or head presence are described by mean ± standard error of the mean (SEM). In Experiment 1, 3 and 4 differences in the proportion of spermatozoa with either the acrosome or head present after preservation was assessed with ANOVA followed by a Post-Hoc Tukey's test using JMP (SAS Institute Inc., Cary, NC, USA). The same method was used in experiment 2 to determine differences in the loss of viability, as a percentage of initial viability, between the control and treatment groups within each time point. Differences in the percentage of DNA degradation following different preservation methods in experiment 3 and 4 was also assessed with ANOVA followed by Post-Hoc Tukeys test using JMP.

3.3 RESULTS

3.3.1 Experiment 1: Freezing with Egg Yolk and 8% Glycerol

S. crassicaudata samples frozen with 8% glycerol and 7.5% egg yolk had no significant head loss (spermatozoa with head present 93.40 ± 0.62%) when compared to control values. But following preservation all spermatozoa were non-viable and visualisation of the acrosome unreliable due to spermatozoa having a malformed sickle shaped head.

3.3.2 Experiment 2: Tolerance to Cryoprotectants

The viability of spermatozoa incubated with Tris-Citrate-Fructose containing 8 to 40% glycerol was not significantly different from that observed in control samples which contained dasyurid sperm medium only (Figure 3-1). This was observed at all time points up to and including three hours and suggests that concentrations of up to 40% glycerol do not cause *S. crassicaudata* spermatozoa to become non-viable.

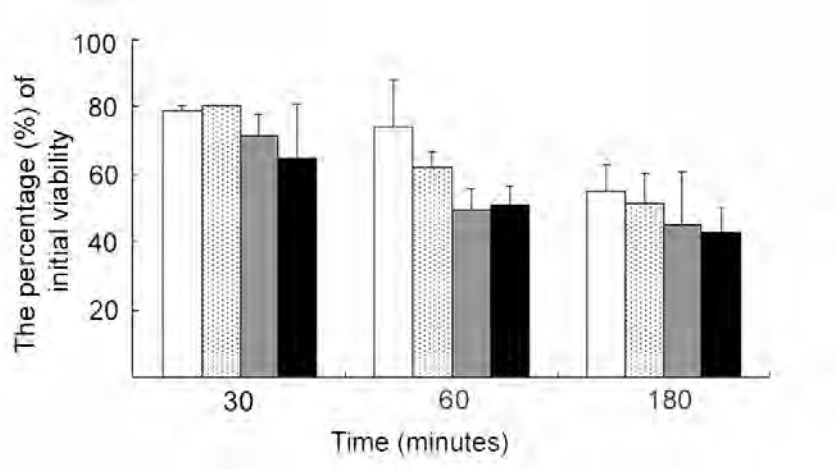

Figure 3-1: The percentage of initial viability ± SEM observed for up to 180 minutes in fat tailed dunnart (*Sminthopsis crassicaudata*) spermatozoa after co-incubation with dasyurid sperm medium (unfilled) or 8% glycerol (stippled), 20% glycerol (grey) or 40% glycerol (black) in Tris-Citrate Fructose buffer.

3.3.3 Experiment 3: Freezing with Increased Glycerol Concentrations

In *S. crassicaudata* acrosome loss was observed in the positive control ($P<0.05$), TCF + 8G ($P<0.001$) and TCF + 16G ($P<0.01$) when compared to the negative control, but there was no significant loss of acrosomes in samples frozen with TCF + 20G or TCF + 40G (Figure 3-2A). There was no significant head loss in any treatment but when assessed with a membrane permeable viability stain, all samples from all treatments were non-viable. There was also no viable spermatozoa present in TCF + 40G samples frozen using the 1.0 or 5.0 ºC min^{-1} controlled rate freezing protocol.

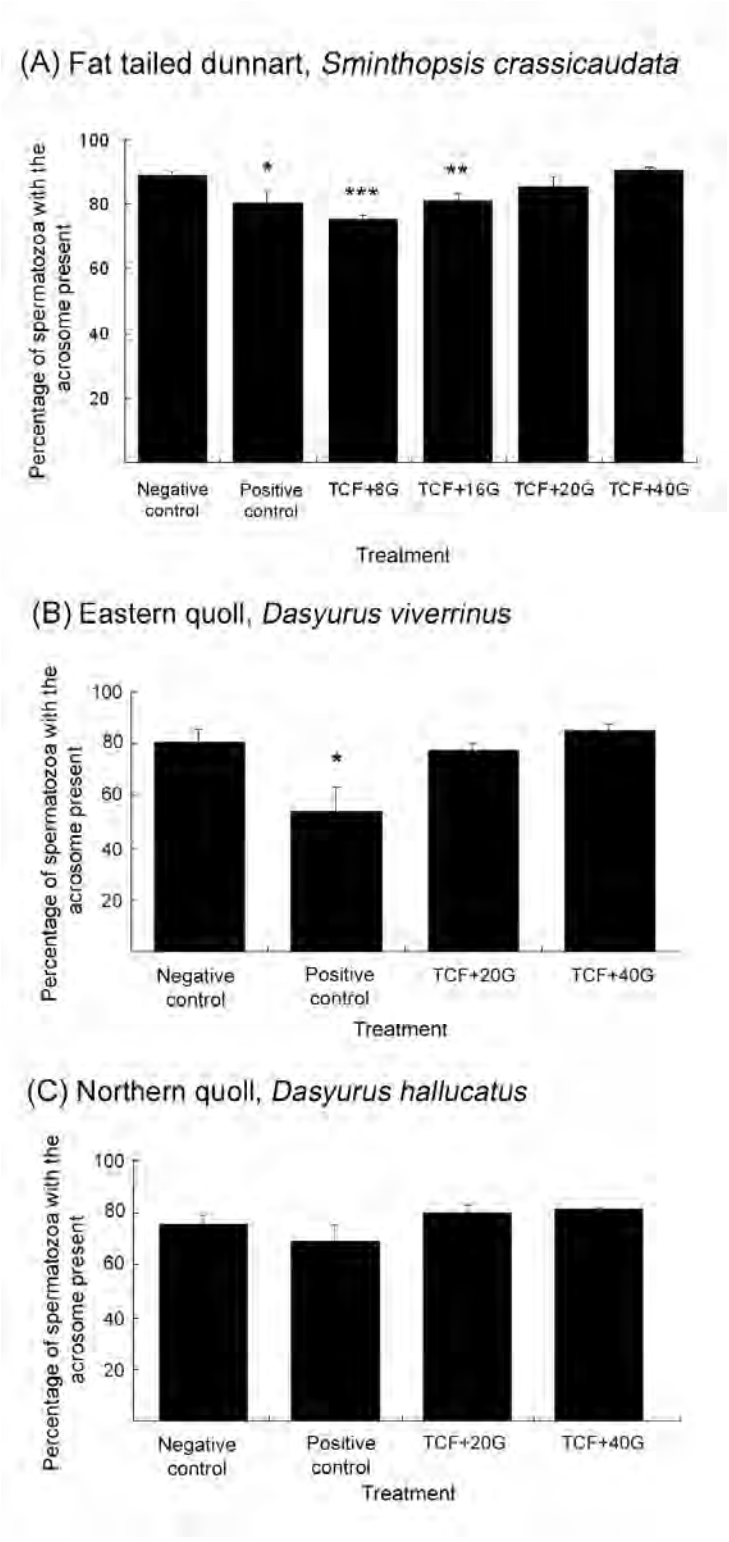

Figure 3-2: Acrosomal presence ± SEM following freezing of (A) fat tailed dunnart (*Sminthopsis crassicaudata*) (B) eastern quoll (*Dasyurus viverrinus*) and (C) northern quoll (*Dasyurus hallucatus*) spermatozoa. Negative controls were fresh and positive controls contained no extender. The extender was comprised of Tris-Citrate Fructose buffer (TCF) with 8 (TCF + 8G), 16 (TCF + 16G), 20 (TCF + 20G) or 40% glycerol (TCF + 40G). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Figure 3-3A and B show the appearance of negative and positive control TUNEL spermatozoa. Frozen samples without cryoprotectant contained 96.50 ± 2.52% normal spermatozoa, which was not significantly different from the percentage of normal spermatozoa in fresh samples (98.50 ± 1.17%). Spermatozoa frozen with TCF + 40G had more variable levels of DNA damage (Figure 3-3C) and only 80.00 ± 9.68% were classified as normal, however this too was not significantly different from control values.

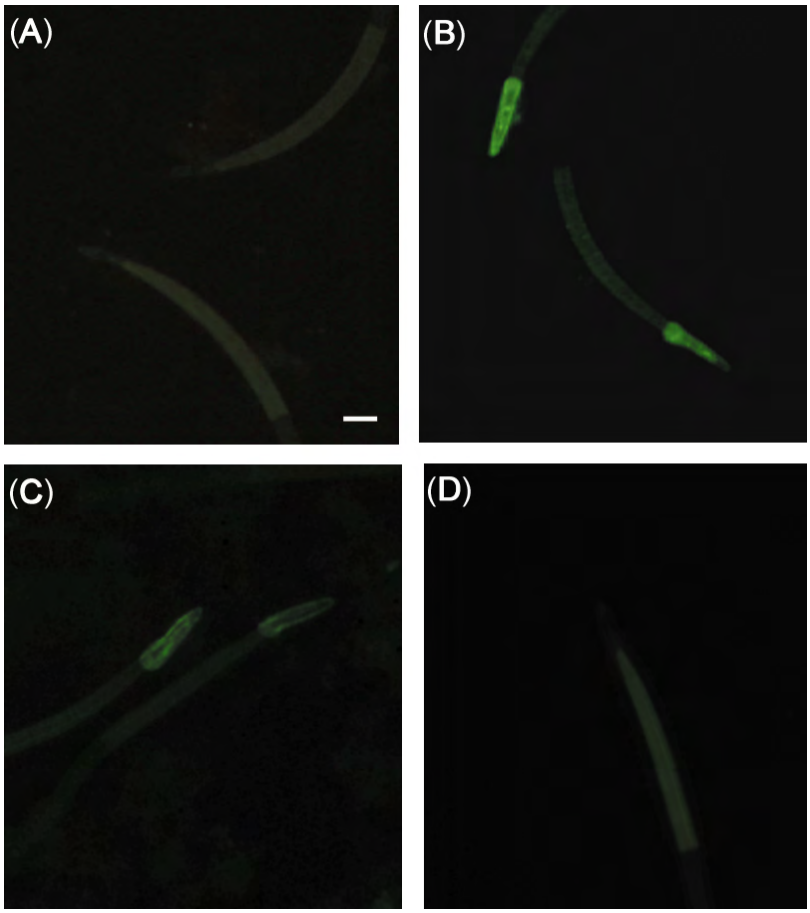

Figure 3-3: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) to indicate DNA damage in fat tailed dunnart (*Sminthopsis crassicaudata*) spermatozoa (A) fresh spermatozoa showing no DNA damage (B) positive control DNase treated spermatozoa indicating damaged DNA (C) DNA damage in frozen spermatozoa (D) freeze dried spermatozoa showing no DNA damage. Bar=10 µm.

Following freezing positive control *D. viverrinus* spermatozoa displayed acrosome loss ($P<0.05$, Figure 3-2B) and head loss ($P<0.05$). Interestingly *D. hallucatus* spermatozoa appeared more robust with no significant reduction in the percentage of spermatozoa with the head or acrosome present (Figure 3-2C).

3.3.4 Experiment 4: Freeze Drying

Freeze drying *S. crassicaudata* spermatozoa resulted in a 20% drop in the percentage of acrosomes which remained intact ($P<0.001$, percentage of spermatozoa with the acrosome present: 70.80 ± 1.00%). Pre-incubation with trehalose conferred no significant improvement to acrosomal stability (percentage of spermatozoa with the acrosome present: 71.90 ± 2.10%) and in neither protocol was significant head loss identified. TUNEL revealed that samples preserved with trehalose maintained 97.88 ± 0.47% normal nuclear morphology (Figure 3-3D) and those preserved without protection were 85.00 ± 4.08% normal, neither treatment significantly differed from the percentage of normal spermatozoa in fresh samples.

3.4 DISCUSSION

This study has shown that dasyurid spermatozoa possess a high tolerance to glycerol and following freezing have normal gross morphology and minimal DNA damage, but are not viable. After freeze drying a high proportion of spermatozoa had normal gross morphology and there was no increase in DNA damage. This is the first assessment of quoll sperm freezing, the first assessment of DNA integrity in frozen or freeze dried dasyurid spermatozoa and the first report of freeze drying spermatozoa from any marsupial species.

S. crassicaudata maintained viability in solutions containing up to 40% glycerol, despite the fact that glycerol is generally used at concentrations of 4 to 10% in eutherian species (Watson 1979). Glycerol can be toxic to eutherian species and macropods inducing cytoskeletal alteration, increased cytoplasmic viscosity and mitochondrial disturbance (Gao *et al.* 1993; McClean *et al.* 2006; McClean *et al.* 2008a). Taggart *et al.* (1996) indicates that *S. crassicaudata* spermatozoa may not have a high tolerance to glycerol as optimal recovery was seen following preservation in only 8% glycerol. The present study demonstrated that concentrations of glycerol as high as 20 and 40% are not toxic and should theoretically protect proteins from dehydration and confer membrane plasticity (Crowe *et al.* 1990). This result is not overly surprising as other non-macropodid marsupials, such as *T. vulpecula*, demonstrate similarly high tolerance to glycerol with optimal cryopreservation solutions containing 17.5% glycerol (Molinia and Rodger 1996; Taggart *et al.* 1996; MacCallum and Johnston 2005). Our results suggest there is no need to limit toxicity by utilising a stepwise addition of cryoprotectants at low temperatures or utilising a combination of cryoprotectants (Gao *et al.* 1995).

Despite the demonstrated tolerance to high concentrations of glycerol we were unable to replicate the low level of viable spermatozoa observed upon thawing by Taggart *et al.* (1996), potentially because of the rate of freezing or thawing. The two-factor hypothesis for cryopreservation of the spermatozoa from eutherian mammals suggests that equilibrium exists between the rate of cooling and water loss (Mazur 1965). To investigate if this was relevant to the spermatozoa of *S. crassicaudata* the present study examined three freezing rates. Neither rapid freezing in liquid nitrogen vapour nor slow cooling at 1.0ºC or 5.0 ºC min^{-1} resulted in viable spermatozoa despite superior results with slow cooling methods in other marsupials (Johnston *et al.* 2006; Phillips *et al.* 2008). A further alternative, which has high application to field studies, is pellet freezing spermatozoa on dry ice (Molinia and Rodger 1996; MacCallum and Johnston 2005). However a pilot study demonstrated that pellet freezing also conferred no advantage in *S. crassicaudata* spermatozoa (data not shown).

Although spermatozoa were not viable this study demonstrated that the gross morphology of spermatozoa was maintained following freezing with high concentrations of glycerol. The membrane stabilising properties of glycerol were capable of preserving the morphological character of normal dasyurid spermatozoa which lack disulphide stabilisation of the acrosomal matrix (Czarny *et al.* 2008) and have a relatively fragile ball-and-socket sperm head attachment (Lin *et al.* 1998). There was no evidence of decondensed chromatin nor an increased head volume as observed in *P. cinereus* and the ring tailed possum (*Pseudocheirus peregrinus*) (Johnston *et al.* 2006; Phillips *et al.* 2008).

We have previously described the fragility of the dasyurid acrosome, relative to the resilience of the acrosome from other marsupials (Sistina *et al.* 1993a; Czarny *et al.* 2008), and reported that the extent of damage does not correlate with the damage seen in eutherians (Oettlé 1986). Nonetheless the resilience of the acrosome in spermatozoa frozen without glycerol, examined as a positive control in the present study, was surprising in light of our earlier findings that almost half the spermatozoa lost their acrosomes following immersion in liquid nitrogen (Czarny *et al.* 2008). Both studies immersed the spermatozoa directly into liquid nitrogen without cryoprotection but in the present study spermatozoa were frozen in 0.25 mL straws and in Czarny *et al.* (2008) spermatozoa were frozen in 1.5 mL tubes. The improved results could potentially be due to the increased surface area of spermatozoa frozen in straws permitting faster and more consistent rates of freezing and thawing (Eriksson and Rodriguez-Martinez 2000).

Nuclear damage was detected with using the TUNEL protocol which labels cleaved double stranded DNA and in the present study it revealed that fresh spermatozoa had consistently low rates of damaged DNA. A similar test, the sperm chromatin dispersion test, has been used in *P. cinereus* where the varied sperm morphotypes displayed different rates of DNA damage, although all were in general higher than that observed in fresh *S. crassicaudata* spermatozoa examined in the present study (Johnston *et al.* 2007). We observed that freezing with or without cryoprotection did not increase DNA damage however there was some indication that individuals had a variable response to freezing in the presence of 40% glycerol. Although these spermatozoa are not useful for conventional breeding or artificial insemination, the apparently normal morphology and lack of DNA damage encourages examination of their fertilising potential because spermatozoa considered non-viable by traditional methods remain capable of siring offspring following ICSI in mice, pigs and rabbits (Wakayama and Yanagimachi 1998; Kwon *et al.* 2004; Liu *et al.* 2004).

As an alternative to freezing, the development of dasyurid freeze drying protocols could be advantageous because of reduced costs, space requirements and safety issues associated with long term liquid nitrogen storage (Kwon *et al.* 2004). The present study demonstrated low rates of acrosome loss and DNA damage in freeze dried samples. Trehalose is proposed to interact with the phospholipid heads of lipid bilayers increasing membrane stabilisation (reviewed in Crowe *et al.* 1990) and although not significant there was a trend for spermatozoa freeze dried in the presence of trehalose to have lower rates of acrosomal loss, head loss and DNA damage compared to spermatozoa freeze dried in the absence of trehalose.

This study has demonstrated that spermatozoa from dasyurids are tolerant of high concentrations of glycerol. Despite the lack of post-thaw viability, normal morphology was maintained in all species when higher glycerol concentrations were used in sperm freezing protocols. Following freeze drying minimal morphological damage was detected and DNA integrity was preserved. The finding that spermatozoa are tolerant of up to 40% glycerol suggests that the lack of post-thaw viability is unlikely to be associated with cryoprotectant toxicity- instead we suggest that the freezing process is damaging to spermatozoa. However the findings that that freeze dried spermatozoa had normal morphology and indicated no DNA damage is encouraging and suggests that freeze drying coupled with the development of ICSI is a reasonable strategy for future examination in studies on the long term preservation of the male gamete from these highly threatened native Australian carnivorous marsupials.

ADDENDUM TO CHAPTER 3: Preliminary studies regarding intracytoplasmic sperm injection in the fat tailed dunnart (*Sminthopsis crassicaudata*)

1 INTRODUCTION

In this chapter dasyurid spermatozoa were preserved using methods which resulted in normal morphology and no DNA damage, but spermatozoa were neither motile nor viable. Hence in order for these preserved spermatozoa to be used in post-preservation fertilisation experiments ICSI would be required.

ICSI was initially developed in echinoderms (Hiramoto 1962) and amphibians (Brun 1974) and subsequent work examined mice and hamsters (Hoshi *et al.* 1994; Kimura and Yanagimachi 1995) before ICSI became a well established ART in humans (Palermo *et al.* 1995). ICSI has since been used in transgenic rats (Hirabayashi *et al.* 2002), agricultural species (Keskintepe *et al.* 2002; Lee and Niwa 2006) and domestic cats (Bogliolo *et al.* 2001). ICSI has also been examined in two marsupials, as a tool to study early developmental processes and contribute towards marsupial ART. In *M. eugenii*, *in vivo* matured oocytes harvested following ovarian stimulation reach the two pronuclei stage following sperm injection but *in vivo* matured oocytes from unstimulated females develop into cleavage stage embryos, suggesting potential effects of the ovarian stimulation protocol on oocyte quality (Magarey and Mate 2003; Magarey and Mate 2004; Richings *et al.* 2004). Oocytes collected from stimulated *V. ursinus* have also been injected with cryopreserved sperm, but only a small percentage underwent cleavage and reached the 100 cell stage (West *et al.* 2007). There have been no published reports of ICSI in dasyurids however the technique has been previously investigated in *S. crassicaudata* with little success due to the large size of the dasyurid spermatozoa and the fragility of the thin zona pellucida (B Breed personal communication 2007). Certainly the large size of the dasyurid spermatozoon (300 µm long) and mature oocyte (210 µm diameter) will introduce unique practical difficulties.

This preliminary study describes progress toward ICSI protocols in *S. crassicaudata*. This is the first description of sperm microinjection in a dasyurid and it describes the source, and use, of oocyte holding and sperm injecting micropipettes which enable the large dasyurid gametes to be manipulated. This study utilised frozen spermatozoa (described earlier in this chapter) and oocytes collected following ovarian stimulation and *in vitro* maturation (described in detail in Chapter 5).

2 METHODS

2.1 Source of Gametes

Epididymal spermatozoa from *S. crassicaudata* were frozen as part of the experiments described earlier in this chapter. The spermatozoa selected for ICSI had been rapidly frozen in liquid nitrogen without cryoprotection and were thawed by immersion in a 35 ºC water bath. All spermatozoa were non-viable when the experiment began but assessment of DNA damage with the TUNEL protocol had shown that this preservation method did not result in damaged sperm nuclei.

Oocytes were harvested using the techniques described in Chapter 5. Briefly retired females (>12 months of age) were stimulated with 1 IU equine serum gonadotrophin (eSG) during the intermediate phase of their reproductive cycle. Oocytes were collected from pre-ovulatory antral follicles four days later and cultured without exogenous hormones for up to 48 hours. This protocol had been shown to result in almost 100% mature first polar body stage oocytes which were thus suitable for use in ICSI.

2.2 Gamete Preparation

Spermatozoa were thawed prior to use and 20 µL was added to 90 µL of sperm handling solution which contained 5% polyvinyl propylene (Fluka, Buchs, Switzerland) and 0.2% bovine serum albumin (Sigma-Aldrich) in Milli-Q water. This medium was optimised to improve sperm handling within the micropipette by reducing the concentration of PVP and BSA. Oocytes were harvested from the oocyte culture medium which contained Dulbecco's Modified Eagle's Medium (DMEM- pH 7.4, Sigma-Aldrich) supplemented with 10% (v/v) FCS, 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Invitrogen) and 2 mM fresh glutamine (Sigma-Aldrich). If required the oocytes were gently stripped using a glass pipette to remove granulosa cells.

The mature oocytes of dasyurid marsupials are comparatively large with a dark yolky cytoplasm, similar to the cytoplasm of porcine and bovine oocytes (Rho *et al.* 1998; Yong *et al.* 2005). Difficulty observing the passage of the spermatozoon into the yolky cytoplasm during microinjection was overcome by polarising the cytoplasm using centrifugation (Wall *et al.* 1985; Rho *et al.* 1998). *S. crassicaudata* oocytes were centrifuged in pre-warmed HEPES buffered (Sigma-Aldrich) oocyte culture medium at 500 *g* (Micro 20 Hettich Zentrifugen, Tuttlingen, Germany) for 10 min and this resulted in oocytes with noticeably polarised cytoplasmic lipids (Figure 1). This preparatory step made visualisation of the spermatozoa within the ooplasm possible and also increased the ease at which the spermatozoon was extruded from the micropipette.

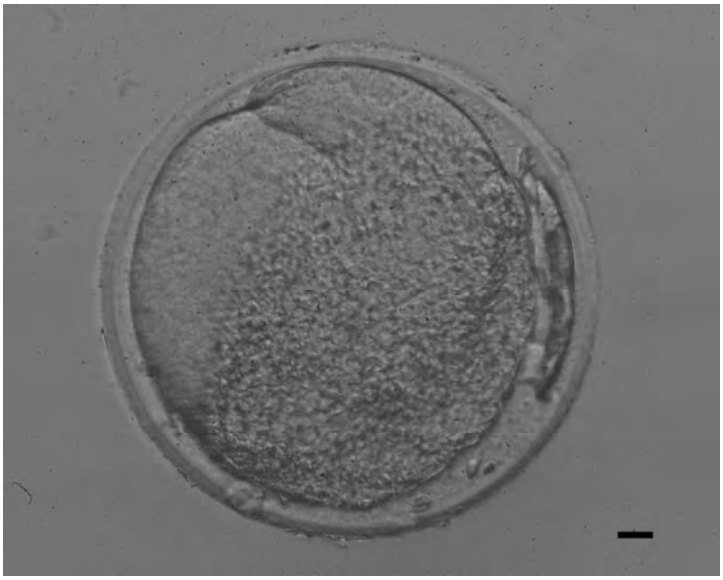

Figure 1: A mature first polar body stage fat tailed dunnart (*Sminthopsis crassicaudata*) oocyte that had been centrifuged to polarise the cytoplasmic lipid and aid in visualisation of the spermatozoon for intracytoplasmic sperm injection. Bar=10 µm.

2.3 Intracytoplasmic Sperm Injection

ICSI was carried out on an Axiovert 200 (Zeiss) fitted with an Intergra TI Micromanipulation Station (Research Instruments Limited, Cornwall, UK) including a heated stage set at 35 ºC. The pipettes were held by two PL30 Tool Holders (Research Instruments Limited) and pressure was applied using an air syringe system (Research Instruments Limited). Injection pipettes had an inner diameter of 8 to 9 µm and a 30 degree angle near the bevelled tip (Humagen, Charlottesville, VA, USA). Oocyte holding pipettes were custom made and had an outer diameter of 220 µm, an inner diameter of 20 µm and a 30 degree angle near the tip which was flat and polished (Humagen).

The microinjection was performed in the lid of a 60 mm polystyrene culture dish (Sigma-Aldrich) which contained an elongated droplet of spermatozoa in sperm handling medium and three droplets, each holding a single oocyte in pre-warmed HEPES buffered oocyte culture medium. An important finding at this preparatory stage was that spermatozoa in the handling solution needed to be added to the ICSI dish at least 15 min before micromanipulation began; this allowed the spermatozoa to sink to the base of the dish which made their collection considerably easier. The droplets were topped with pre-warmed and equilibrated mineral oil (Sigma-Aldrich).

To perform ICSI a single spermatozoon was aspirated tail first into the microinjection pipette. At this point it was important not to allow the spermatozoon to travel too far up into the microinjection pipette as it would become trapped. The pipettes were moved into an oocyte droplet and the presence of the spermatozoon was verified. An oocyte was located and held against the holding pipette with suction. The holding pipette, and oocyte, was lifted off the surface of the culture dish and the suction reduced so that the oocyte could be rotated with the assistance of the injection micropipette. The oocyte was rotated until the clear polarised area was apparent then the suction was re-applied to the oocyte for sperm injection. The position of the spermatozoon was re-checked and the micropipette was pushed through the zona pellucida with negative pressure applied against the ooplasm. When the ooplasm ruptured and yolky cytoplasm rushed into the micropipette the pressure was reversed causing the spermatozoon, and extruded cytoplasm, to be pushed into the oocyte. When the sperm tail was observed in the cytoplasm the micropipette was carefully removed from the cytoplasm. The oocyte was released from the holding pipette and the procedure was repeated for the remaining two oocytes before the culture dish was returned to a desktop incubator (MINC benchtop incubator, Cook, Bloomington, IN, USA) set at 35 ºC with 5% CO_2 in air for up to 1 hour. Injected oocytes were then transferred to fresh oocyte culture medium and cultured (35 ºC, 5% CO_2 in air) for 16 to 20 hours.

2.4 Assessment of Sperm Nuclei

In order to determine if sperm nuclei had decondensed the injected oocytes were stained with Hoechst (H33342, Sigma-Aldrich). Oocytes were washed in PBS, stained for 20 min in 10 µg mL^{-1} H33342 and then washed three times in PBS as described by Mate and Buist (1999). Oocytes were mounted on slides with the coverslip supported by a 9:1 mixture of Vaseline and candle wax and visualised using an inverted microscope (Zeiss) at x200 and x400 using filter set 2 (exciter filter 365 nm, emission filter 420 nm). Sperm heads were only classed as diffuse if they were unable to be brought into focus, even when the oocytes were rotated.

3 RESULTS

Of eight oocytes examined 16 hours after microinjection five contained a spermatozoa with condensed nuclei (Figure 2A,C), and three oocytes contained spermatozoa with decondensed nuclei. At 20 hours post-ICSI five oocytes were examined and all contained spermatozoa with diffuse decondensed nuclei (Figure 2B,D). After injection all oocytes appeared enlarged but by 16 hours oocytes had returned to normal size. There was no evidence of the polarised cytoplasmic lipid at either time point and no oocytes had normal female pronuclei.

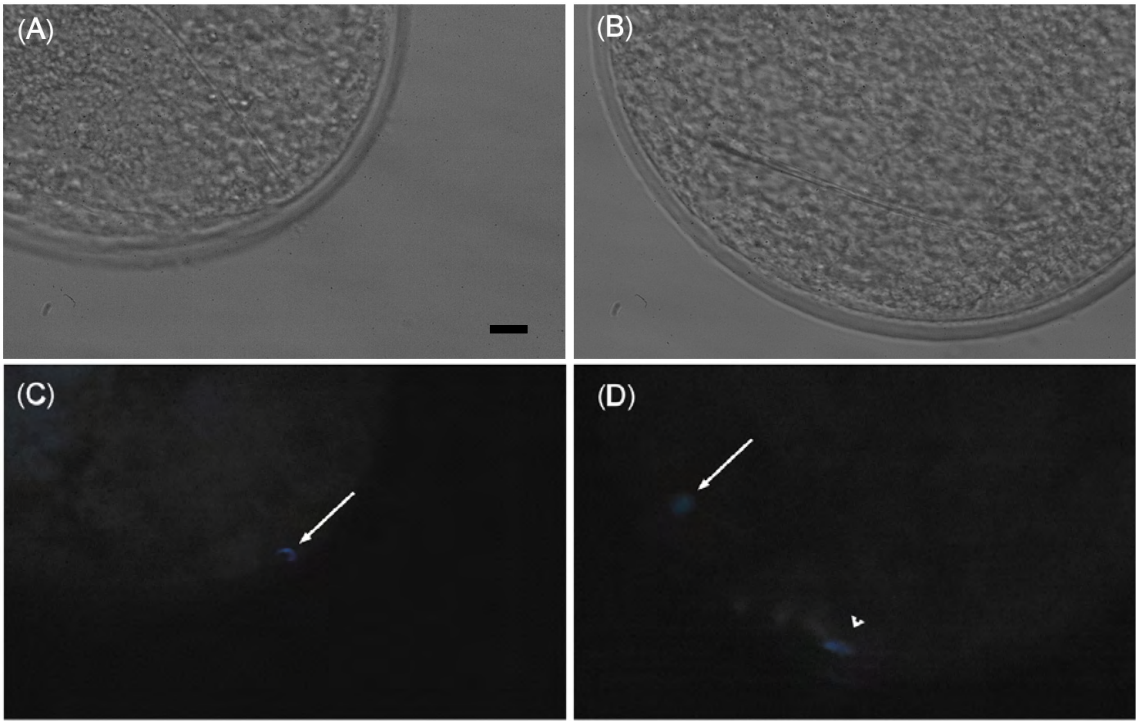

Figure 2: Hoechst stained fat tailed dunnart (*Sminthopsis crassicaudata*) oocytes which had undergone intracytoplasmic sperm injection (ICSI) observed with light (A,B) or fluorescent (C,D) microscopy. (A,C) were examined at 16 hours post-ICSI and show condensed sperm nuclei (arrow) but (B,D) were examined at 20 hours post-ICSI and show decondensed sperm nuclei with a diffuse appearance (arrow) and a polar body (arrowhead). Bar=10 µm.

4 DISCUSSION

This preliminary study represents the first demonstration of ICSI in a dasyurid marsupial. The finding that spermatozoa which were frozen without cryoprotection do not immediately decondense spontaneously is encouraging as it suggests that despite being non-viable the amount of damage to the nuclei, which lacks disulphide stabilisation (Retief *et al.* 1995a), is limited. Another encouraging finding was that oocytes produced by ovarian stimulation have sufficient intracellular concentrations of sperm nuclear decondensation factors to cause the brightly stained condensed sperm nuclei to become diffuse.

Ultrastructural studies of *S. crassicaudata* fertilisation have shown that when the spermatozoon initially enters the oocyte it has a well defined streamlined head (Breed and Leigh 1988). As it decondenses the nuclear material becomes “whispy” and it is fully decondensed when it appears circular, expanded and dispersed (Breed and Leigh 1988; Breed and Leigh 1990; Breed 1994b).

In this study only a proportion of sperm heads had decondensed when injected oocytes were examined 16 hours post-ICSI. When assessed at 20 hours, however 100% of the injected sperm heads had decondensed. This time frame is longer than observed in other marsupial studies (Rodger and Bedford 1982a; Taggart *et al.* 1993) and in *M. eugenii* oocytes fertilised with ICSI, sperm decondensation was observed within 10 hours of sperm injection (Magarey and Mate 2003). However ultrastructural studies have shown that sperm decondensation does not occur immediately upon entry into the oocyte in *S. crassicaudata* (Breed and Leigh 1988). This has been suggested to relate to the long length of the dasyurid spermatozoon (300 µm) requiring more time to enter the oocyte and the fact that the nuclei do not begin to decondense until the entire sperm tail is inside the ooplasm (Breed 1996). This delayed sperm head decondensation is proposed to be controlled by decreased concentrations of sperm nuclear decondensation factors within the oocyte (Breed 1996).

One of the major difficulties encountered in the development of the protocol was the inability to visualise the spermatozoon as it entered the yolky ooplasm. Previous studies in agricultural species have overcome this by centrifuging the oocytes to polarise the lipid (Wall *et al.* 1985; Rho *et al.* 1998). This was used effectively in *S. crassicaudata* oocytes and enabled better visualisation of the sperm, but also increased the ease with which the spermatozoon was extruded from the injecting micropipette, an issue which was problematic in previous dasyurid ICSI attempts (B Breed personal communication 2007). However although yolk polarisation is a useful tool it has demonstrated some negative developmental effects in the pig (Yong *et al.* 2005). An alternative to yolk polarisation could be the use of a non-toxic stain which would allow better visualisation of the spermatozoon as it left the injection pipette.

Another area requiring improvement relates to the amount of medium which is injected into the oocytes with the spermatozoa. Post-injection the oocytes were enlarged and although they returned to their regular size it is likely that this size fluctuation may cause damage. The injection of additional medium was unavoidable with this protocol, as the volume of liquid in the micropipette lumen is injected along with the 300 µm long spermatozoon. Assuming the oocyte is a perfect sphere (volume calculated by $^4/_3 x \pi x r^3$, where r = radius) and the injecting micropipette is a perfect cylinder (volume calculated by $\pi x r^2 x h$, where r = radius and h = height), the injection of the spermatozoa would include an additional 0.019 nL of medium, which is an additional 0.4% of the oocyte's volume. This study used large 9 µm injection micropipettes, which were perhaps unnecessary given that the dasyurid spermatozoon is only 2 µm in

diameter. A reduction in microinjection pipette diameter to 7 µm would theoretically halve the volume of medium injected in future studies. A smaller injection pipette would also be advantageous because it would decrease the size of the point of entry hole in the oocyte, although this was not perceived as a problem in the present study. These issues suggest that reducing the injection micropipette size is one of the first improvements which could be made to the current protocol. However further studies should also examine ICSI in naturally grown oocytes using immobilised fresh sperm, as optimising gamete quality may yield improved ICSI outcomes.

This pilot study provides the first evidence that ICSI is likely to be possible in dasyurid marsupials. It has demonstrated decondensation of the nuclei from spermatozoa frozen without cryoprotection in oocytes collected from artificially stimulated females. Although these results are preliminary, this study has successfully established the practicalities required for *S. crassicaudata* gamete manipulation (equipment, media and preparation) which will be fundamentally important in underpinning the development of ICSI for dasyurid marsupials.

CHAPTER 4

The spermatozoa of the dasyurid marsupial, *Sminthopsis crassicaudata*, are highly susceptible to cold shock

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I J. C. Rodger attest that the Research Higher Degree candidate Natasha Czarny had an integral role in the experimental design, carried out the research, analysed the data and prepared the publication entitled "The spermatozoa of the dasyurid marsupial, *Sminthopsis crassicaudata*, are highly susceptible to cold shock". I consulted and advised on the experimental design, interpretation of results and preparation of the manuscript.

Name……..

Signature…….. Date……

Candidate signature…….(Natasha Czarny) Date……

CHAPTER 4: The spermatozoa of the dasyurid marsupial, *Sminthopsis crassicaudata*, are highly susceptible to cold shock.

4.1 INTRODUCTION

Dasyurid marsupials are native Australian carnivores which are essential to the maintenance of healthy ecosystems, but many species within this family are threatened by introduced pests, habitat destruction or disease (reviewed by Jones *et al.* 2003). One tool used for the conservation of wildlife is assisted reproduction involving the preservation of gametes which can be reintroduced into the population at a later date (Holt 2001; Howard and Wildt 2009). Although recent progress has been made with the preservation of dasyurid oocytes (Czarny *et al.* 2009b) there has been little progress with the preservation of dasyurid spermatozoa, which lack disulphide stabilisation of both the nucleus and acrosome, despite their demonstrated tolerance to high concentrations of cryoprotectants (Retief *et al.* 1995a; Taggart *et al.* 1996; Czarny *et al.* 2008 and Chapter 3; Czarny *et al.* 2009a).

For 30 years one of the paradigms of marsupial sperm biology has been that spermatozoa do not suffer cold shock as observed in pigs and sheep (White 1993; Rodger *et al.* 2009). Spermatozoa which are affected by cold shock become immotile and cannot recover when warmed, this occurs due to physical changes including structural membrane alterations, increases in cell permeability and swelling (White 1993). Cold shock as defined by Mann (1964) occurs following a rapid exposure to approximately 0 ºC in a pre-chilled tube held in iced water.

Early studies on a marsupial, the brush tailed possum (*Trichosurus vulpecula*), indicated that phalangerid spermatozoa did not suffer cold shock following this rapid cooling method (Mann 1964; Rodger and White 1978). However subsequent studies which suggest the lack of cold shock in marsupials used less stringent methods, sometimes only examining refrigerated temperatures. Taggart *et al.* (1996) suggested that the spermatozoa of the fat tailed dunnart (*Sminthopsis crassicaudata*), long footed potoroo (*Potorous longipes*) and ringtail possum (*Pseudocheirus peregrinus*) are cold shock tolerant as they survived several days in the refrigerator at 4 ºC held within the scrotum. Common wombat (*Vombatus ursinus*) spermatozoa are also said to be cold shock tolerant as there was no reduction in motility or viability following rapid exposure to 0 ºC (SD Johnston personal communication 2008; Miller *et al.* 2004). Macropod spermatozoa survive relatively rapid cooling to -7 ºC at 10 ºC minute^{-1} (Holt *et al.* 1999)

and thus appear to be cold shock tolerant despite their relatively poor record of cryopreservation (McClean and Johnston 2003; McClean *et al.* 2008b). The assessment of koala (*Phascolarctos cinereus*) spermatozoa was the first to hint at cold induced damage as a possibly significant factor because a 12% decrease in motility was observed following chilling in a refrigerator at 5 ºC for 10 minutes (Johnston *et al.* 2000).

Despite the apparent evidence that the spermatozoa of several marsupial species do not suffer cold shock many studies still choose to include egg yolk in their preservation protocols (Taggart *et al.* 1996; MacCallum and Johnston 2005; Johnston *et al.* 2006; Phillips *et al.* 2008). Egg yolk contains phospholipids which assist in membrane stabilisation to protect against the negative effects of cold shock (Watson and Martin 1975; Watson 1976). The protective function of egg yolk is provided by the low density lipoprotein fraction which interacts with and stabilises lipid bilayers (White 1993).

This investigation was carried out following the findings that spermatozoa from the model dasyurid *S. crassicaudata*, the eastern quoll (*Dasyurus viverrinus*) and the northern quoll (*Dasyurus hallucatus*) were immotile and non-viable after freezing using a variety of glycerol concentrations and freezing protocols (Czarny *et al.* 2009a and Chapter 3). This study was initiated to determine whether the extremely poor response of dasyurid spermatozoa to freezing was due too unexpected susceptibility to cold shock and whether the inclusion of egg yolk in the medium and slower cooling protocols allows *S. crassicaudata* spermatozoa to survive to 0 ºC.

4.2 MATERIALS AND METHODS

4.2.1 Husbandry

S. crassicaudata were housed onsite at the University of Newcastle (Australia) in male only bachelor groups of up to five individuals in opaque polypropylene boxes (420 mm x 280 mm x 160 mm) with a recycled paper floor covering, shelters and nesting material. They had *ad. libitium* access to food (IAMS chicken adult cat food, Dayton, OH, USA) and water via dripper bottles and plastic bowls. The animals were exposed to a 16 hour light, 8 hour dark light cycle to promote continuous sperm production.

The use of protected native species was licensed by New South Wales National Parks and Wildlife Service (Australia) and all experiments were approved by Newcastle University Animal Care and Ethics Committee.

4.2.2 Sperm Collection

For each experiment at least four adult male *S. crassicaudata* were euthanised by CO_2 inhalation. Testes were dissected and processed within 30 minutes (min), each epididymidis was handled separately to avoid contamination with low quality spermatozoa (Czarny *et al.* 2009a). Approximately 4 mm of the caudal, distal corpus or caput epididymidis was placed in 300 µL of dasyurid sperm medium (pH 7.4, osmolarity 310 mOsm L^{-1}) which was comprised of Tris Buffered Saline (TBS- 150 mM NaCl, 2 mM KCl, 25 mM Tris, all components were purchased from Sigma-Aldrich, St Louis, MO, USA) supplemented with 4% (v/v) fetal calf serum (FCS) (Trace Biosciences, Castle Hill, NSW, Australia). The epididymal sections were incubated for 30 min at 35 ºC on a custom built heat tray to extrude spermatozoa. We examined all regions of the epididymis as the literature suggested that tolerance to cryopreservation changes throughout the epididymis (Simpson *et al.* 1987; McClean *et al.* 2006), as do membrane lipids which are associated with tolerance to cold shock (Miller *et al.* 2004).

The percentage of motile spermatozoa was assessed visually on a microscope with a 35 ºC heated stage (Zeiss Axiovert 35, Jena, Germany) and did not require forward progression to be considered motile (Holt *et al.* 1999). Dasyurids produce low concentrations of spermatozoa and the total yield from an individual contains 6.9 x 10^5 spermatozoa (Breed *et al.* 1989). In the present study sperm concentration was adjusted to approximately 100,000 spermatozoa mL^{-1} using dasyurid sperm medium.

4.2.3 Experiment 1- Cold Shock

This experiment replicated the cold shock tests carried out by Mann (1964). Caudal, distal corpus and caput spermatozoa (n=6) were slowly diluted 1:1 with pre-warmed medium to a final concentration of 0 (sperm medium), 5% (pH 7.3, osmolarity 304 mOsm L^{-1}) or 20% (pH 6.6, osmolarity 282 mOsm L^{-1}) (v/v) egg yolk from eggs laid the previous day. The yolk solutions were clarified by centrifuging at 15,000 *g* (Micro 20 Hettich Zentrifugen, Tuttlingen, Germany) for 30 min prior to being combined with spermatozoa (Molinia *et al.* 1994; Holt *et al.* 1996). After a 10 min incubation at 35 ºC sperm motility was assessed in a multiwell culture dish, then spermatozoa were cold shocked by rapid cooling in an iced water bath as shown in Figure 4-1. A 0.5 mL polypropylene tube (Sarstedt, Nümbrecht, Germany) was pre-cooled to 0.5 ºC in a beaker containing an iced water slurry resting in a container of ice. A 50 µL aliquot of sperm suspension was transferred into the cold tube and rotated in the iced water slurry between the thumb and forefinger for 2 to 3 min. Then the sperm suspension was transferred back into the culture dish and re-warmed for 10 min on a heated tray at 35 ºC before the motility was re-assessed.

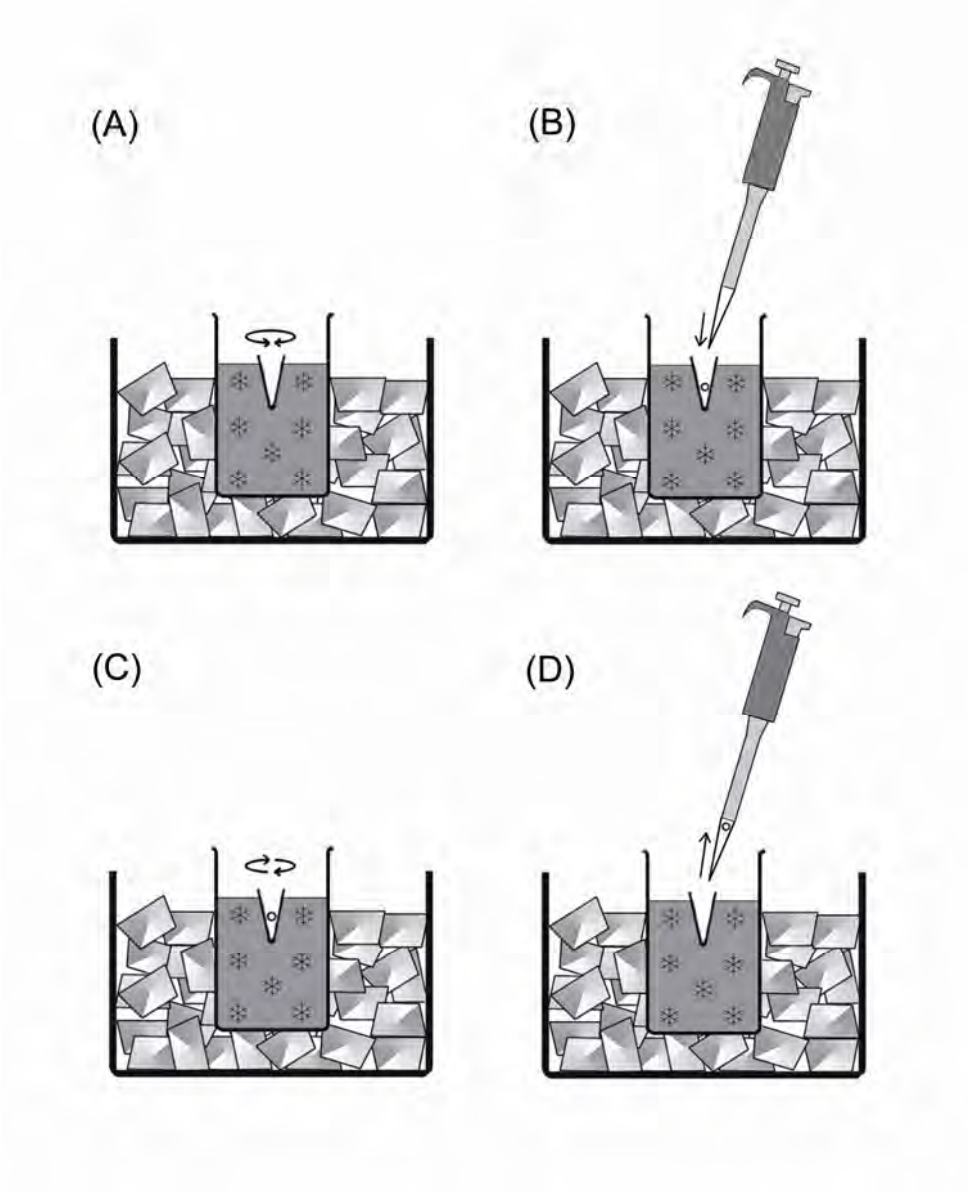

Figure 4-1: A pictorial representation of the cold shock procedure described by Mann (1964). (A) a tube is pre-cooled by rotating in an beaker which is filled with an iced water slurry and surrounded by ice and then (B) spermatozoa are added to the cooled tube. (C) The tube is then rotated within the iced water slurry for several minutes to ensure rapid cooling of the spermatozoa and then (D) spermatozoa are removed from the tube and warmed for assessment.

4.2.4 Experiment 2- Cold Induced Damage at 4 ºC

This experiment examined whether cold induced damage occurred when spermatozoa were rapidly cooled to 4 ºC, and if egg yolk conferred protection. Caudal and distal corpus spermatozoa (n=8 to 10) were slowly diluted 1:1 with pre-warmed sperm medium to a final concentration of 0 (sperm medium), 5%, 10% (pH 6.8, osmolarity 270 mOsm L^{-1}), 15% (pH 6.8, osmolarity 302 mOsm L^{-1}) or 20% (v/v) clarified yolk.

After a 10 min incubation at 35 ºC sperm motility was assessed then 50 µL of sperm suspension was transferred to 0.5 mL tubes and exposed to 4 ºC water in a calibrated refrigerated cooling unit for 10 min (MultiTemp III, Pharmacia Biotech, Uppsala, Sweden). The motility of cooled spermatozoa was assessed following return to the culture dish and re-warming.

4.2.5 Experiment 3- Programmed Slow Cooling

This experiment examined if cold induced damage occurred at 0 ºC if the temperature was slowly reduced at a rate of 0.5 ºC min^{-1}, and if egg yolk conferred protection. Caudal and distal corpus spermatozoa (n=6) were slowly diluted 1:1 with pre-warmed medium to a final concentration of 0 (sperm medium), 10% or 20% (v/v) clarified yolk.

After a 10 min incubation at 35 ºC, 40 µL of sperm suspension was loaded into 0.25 mL cryostraws (IMV, L'Aigle, France) which were sealed with dampened polyvinyl alcohol (PVA) (Sigma-Aldrich). Straws were placed in a controlled rate freezer (CL 863 Freeze Control, Cryologic, Mulgrave, Australia) which was programmed to reduce the temperature at 0.5 ºC min^{-1} from a starting temperature of 20 ºC. At 10, 6, 4, 2 and 0 ºC straws were removed and spermatozoa were returned to the culture dish for re-warming and assessment of motility.

4.2.6 Statistical Analysis

Motility scores are described by mean ± standard error of the mean (SEM). Prior to cooling in experiment 2, significant differences between all the media were assessed with ANOVA using JMP (SAS Institute Inc., Cary, NC, USA). Differences in the motility of spermatozoa mixed with different yolk concentrations after cooling was determined in all experiments using ANOVA followed by a Post-Hoc Tukey's test in JMP. The difference between pre and post cooling motility was assessed for all the media using pairwise T-tests in Microsoft Excel. All statistics were carried out on transformed data.

4.3 RESULTS

4.3.1 Experiment 1- Cold Shock

Table 4-1 shows that following exposure to 0.5 ºC no caudal or distal corpus spermatozoa were motile in any medium. The percentage of caput spermatozoa which were motile upon collection was very low (range: 3.0 to 7.0%) but Table 4-1 shows that approximately 2% of caput spermatozoa remained motile following cold shock.

Table 4-1: The percentage ± SEM of fat tailed dunnart (*Sminthopsis crassicaudata*) spermatozoa from three regions of the epididymis in three different media prior to, and following, cold shock.

	Percentage of spermatozoa motile prior to cold shock			Percentage of spermatozoa motile following cold shock		
	Sperm medium	5% yolk	20 % yolk	Sperm medium	5% yolk	20 % yolk
Caput	4.8 ± 0.5	5.2 ± 0.4	4.3 ± 0.5	1.8 ± 0.4	1.7 ± 0.5	1.5 ± 0.6
Corpus	52.3 ± 2.1	57.5 ± 2.5	52.5 ± 2.5	No motile spermatozoa		
Cauda	50.8 ± 6.9	51.7 ± 4.0	50.8 ± 4.2	No motile spermatozoa		

4.3.2 Experiment 2- Cold Induced Damage at 4 ºC

Figure 4-2A and B show that across the full range of yolk media there was no significant difference in pre-cooling sperm motility. Upon re-warming after rapid exposure to 4 ºC the percentage of motile spermatozoa in sperm medium was reduced to 2.0 ± 1.3% for the corpus and 1.1 ± 1.0% for the caudal spermatozoa. These values were lower than those observed for corpus spermatozoa in all the media containing yolk ($P<0.001$, Figure 4-2A) and caudal spermatozoa in either 5% yolk ($P<0.01$) or 10%, 15% and 20% yolk ($P<0.001$, Figure 4-2B). The percentage of motile spermatozoa in all media decreased following exposure to 4 ºC ($P<0.05$ to 0.001), although approximately three quarters of the spermatozoa remained motile when cooled in the presence of 10% egg yolk (distal corpus: 79.1 ± 3.2%, caudal: 66.7 ± 5.3%). There was a trend to suggest that 20% egg yolk is detrimental to both pre- and post-cooling sperm motility, but this was not significant.

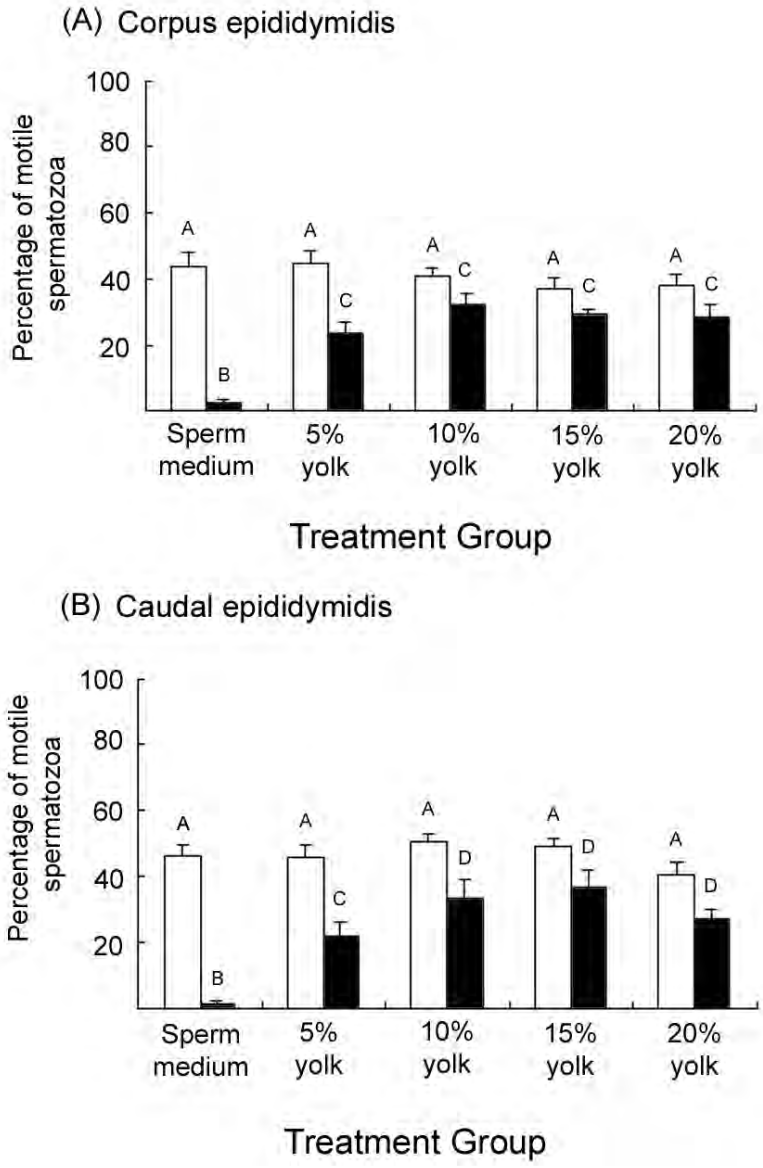

Figure 4-2: The percentage ± SEM of motile (A) corpus or (B) caudal fat tailed dunnart (*Sminthopsis crassicaudata*) spermatozoa before (unfilled) or after (filled) rapid cooling to 4 ºC in sperm medium or 5%, 10%, 15% or 20% clarified egg yolk. Values with a different letter are significantly different. P at least <0.01.

4.3.3 Experiment 3- Programmed Slow Cooling

The percentage of motile spermatozoa from the distal corpus was lower in sperm medium than in 10% ($P<0.001$) or 20% ($P<0.01$) egg yolk once the temperature decreased to 4 ºC (Figure 4-3A). However for caudal spermatozoa there was no difference between samples in sperm medium and those in 10% or 20% egg yolk until the temperature decreased to 2 ºC ($P<0.001$, Figure 4-3B). Throughout the experiment there was no decline in the percentage of motile spermatozoa from either region in either yolk media. Spermatozoa in sperm medium lost motility between 4 and 2 ºC (corpus $P<0.05$ and caudal $P<0.01$) and at 0 ºC only 5% of spermatozoa were motile.

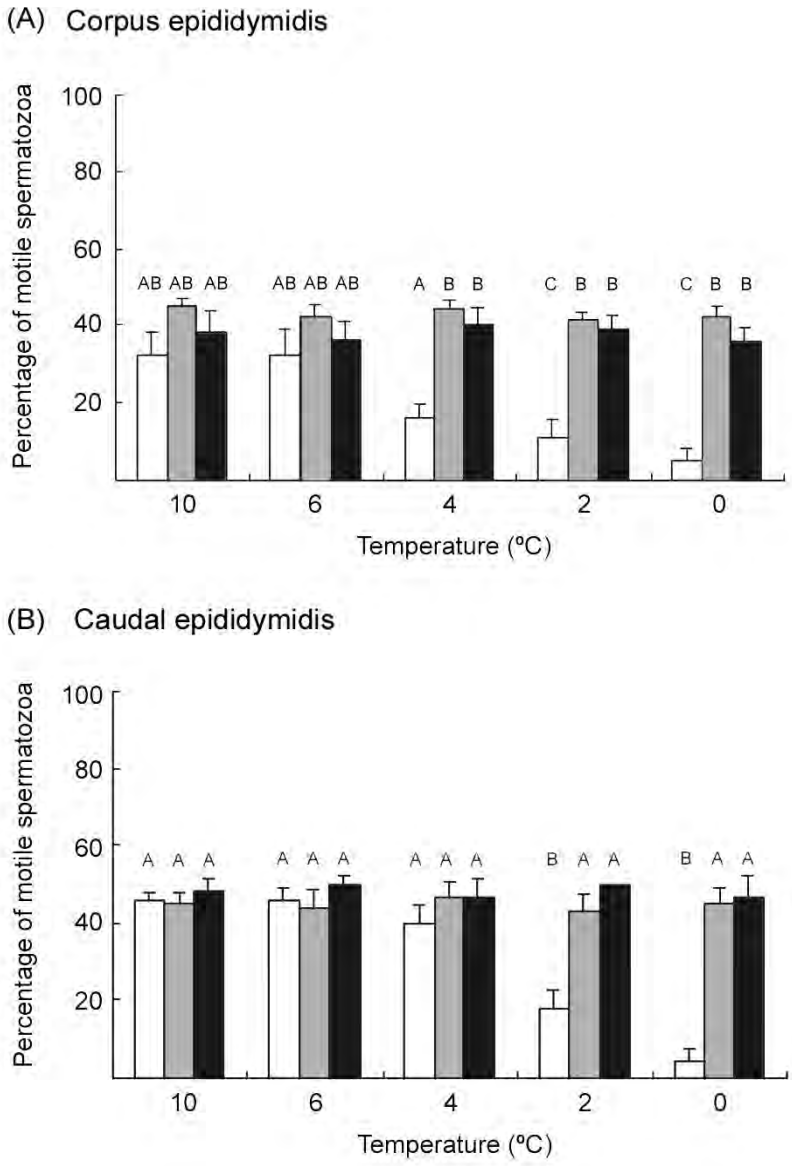

Figure 4-3: The percentage ± SEM of motile (A) corpus or (B) caudal fat tailed dunnart (*Sminthopsis crassicaudata*) spermatozoa following slow cooling in sperm medium (unfilled), 10% (grey) or 20% (black) clarified egg yolk. Values with a different letter are significantly different. P at least <0.01.

4.4 DISCUSSION

This is the first study to demonstrate a high susceptibility to cold shock in the spermatozoa of a marsupial. We have described complete loss of motility in distal corpus and caudal, but not caput, spermatozoa following a rapid drop in temperatures to near 0 ºC, and shown that the addition of egg yolk to the media cannot overcome this. However, egg yolk is beneficial when spermatozoa are rapidly cooled to 4 ºC and the combined effect of slow cooling at 0.5 ºC min^{-1} and inclusion of egg yolk allows *S. crassicaudata* spermatozoa to be cooled to 0 ºC and still maintain high motility. We have also observed that at least 10% egg yolk was required to create a beneficial effect and that *S. crassicaudata* spermatozoa were not damaged by incubation with up to 20% egg yolk. Recognition of the susceptibility of *S. crassicaudata* spermatozoa to cold shock has important consequences for the ongoing studies of sperm cryopreservation in dasyurid marsupials.

Although several previous studies have discussed cold shock in marsupial spermatozoa we believe that true cold shock, in response to a very rapid drop in temperature to near 0 ºC, has only been assessed for *T. vulpecula, V. ursinus* and macropods (Rodger and White 1978; Holt *et al.* 1999; Miller *et al.* 2004). For example, Taggart *et al.* (1996) suggest that spermatozoa from dasyurids, potoroids and phalangerids are cold shock tolerant but tested this by placing the whole scrotum in the refrigerator. The insulative properties of the scrotum would have slowed the rate of cooling, making this process equivalent to slow cooling to 4 ºC. Previous studies in our laboratory have also reported that testicular tissue can be wrapped in gauze and transported on ice for one hour and the spermatozoa remains motile (Czarny *et al.* 2008). Other studies examine cooling of spermatozoa in a refrigerator (Johnston *et al.* 2000) which is likely to be equivalent to our slow cooling experiments but not continue below 4 or 5 ºC. Our data suggested significant damage occurred between 4 and 2 ºC which would not have been detected by studies examining refrigerated samples. In the present study our methods differed slightly from that of Mann (1964) by using a lower volume of spermatozoa, due to the lower number of spermatozoa in *S. crassicaudata* (Breed *et al.* 1989), and by using a polypropylene tube instead of glass. However most critically, the method had the key feature of the original technique described by Mann (1964)- essentially an immediate cooling to near 0 ºC in an iced water slurry.

Spermatozoa from several eutherian mammals including cattle, pigs, sheep and horses are susceptible to cold shock in response to rapid exposure to low temperatures (Mazur 1965; Darin-Bennett *et al.* 1973; Watson *et al.* 1987; White 1993). In several of these species cold shock can be alleviated by the addition of egg yolk or low density lipoprotein (White 1993). This was observed in the present study with egg yolk having a protective effect when spermatozoa were rapidly cooled to 4 ºC and slowly cooled to 0 ºC. Egg yolk did not confer protection when spermatozoa were rapidly cooled to 0.5 ºC. The mechanism by which egg yolk is protective is not well understood but phospholipids are suggested to assist in membrane stabilisation (Watson and Martin 1975; Watson 1976). In the present study we observed that egg yolk was beneficial when used at a concentration of 10% to 20% and this is consistent with studies on several eutherian species (Cochran *et al.* 1984; De Leeuw *et al.* 1993). However in the cold shock prone ram, some extenders with egg yolk concentrations greater than 13.5% are sub optimal (Molinia *et al.* 1994) and in the Mohor gazelle (*Gazella dama mhorr*) 20% egg yolk is also suggested to be damaging (Holt *et al.* 1996). Our study indicated that 20% egg yolk may have some detrimental effect but this was not statistically valid. Nonetheless we suggest that future protocols use 10% egg yolk to avoid any possible negative effects of high yolk concentrations.

Slow cooling protocols can also overcome cold shock in the spermatozoa of some susceptible eutherians. Our study demonstrated that *S. crassicaudata* spermatozoa in egg yolk could not survive rapid cooling to 0.5 ºC, but could survive if the rate of cooling was slowed to 0.5 ºC min^{-1}. In the black footed ferret (*Mustela nigripes*) the cooling rate is required to be as low as 0.2 ºC min^{-1} with damage apparent when increased to 1 ºC min^{-1} (Santymire *et al.* 2007). Similar requirements are observed in domestic cats where slow cooling at 0.5 ºC min^{-1} is required to avoid morphological damage (Pukazhenthi *et al.* 1999). During cryopreservation slow cooling can be detrimental as spermatozoa are exposed to the potentially toxic cryoprotectants for longer (Mazur 1965). This is unlikely to be an issue for *S. crassicaudata* spermatozoa as a previous study in our laboratory has demonstrated that glycerol is not toxic to dasyurid spermatozoa (Czarny *et al.* 2009a and Chapter 3).

This study also demonstrated that caput spermatozoa, still carrying the cytoplasmic droplet, were as not as sensitive to cold shock compared to more mature spermatozoa when rapidly cooled to 0.5 ºC without egg yolk. Tolerance to cold shock in immature spermatozoa has been previously reported in eutherian livestock (Simpson *et al.* 1987; White 1993) and may be related to changes in the composition of membrane lipids during epididymal transit. However the relationship between of sperm membrane fatty acid saturation and tolerance to cold shock in marsupials is controversial and requires further examination. In addition the motility assessment was difficult due to the low initial motility values in the immature caput spermatozoa. Subsequent studies could reexamine cold shock in spermatozoa from the caput epididymidis, replacing motility with cell viability as an indicator of cold shock damage.

This study presents the first evidence that a high susceptibility to cold shock does occur in at least one marsupial. However, this can be overcome and spermatozoa can be successfully cooled to 0 ºC if combined with egg yolk and slowly cooled. These results now focus research on what may be the key factor in successful sperm cryopreservation in dasyurid marsupials- surviving cold shock and subsequent freezing protocols which acknowledge the necessity for slow cooling. The present study also suggests the need for more stringent, and consistent, methodology in determining the susceptibility to cold shock in the spermatozoa of other marsupials, and argues that cold shock may be a limiting factor in those marsupial species where sperm motility after cryopreservation remains poor.

CHAPTER 5

Comparison of the production, quality and *in vitro* maturation capacity of oocytes from untreated cycling and intermediate phase eSG treated fat tailed dunnarts (*Sminthopsis crassicaudata*)

PUBLISHED ARTICLE

Czarny, N. A., Garnham, J. I., Harris, M. S. and Rodger, J. C. Comparison of the production, quality and *in vitro* maturation capacity of oocytes from untreated cycling and intermediate phase eSG treated fat tailed dunnarts (*Sminthopsis crassicaudata*). *Reproduction* **138,** 23-31.

We J. I. Garnham, M. S. Harris and J. C. Rodger attest that the Research Higher Degree candidate Natasha Czarny had an integral role in the experimental design, carried out the research, analysed the data and prepared the publication entitled "Comparison of the production, quality and *in vitro* maturation capacity of oocytes from untreated cycling and intermediate phase eSG treated fat tailed dunnarts (*Sminthopsis crassicaudata*)". We advised on the experimental design, interpretation of results and preparation of the manuscript.

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Candidate signature…….(Natasha Czarny) Date……

CHAPTER 5: Comparison of the production, quality and *in vitro* maturation capacity of oocytes from untreated cycling and intermediate phase eSG treated fat tailed dunnarts (*Sminthopsis crassicaudata*)

5.1 INTRODUCTION

The fat tailed dunnart (*Sminthopsis crassicaudata*) is a small (10 to 20 g), short lived and generally solitary dasyurid marsupial found in central and southern Australia (Morton 1995). Sexual maturity in females is reached at three months of age and in captivity photoperiod manipulation allows continuous breeding until females reach 30 months of age (Smith *et al.* 1978; Bennett *et al.* 1990). The reproductive cycle lasts 31 days and in this polyovular species approximately 14 oocytes are ovulated, although up to 24 embryos have been observed (Smith and Godfrey 1970; Bennett *et al.* 1990). After a gestation period of thirteen days females give birth to supernumerary young, as only eight to ten teats are present (Godfrey and Crowcroft 1971; Bennett *et al.* 1990). The young who successfully attach to a teat are weaned at approximately 70 days (Godfrey and Crowcroft 1971).

The relative ease of housing, breeding and handling of *S. crassicaudata* makes them good experimental models for the study of assisted reproductive techniques (ART) for application to larger threatened dasyurids such as the Tasmanian devil (*Sarcophilus harrisii*) which is 6 to 8 kg and the northern quoll (*Dasyurus hallucatus*) which weighs 350 to 1200 g. There is an increasing role for the use of ART in marsupial conservation but difficulties arise in species where our understanding of reproductive physiology is limited. A key issue for the implementation of ART is the development of protocols to stimulate or synchronise female reproductive activity (Rodger *et al.* 2009)

The first study to report ovarian stimulation protocols in *S. crassicaudata* examined adults (greater than four months of age) and used 20 IU equine serum gonadotrophin (eSG), resulting in ovulation and mating but not live births (Smith and Godfrey 1970). Subsequent studies examined females at random points in their reproductive cycle and found that 3 to 5 month old females treated with 1 or 5 IU eSG ovulated within 5 to 6 days (Rodger *et al.* 1992a). However results were variable as only a low proportion of females ovulated and at the higher dose overstimulation was recorded (Rodger *et al.* 1992a). Additional investigations indicated that doses as low as 1.5 IU caused overstimulation and luteinisation of follicles in animals 3 to 6 months of age (M Smola

and JC Rodger, unpublished observations). Although *S. crassicaudata* embryos have been produced (Breed and Leigh 1996) and a single litter has been born (Rodger *et al.* 1992a) following the use of these protocols an unquantified proportion of stimulations were reported to have failed. Although reduced reliability is acceptable for morphological studies (Anderson and Breed 1993; Breed and Leigh 1996), it is less appropriate for the development of ART.

An explanation for the variable results may be the presence or absence of active corpora lutea (CL). In marsupials CL persist through the majority of the luteal phase and do not regress when the female is treated with exogenous hormones (Tyndale-Biscoe *et al.* 1974). To avoid these CL effects, ovarian stimulation has been carried out in juvenile females (less than 18 weeks of age) (Smith and Godfrey 1970). Alternatively reproductive monitoring can be carried out to avoid the luteal phase and studies in the stripe faced dunnart (*Sminthopsis macroura*) demonstrate superior results when females are stimulated during the intermediate or follicular phase (Hickford *et al.* 2001; Menkhorst *et al.* 2007). Reproductive monitoring involves examining the presence of urinary cornified epithelial cells (CEC) which are an indicator of elevated oestradiol concentrations and thus the late follicular phase (de Brux 1958). CEC can remain elevated for several days prior to an influx of leukocytes (Woolley 1990) and this period of time will be defined as the “cytological oestrus” for the purpose of this study. Monitoring CEC in *S. crassicaudata* has demonstrated that oestrus and mating occurs at times of increased CEC and that early zygotes are found during periods of high CEC (Godfrey 1969a; Godfrey and Crowcroft 1971; Bennett *et al.* 1979; Selwood 1987). However the precise timing of ovulation and its relationship to elevated CEC is known.

For studies examining oocyte-based ART for marsupials the collection of ovulated oocytes from the reproductive tract is not an option due to the secretion of the oviductal mucoid and shell coat which surrounds the oocyte and acts as a block to sperm penetration (Rodger 1990). Alternatively oocytes from pre-ovulatory ovarian antral follicles may be collected and reach nuclear maturation *in vitro*. In naturally cycling eutherians and marsupials *in vitro* maturation of oocytes from pre-ovulatory antral follicles is spontaneous and requires no exogenous hormonal support (Pincus and Enzmann 1935; Selwood and VandeBerg 1992). Spontaneous maturation also occurs in most marsupials following stimulation with eSG, but not FSH (Mate and Rodger 1993a; Mate and Buist 1999; Glazier *et al.* 2002). However in *S. macroura* eSG stimulated oocytes collected from antral follicles do not reliably reach nuclear maturation (Merry *et al.* 1995) and the addition of luteinising hormone (LH) to culture medium only resulted in maturation rates of 60% (Maleszewski and Selwood 2004).

This study aimed to develop high yield protocols for the collection of first polar body stage oocytes from two age classes (≤12 months and >12 months) of *S. crassicaudata*. To achieve this we used urinary CEC and the influx of leukocytes to examine ovarian activity, oocyte maturation and oocyte quality in the unstimulated cycle. Subsequently we stimulated females with eSG during the intermediate phase, as defined by CEC and leukocytes. The development of the ovary and nuclear status of oocytes was assessed three, four and five days later. Oocytes were placed also cultures without exogenous hormones and their nuclear development was assessed for up to 48 hours.

5.2 MATERIALS AND METHODS

5.2.1 Husbandry

S. crassicaudata were sourced from the Small Marsupial Facility at the University of Newcastle (Australia) and originated from the long established colony at the University of Adelaide (Australia). Females had no access to males and were housed in groups of up to four in opaque polypropylene boxes (420 mm x 280 mm x 160 mm) with a recycled paper substrate, shelters and paper to build nests. They had *ad. libitium* access to food (IAMS chicken adult cat food, Dayton, OH, USA) and water. The animals were exposed to a 16 hour light, 8 hour dark light cycle to promote constant breeding (Smith *et al.* 1978; Bennett *et al.* 1990). This study used breeding age females (≤12 months of age) and those who had been retired from the colony's breeding population (>12 months of age). Retired females were still capable of natural breeding and represent a readily available pool of oocyte donors for experimental studies while not compromising the sustainability of the breeding colony.

The use of protected native species was licensed by New South Wales National Parks and Wildlife Service (Australia) and all experiments were approved by Newcastle University Animal Care and Ethics Committee.

Reproductive state was assessed every second day by collection of urine onto glass slides overlaid with a glass coverslip (n=86). Samples were examined on a microscope at x250 and the proportion of CEC was assessed across no less than five fields of view. A three point scoring system was used to assess the presence of CEC (score 1: 1 to 2 cells per field of view, score 2: 3 to 8 cells per field of view and score 3: >8 cells per field of view). When samples contained score 2 CEC animals were checked daily until they reached score 3 followed by an influx of leukocytes- this day was designated as day 0 (D0). The success of CEC monitoring was determined by the proportion of monitored females for which oestrus was correctly nominated and reasons for exclusion from the protocol, such as reluctance to urinate or illness, were recorded.

5.2.2 Gross Morphology

Females were euthanised between 8 and 11 am by CO_2 inhalation and the reproductive tract was transferred into warm (35 ºC) phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) under a dissection microscope. The length and width of the uterus was measured with an eyepiece graticule and the uterine area was calculated as an index of uterine size using the formula for the area of an oval (π x half the width x half the length). Ovaries were removed, washed in warm PBS and transferred to bench medium which was comprised of HEPES buffered Dulbecco's Modified Eagle's Medium (H-DMEM pH 7.4, Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% (v/v) FCS (Trace Biosciences, Castle Hill, NSW, Australia), 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Invitrogen).

5.2.3 Follicular Morphology

The diameters of CL and follicles were measured with an eyepiece graticule CL were measured within the ovary but individual follicles were isolated by dissection, using a 27.5 gauge needle mounted on a 0.5 mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Follicles were classed as preantral if no space between the oocyte and the follicle was observed and antral when a crescent shaped space was seen.

5.2.4 Oocyte Collection and Staining

Syringe mounted needles were used to flush ovulated oocytes from the uterus or extrude granulosa cell-oocyte complexes (GOC) from the isolated follicles. Oocytes were either used for culture without further treatment (see below) or stripped of granulosa cells using a glass pipette and stained to determine the state of maturation. The degree of granulosa cell adhesion was recorded then oocytes were washed in PBS, stained for 20 minutes (min) in 10 µg mL^{-1} Hoechst (H33342, Sigma-Aldrich) and then washed three times in PBS (Mate and Buist 1999). To aid in visualisation the oocytes were mounted on slides with the coverslip supported by a 9:1 mixture of Vaseline and candle wax.

Nuclear maturation was assessed by viewing on an inverted microscope (Zeiss, Jena, Germany) at x200 and x400 using filter set 2 (exciter filter 365 nm, emission filter 420 nm). Oocytes were classed as germinal vesicle (GV), germinal vesicle breakdown (GVBD), first polar body stage (PB1), parthenogenetic or fragmented.

5.2.4.1 Experiment 1- Timetable of reproductive events in natural cycles

To determine the best time for stimulation with eSG three time points in the reproductive cycle were examined- day 0 (D0) represented the oestrous period, day 16 (D16) represented the intermediate phase and day 20 (D20) represented the follicular phase. At D0, 16 and 20 retired females (> 12 months of age) were assessed as described above. Breeding females (≤ 12 month of age) were also assessed at D0 and D20 to identify the presence of age related differences in the number of oocytes ovulated and antral follicles. In all females the size and contents of the uterus, size and state of the ovaries including CL and follicles, the adherence of granulosa cells and nuclear maturation of oocytes were examined.

Initial experiments indicated high variation in the oocyte quality of D0 females which appeared to be related to the period of time that elevated CEC were observed prior to the influx of leukocytes- defined as the length of the cytological oestrus. To further examine this aspect, oocyte quality was compared in D0 females which were grouped according to the length of their cytological oestrus being two (n=3), three (n=3) or four or more (n=3) days. To determine the frequency of occurrence, the length of cytological oestrus was assessed for each of the 75 cycles where oestrus was determined.

5.2.4.2 Experiment 2- Stimulation of females with eSG

Females were given 1 IU eSG (Folligon, Intervet, Boxmeer, Holland) i.p. whilst anesthetised with 4% isofluorane (Virbac Animal Health, Peakhurst, NSW, Australia) between 8 and 11 am on D16 of their cycle. The welfare of stimulated females was examined daily with a specifically developed *S. crassicaudata* nine point monitoring scale which assessed body condition (such as coat condition and tail width) and behavioural aspects (such as shallow breathing and isolation from cage mates). Females were euthanised three (D16+3), four (D16+4) and five (D16+5) days following stimulation and processed as described above. Each group contained at least four individuals and breeding and retired females were assessed separately.

Oocyte maturation without exogenous hormone supplementation was carried out on GOC from breeding (n≥4) and retired (n≥3) D16+3 and retired D16+4 (n≥5) females. GOC were pipetted by mouth from bench medium into sterile culture medium containing Dulbecco's Modified Eagle's Medium (DMEM- pH 7.4, Sigma-Aldrich) supplemented with 10% (v/v) FCS, 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Selwood 1987; Merry *et al.* 1995). Nuclear maturation of the stripped oocytes was assessed upon collection and for up to 48 hours using H33342 as described above.

5.2.5 Statistical Analysis

Mean values were presented ± standard error of the mean (SEM) and significant differences were determined using T-tests in Microsoft Excel or analysis of variance (ANOVA) followed by post-hoc Tukey's tests in JMP (SAS Institute Inc. Cary, NC, USA) for parametric data. Oocyte maturation data did not follow a normal distribution and was assessed with the non parametric Kruskal-Wallis one way ANOVA in JMP.

5.3 RESULTS

Cytological oestrus was identified in 72 (84%) of the 86 animals monitored. General health issues or pouch tumours (identified as benign fibroadenoma or malignant adenocarcinoma) caused ten animals to be removed from the experiment and fourteen animals were unable to be monitored as they regularly defecated upon handling or would not urinate.

5.3.1 Experiment 1- Timetable of Reproductive Events in Natural Cycles

In natural cycles females assessed at D0 had recently ovulated and their uterine tissues were large, turgid and well vascularised (Table 5-1). There was no significant difference in the number of oocytes ovulated in the breeding (10.60 ± 1.16 oocytes female^{-1}, n=5) and retired (11.75 ± 1.94 oocytes female^{-1}, n=8) females. At D16 uterine tissues were less vascularised and smaller ($P<0.05$, Table 5-1) and the ovary was dominated by large CL (Table 5-1). No antral follicles were observed and oocytes collected from preantral follicles were tightly surrounded by granulosa cells which could not be removed by manual pipetting (Figure 5-1A). By D20 the CL had an irregular, creased surface and were smaller ($P<0.001$, Table 5-1). A small number of antral follicles were observed and the number of antral follicles observed in breeding (4.75 ± 0.59, n=4) and retired (3.4 ± 0.08, n=5) females was not significantly different; hence the data was combined for subsequent analysis. Oocytes extruded from antral follicles were surrounded by granulosa cells which when removed revealed GV stage oocytes (n=32, Figure 5-1B,C).

Table 5-1: Reproductive attributes of naturally cycling fat tailed dunnarts (*Sminthopsis crassicaudata*) at Day 0 (n=13), determined by the presence of leukocytes following a period of high cornified epithelial cells in urine samples, day 16 (n=4) and day 20 (n=9). Values with different letters are significantly different. P at least < 0.05.

	Uterine size (mm^2)	Corpora lutea diameter (µm)	Antral follicles	Ovulation
Day 0	51.57 ± 9.07 ^{A}	396.20 ± 12.55 ^{A}	No	Yes
Day 16	13.27 ± 0.68 ^{B}	525.28 ± 14.62 ^{B}	No	No
Day 20	8.69 ± 0.86 ^{B}	397.55 ± 8.73 ^{A}	Yes	No

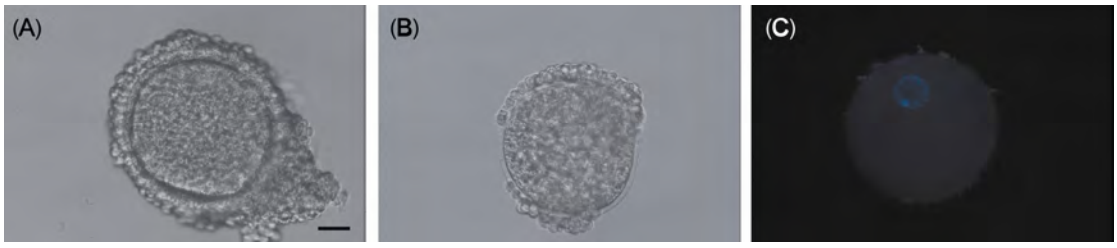

Figure 5-1: Fat tailed dunnart (*Sminthopsis crassicaudata*) granulosa cell-oocyte complex (GOC) from a preantral follicle collected (A) at day 16 with tightly bound granulosa cells or (B) at day 20 GOC where the granulosa cells are able to be removed to (C) reveal a germinal vesicle stage oocyte following staining with Hoechst 33342. Bar=40 µm.

5.3.2 Effect of the Length of Cytological Oestrus on Ovulated Oocyte Quality

Females with a two day cytological oestrus had oocytes with a visible yolk mass, two polar bodies and female pronuclei (n=24) (Figure 5-2A,B). Cleavage was rare but if present it represented parthenogenetic activation with both cells indicating morphologically normal nuclear material. Females who had a three day cytological oestrus were mostly pre-cleavage or parthenogenetic (Figure 5-2C,D) but 15% (n=26) showed fragmentation and contained segments lacking nuclear material. In females who had a cytological oestrus which lasted four or more days, 45% of oocytes were fragmented (n=36) (Figure 5-2E,F).

The length of cytological oestrus was determined in 52 (69%) of the 75 cycles where oestrus was determined due to the alternate day sampling regime (n=72, three animals cycled twice). There was no discernable pattern in the proportion of females which had a cytological oestrus for two, three or four or more days although the latter was the most common occurring in 22 (42%) of the 52 defined cycles.

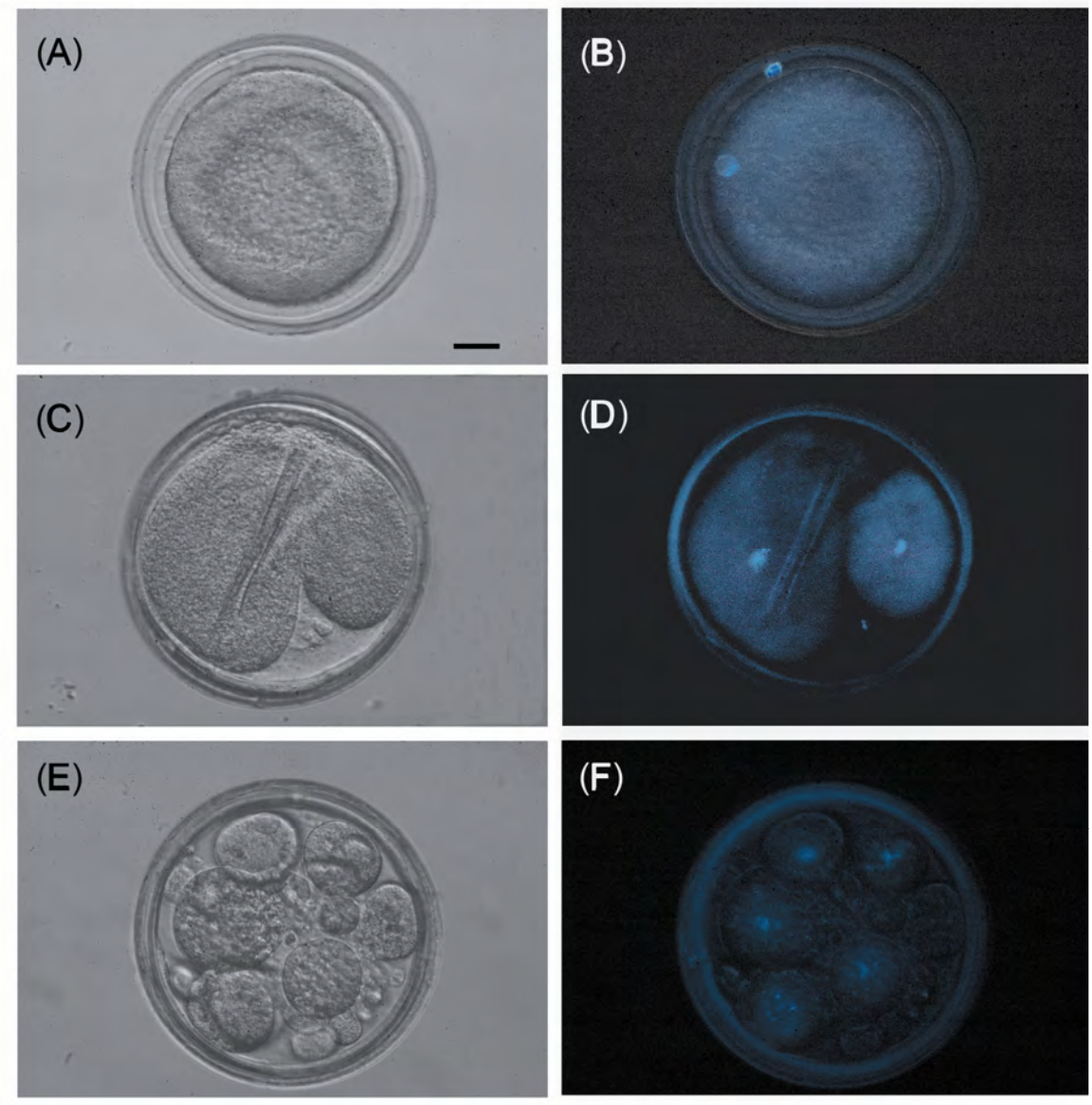

Figure 5-2: Morphology of naturally ovulated oocytes from the fat tailed dunnart (*Sminthopsis crassicaudata*) collected after a cytological oestrus which lasted: (A,B) two days showing polar body extrusion and a pronuclei, (C,D) three days showing parthenogenesis with nuclear material in each cell or (E,F) four or more days showing fragmentation seen with (A,C,E) light microscopy or (B,D,F) following staining with Hoechst 33342. Bar=40 µm.

5.3.3 Experiment 2- Stimulation of Females with eSG

Stimulation increased the number of antral follicles in both breeding and retired females at D16+3 ($P<0.001$ and $P<0.05$ respectively) and D16+4 ($P<0.001$) when compared to unstimulated D20 females but retired females had less antral follicles than breeding females ($P<0.05$ and $P<0.01$) (Figure 5-3).

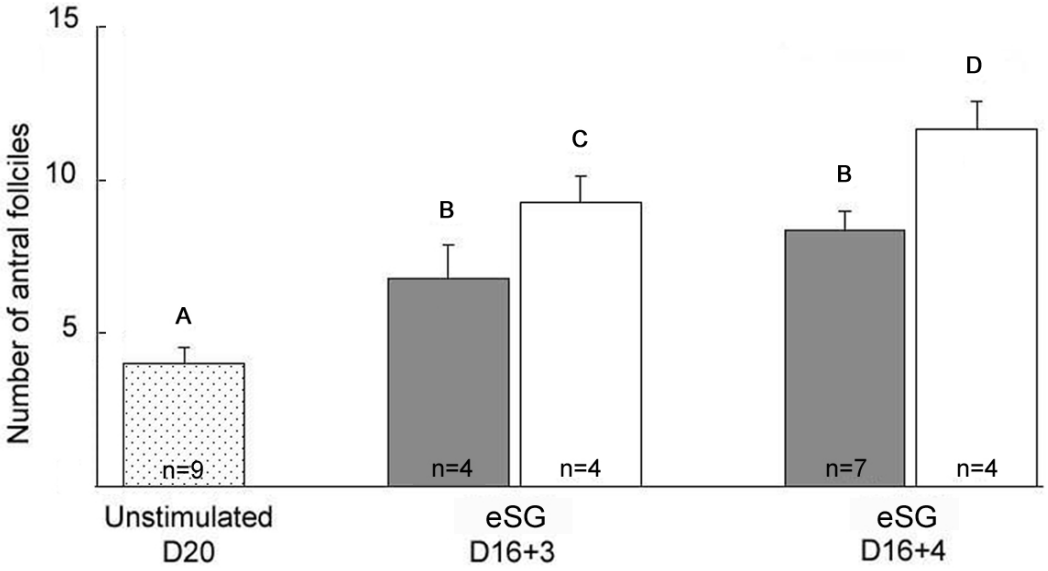

Figure 5-3: The number of antral follicles the fat tailed dunnart (*Sminthopsis crassicaudata*). Data is combined at day 20 (D20) of an unstimulated cycle and presented separately from retired females (>12 months of age, filled bars) and breeding females (≤12 months of age, unfilled bars) either three (D16+3) or four (D16+4) days following stimulation with 1 IU equine serum gonadotrophin on day 16 of their cycle. Values with different letters are significantly different. P at least <0.05.

Stimulation increased the size of antral follicles in both breeding and retired females. Antral follicles from D16+3 ($P<0.01$) and D16+4 ($P<0.001$) were larger than those from unstimulated D20 females and D16+4 antral follicles were larger than those from D16+3 breeding ($P<0.001$) and retired females ($P<0.01$) but the D16+4 antral follicles from retired females were smaller than those from younger breeding females ($P<0.001$, Figure 5-4). No ovulation was observed in D16+3 or D16+4 breeding or retired females.

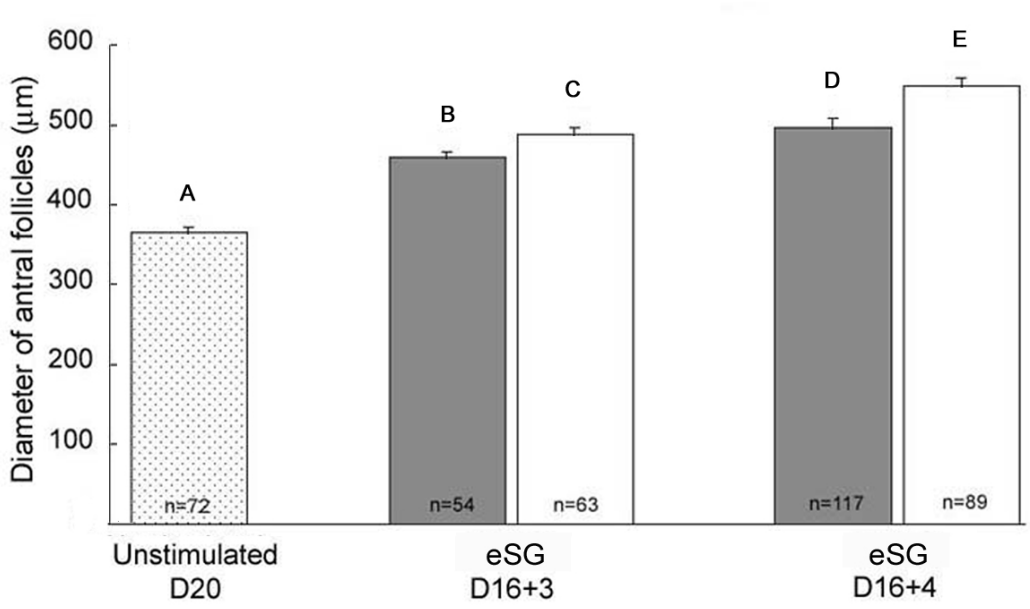

Figure 5-4: The diameter of antral follicles in the fat tailed dunnart (*Sminthopsis crassicaudata*). Data is combined at day 20 (D20) of an unstimulated cycle and presented separately from retired females (>12 months of age, filled bars) and breeding females (≤12 months of age, unfilled bars) either three (D16+3) or four (D16+4) days following stimulation with 1 IU equine serum gonadotrophin on day 16 of their cycle. Values with different letters are significantly different. P at least <0.01.

5.3.4 *In Vitro* Maturation

Upon collection from breeding females three days following eSG stimulation oocytes were GV or GVBD (n=23) and after 48 hours in culture only 20.83 ± 20.83% demonstrated nuclear maturation to the PB1 stage (n=18). Prior to culturing only 17.50 ± 9.38% of oocytes from retired females had undergone GVBD (n=22) and after 48 hours 38.63 ± 8.61% had reached the PB1 stage, with none remaining at the GV stage (n=26). Oocytes harvested from breeding females at D16+4 were all at the PB1 stage (n=33) upon collection and appeared normal with no evidence of fragmentation (Figure 5-5A,B). In retired females collected at D16+4, 77.06 ± 8.68% of oocytes had undergone GVBD but only 6.67 ± 6.67% had reached the PB1 stage (n=34). Following *in vitro* culture for 24 hours the proportion of PB1 oocytes increased to 62.06 ± 14.02% ($P<0.01$, n=34) and by 48 hours 98.18 ± 1.92% of oocytes had achieved nuclear maturation to the PB1 stage (n=26). There was no evidence of fragmentation of *in vitro* matured oocytes in either breeding or retired females (Figure 5-5C,D).

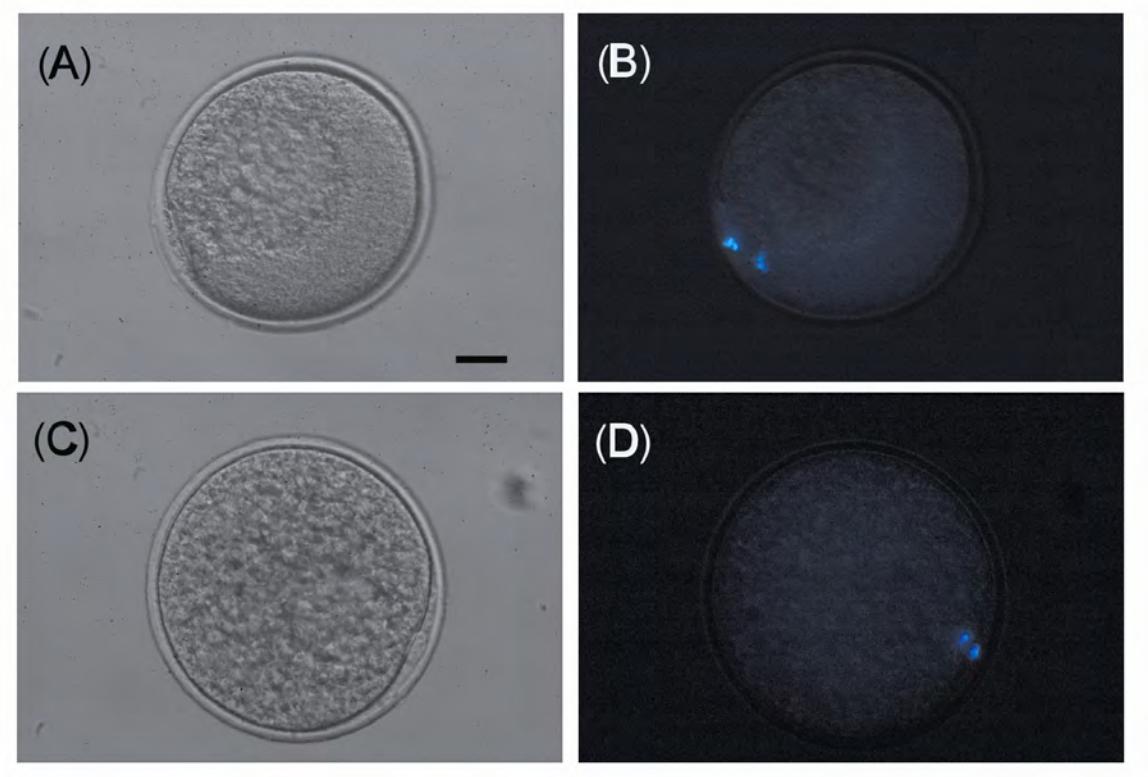

Figure 5-5: First polar body stage oocytes from a breeding age (≤12 months of age) fat tailed dunnart (*Sminthopsis crassicaudata*) (A,B) upon collection, or (C,D) a retired (>12 months of age) female *S. crassicaudata* following 24 hours *in vitro* culture after stimulation with 1 IU equine serum gonadotrophin on D16 of their cycle seen with (A,C) light microscopy or (B,D) following staining with Hoechst 33342. Bar=40 μm.

5.3.5 Induced Ovulation

Ovulation was observed in five out of six breeding D16+5 females but oocytes were low quality demonstrating evidence of cytoplasmic breakdown, blebbing and non-specific cytoplasmic staining (Figure 5-6). Only two out of six retired females had ovulated by D16+5, and oocytes were again degraded. Hormone administration was confirmed in D16+5 retired females whom had not ovulated by the presence of highly vascularised uterine tissues which were larger than that of D0 retired females (84.26 ± 9.56 mm^2, n=4, $P<0.05$). The rate of ovulation in breeding (17.00 ± 2.17 oocytes female^{-1}, n=5) and retired females (17.5 ± 0.50 oocytes female^{-1}, n=2) was not significantly different. However both were greater than the natural rate of ovulation for retired ($P<0.01$) and breeding females ($P<0.05$) as presented in Experiment 1.

Figure 5-6: Degraded ovulated oocytes from female fat tailed dunnarts (*Sminthopsis crassicaudata*) stimulated with 1 IU equine serum gonadotrophin on D16 of their cycle and collected from the uterus five days later viewed with a light microscope (A) or following staining with Hoechst 33342 (B,C). Bar=50 µm.

During this study there was no evidence of overstimulation, typified by opaque follicles still containing oocytes, or negative effects of the stimulation protocol on *S. crassicaudata* with regular activity, grooming and socialisation maintained. However increased aggression towards researchers typified by vocalisations and biting was observed following eSG treatment as is seen during the normal cytological oestrous period (personal observation).

5.4 DISCUSSION

The results from this study provide the first detailed description of follicular development at several points during the oestrous cycle in unstimulated and eSG stimulated *S. crassicaudata*. We demonstrate that a significantly larger cohort of oocytes can be harvested from antral follicles following stimulation with 1 IU eSG and that if collected four, but not three, days post stimulation they reliably undergo nuclear maturation to the PB1 stage without additional hormone supplementation. We have also shown that females older than 12 months of age have a reduced response to eSG stimulation, but this difference is not apparent in non-stimulated cycles.

5.4.1 Cornified Epithelial Cell Monitoring

The period of increased CEC, dictated by elevated oestradiol concentrations that result in the desquamation of vaginal epithelial cells, was used in this study to designate cytological oestrus (de Brux 1958; Regli and Kress 2002). Monitoring CEC in *S. crassicaudata* had been described by Godfrey (1969a), Godfrey and Crowcroft (1971) and Bennett *et al.* (1979) who reported oestrus and mating at times of increased CEC. But the present study was the first to specifically examine the timing of ovulation.

Our findings demonstrated that the presence of leukocytes in urine samples did not indicate the day of ovulation as seen in *S. macroura* (Selwood and Woolley 1991). Nor did ovulation coincide with a decrease in CEC as observed in another dasyurid, the brown antechinus (*Antechinus stuartii*) (Selwood 1980). Instead the increasing degradation of ovulated oocytes from females with an increasingly long cytological oestrus indicated that ovulation occurs when CEC are initially elevated. This concept is supported by the observations of Smith and Godfrey (1970) who describe the onset of oestrus marked by increasing CEC and ovulation occurring soon after this point in *S. crassicaudata.* Furthermore Selwood (1987) describe *S. crassicaudata* having early zygotes during periods of high CEC and 4 to 16 cell embryos following the decline of CEC. Finally, preliminary investigations into specifically timed *S. crassicaudata* breeding trials have indicated that pairs mate on the first day of score 3 CEC but not once leukocytes are observed (data not shown). Hence we suggest that the influx of leukocytes is indicative of the end of the oestrous period. Although not indicative of ovulation, in this study the presence of leukocytes was maintained as D0 as it allows allocation of a defined time point without retrospective assessment. The underestimation in timing of events caused by the nominated D0 falling one to four days after ovulation means that females may be further into the intermediate phase than initially assumed. However, the potential variability in endocrine status was not reflected in the response to eSG treatment, which was highly consistent.

5.4.2 Parthenogenesis and Fragmentation in Naturally Ovulated Oocytes

The parthenogenetic activation seen in oocytes from females with a cytological oestrus of three days was not unexpected, nor is the degradation and fragmentation which occurs after a four day cytological oestrus. Parthenogenesis is reported in 35% of uterine oocytes collected from eSG stimulated *S. crassicaudata* and fragmentation is reported for those which have undergone more than two divisions (Anderson and Breed 1993). This has been suggested to relate to inbreeding within the *S. crassicaudata* captive colony (Anderson and Breed 1993) and the increased rates of parthenogenetic activation observed in the present study may represent further inbreeding pressure within the *S. crassicaudata* colony 15 years on. Nonetheless the colony's ongoing ability to breed naturally indicates that oocytes are still capable of fertilisation and production of live young.

5.4.3 Timetable of Events in Natural Cycles

Given that the reproductive cycle of *S. crassicaudata* lasts 31 days with a gestational period of 13.5 days (Godfrey and Crowcroft 1971; Bennett *et al.* 1990) we suggest that if D0 is nominated as the end of oestrus then D16 females will be within the intermediate phase. This was observed experimentally with D16 females indicating no follicular development. D16 females also demonstrated the largest CL but it is unlikely that these were still secreting progesterone as they were smaller in size than mid to late-luteal phase CL from other dasyurids (Woolley 1966; Selwood and Woolley 1991). In addition progesterone concentrations decline before regression of the CL which can occur at parturition or the end of a non-pregnant cycle (Hinds and Selwood 1990; Woolley 1990; Selwood and Woolley 1991). The smaller size of the CL and the evidence of consistent patterns of dasyurid luteal regression in the literature suggested that the D16 ovary was not governed by progesterone and would be receptive to exogenous stimulating factors as described in studies of *S. macroura* (Hickford *et al.* 2001; Menkhorst *et al.* 2007).

Females examined at D20 were expected to be in the follicular phase and have a young cohort of small hormone dependent antral follicles. This was demonstrated experimentally in the current study which showed that oocytes were at the GV stage and had not begun nuclear maturation. Furthermore the CL were rapidly shrinking which has also been previously reported in other dasyurids (Hinds and Selwood 1990; Selwood and Woolley 1991).

The observation that mechanical removal of granulosa cells from the D16 oocytes was not possible, but that their removal from D20 oocytes was, is likely to be due to immature GOC maintaining cellular processes from granulosa cells which form gap and tight junctions through the zona pellucida for endocrine and nutritional support of the oocyte (Breed and Leigh 1990). This was not observed in more mature GOC in preparation of the complete removal of granulosa cells prior to ovulation (Breed and Leigh 1988). Removal of adherent granulosa cells with hyaluronidase was described in *S. macroura* (Merry *et al.* 1995) but was not able to be reliably achieved in the present study (data not shown), assumedly because marsupials do not utilise hyaluronic acids within their surrounding cell layers (Chapman and Breed 2006). Nonetheless subsequent trials have shown treatment with the protease trypsin to be more successful (2.5 g mL^{-1} for 5 min, NA Czarny, unpublished observations).

5.4.4 Stimulation of Females with eSG

Superovulation has been used in monovular marsupials such as the Tammar wallaby (*Macropus eugenii)* and brushtail possum (*Trichosurus vulpecula*) to overcome the selection of one dominant follicle (Rodger and Mate 1988; Molinia *et al.* 1998b) but these techniques may also be used with polyovular dasyurids to increase, control and manipulate oocyte maturation (Rodger *et al.* 1992a; Merry *et al.* 1995). This study demonstrated that a timed 1 IU eSG stimulation protocol resulted in the recruitment of significantly more follicles than unstimulated cycles, and this increase was greater in younger (≤12 month of age) breeding females. Oocytes from breeding females harvested four days following eSG stimulation were mature and those collected from retired females were reliably cultured to the PB1 stage within 48 hours.

5.4.5 *In Vitro* Maturation

In vitro maturation is an important step towards the generation of protocols for the collection of gametes for future studies of *in vitro* fertilisation or intracytoplasmic sperm injection. This is the first report of a reliable protocol for harvesting GVBD oocytes in *S. crassicaudata* and their subsequent culture to the PB1 stage in the absence of endocrine support. The ability of oocytes collected four days following stimulation to undergo nuclear maturation to the PB1 stage is not unexpected as maturation of oocytes from antral follicles in natural cycles and eSG stimulated females is spontaneous and requires no additional hormonal support in many species, including marsupials (Pincus and Enzmann 1935; Selwood and VandeBerg 1992; Mate and Rodger 1993a; Glazier *et al.* 2002). We also show that the less mature oocytes

collected three days following stimulation with eSG could not reliably reach the PB1 stage within 48 hours in the absence of exogenous hormones, similar to the outcomes in granulosa cell enclosed oocytes from *S. macroura* (Merry *et al.* 1995). Subsequent experiments are suggested to determine the maturation potential of these follicles following longer *in vitro* maturation or the addition of exogenous LH.

Oocytes harvested from breeding females four days following stimulation were the most mature, with 100% at the first polar body stage, those harvested from smaller antral follicles of retired females were predominantly GVBD. Although there is a significant size difference between antral follicles in these two datasets, GVBD oocytes were collected from follicles as large as 650 µm and first polar body stage oocytes were collected from follicles as small as 437 µm. This variation in follicle size makes establishing a threshold follicle size for collection of first polar body stage oocytes difficult, however the production of first polar body stage oocytes can still be readily achieved by the robust *in vitro* maturation protocol or use of breeding age females.

5.4.6 The LH Surge and Induced Ovulation

Although eSG mimics the effect of FSH and LH, some marsupials require an additional drug to induce the resumption of meiosis and ovulation (Rodger and Mate 1988; Jungnickel *et al.* 2000). In *S. crassicaudata* the eSG stimulation was reported sufficient to generate an endogenous LH surge (Rodger *et al.* 1992a). This is supported by the present study as the resumption of meiosis (observed as GVBD) occurs as early as three days following stimulation, presumably in response to the LH surge.

LH activity is also indicated by the high rate of ovulation observed in breeding females when examined five days following stimulation. However the response to stimulation was less consistent in retired females where only two out of six females ovulated. Nonetheless these females may have ovulated given an extra day as their uterine tissues demonstrated increased vascularisation, an effect of increased concentrations of luteinising hormone. Previous studies saw Rodger *et al.* (1992a) stimulate adult *S. crassicaudata* with 1 IU eSG during undefined stages of the reproductive cycle and found ovulation occurred in most females within six, but not five, days. Variable rates of ovulation were also observed with an un-timed 1 IU eSG protocol examining animals which were on average 12 months of age (Anderson and Breed 1993; Breed *et al.* 1994b; Breed and Leigh 1996). Finally, similar results were observed in *S. macroura* which were induced in the intermediate phase, where although the timing of ovulation was improved from prior studies, it varied by up to four days (Menkhorst *et al.* 2007).

In the females which did ovulate, the oocytes were degraded and blebbing with non-specific nuclear staining. Although eSG has a long half life and can result in overstimulation (Smith and Godfrey 1970; Rodger *et al.* 1992a; Hickford *et al.* 2001), this is unlikely to be the case in the present study as the dose used was up to 20 times lower than that used in previous *Sminthopsis* studies (Smith and Godfrey 1970; Menkhorst *et al.* 2007). We also observed no luteinised follicles or negative effects which were examined on a nine-point animal monitoring scale. Our low dose was however sufficiently high to stimulate the growth of increased numbers of follicles and initiate their maturation. Instead, degraded ovulated oocytes may be the result of a delayed LH surge causing oocytes to be retained in the follicle for longer than necessary (Malhi *et al.* 2006). It would be of interest to increase our understanding of this by examining the endocrine profiles or CEC patterns of stimulated females in subsequent studies as examined following stimulation in *M. eugenii* (Jungnickel and Hinds 2000).

5.4.7 Improvement to the Protocol

The present study describes increased consistency following stimulation with eSG at D16 compared to previous untimed studies (Smith and Godfrey 1970; Rodger *et al.* 1992a; Anderson and Breed 1993; Breed and Leigh 1996). Furthermore we found age based differences in the response to stimulation which may also have contributed to the variation observed in previous studies. This age based difference in ovarian activity was not observed in natural cycles but following stimulation older females had fewer and smaller antral follicles and their oocytes were less mature than those of younger females. Our results are not surprising as *S. crassicaudata* is a short lived species with a limited reproductive life and a reduced response to ovarian stimulation with increased age is also observed in other species. Older cows have a reduced number of recruited follicles after superovulation (Malhi *et al.* 2006) and older women have a reduced response to exogenous stimulatory hormones (Jacobs *et al.* 1990; Dew *et al.* 1998).

This age factor is an important finding which unexpectedly reveals an easily avoidable source of variation observed in previous studies examining ovarian stimulation in *S. crassicaudata*. Our results have the additional benefit of describing the use of retired animals for experimental applications, thus developing a tool for more efficient use of oocyte donors and improved colony management.

5.4.8 Conclusion

This study has resolved two major sources of variation in ovarian stimulation protocols. We have increased the reliability of the response to stimulation by treatment during the intermediate phase and we describe a reduced response to stimulation in older females. An understanding of these aspects has led to the development of a high yield protocol which enables the collection of oocytes able to be cultured to the PB1 stage without exogenous hormones. Oocytes demonstrating nuclear maturation can be used in future studies of ART in dasyurid marsupials including the molecular and physical aspects of fertilisation, stimulation of non-seasonal breeding and synchronisation of oestrous cycles for subsequent embryo transfer.

CHAPTER 6

Dissociation and preservation of preantral follicles and immature oocytes from female dasyurid marsupials

PUBLISHED ARTICLE

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We M. S. Harris and J. C. Rodger attest that the Research Higher Degree candidate Natasha Czarny had an integral role in the experimental design, carried out the research, analysed the data and prepared the publication entitled “Dissociation and preservation of preantral follicles and immature oocytes from female dasyurid marsupials”. We advised on the experimental design, interpretation of results and preparation of the manuscript.

Name……..

Signature…….. Date……

Name……..

Signature…….. Date……

Candidate signature…….(Natasha Czarny) Date……

CHAPTER 6: Dissociation and preservation of preantral follicles and immature oocytes from female dasyurid marsupials.

6.1 INTRODUCTION

Dasyurids are carnivorous marsupials which play the important role of native predators in Australian ecosystems. However almost 40% of the dasyurid family is considered threatened, including the northern quoll (*Dasyurus hallucatus*) a species in which entire populations are lost when the poisonous introduced cane toad (*Bufo marinus*) enters their environment (Oakwood 2004) and the Tasmanian devil (*Sarcophilus harrisii*) which suffers Tasmanian Devil Facial Tumour Disease (Siddle *et al.* 2007). In addition to ecosystem management and captive breeding programs, assisted reproductive techniques (ART), including genome resource banking, can contribute to conservation by preserving the genetic diversity presently in the environment.

Successful ART has been carried out in wild bovine, canine and feline species which have closely related well studied models (Farstad 2000; Lanza *et al.* 2000). Recently these techniques have been applied to more diverse wildlife (Pukazhenthi and Wildt 2004), including marsupials (Rodger *et al.* 2009). One aspect of ART, yet to be examined in dasyurids, is the preservation of the female gamete. The mammalian ovary contains large quantities of preantral follicles which are present throughout the oestrous cycle of reproductively active females, unlike antral follicles which only grow during times of oestrus (Shaw *et al.* 2000). The relative stability of preantral follicles justifies studies into the development of genome resource banking but in order to achieve this, viable protocols for their transport, isolation, preservation and *in vitro* culture must first be developed.

Tissues destined for genome resource banks are often collected in the field or at zoological institutions which are isolated from scientific laboratories; hence it is important to establish the environmental conditions in which tissues should be transported. Although low temperature storage is beneficial because it limits cellular metabolism, oxidative damage, edema and autolysis (Churchill 2004) it can also be detrimental to the survival of oocytes. Cooled mature oocytes suffer spindle depolymerisation which results in chromosomal abnormalities (Sathananthan *et al.* 1992; Liu *et al.* 2003) and immature follicles exposed to low temperatures degenerate or have reduced developmental competence in some agricultural species (Didion *et al.* 1990; Wu *et al.* 1999). There are no published reports on the effect of cold storage on immature follicles in dasyurids.

Although the female gamete is traditionally preserved in ovarian slices, individual isolated follicles may also be examined (Carroll *et al.* 1990; Jewgenow *et al.* 1998; Shaw *et al.* 2000). Isolation of ovarian follicles may be achieved by enzymatic methods which allow the collection of very small preantral follicles, has a high yield and has proven successful in several laboratory species (Roy and Greenwald 1985; Eppig and Schroeder 1989; Telfer 1996). However mechanical isolation is required in agricultural species which have tough ovarian connective tissue (Telfer 1996) or suffer damage from the enzymatic cocktail (Nicosia *et al.* 1975; Figueiredo *et al.* 1993). Enzymatic dissociation has not been examined in any marsupial but mechanical methods have been used to isolate large preantral follicles from the grey short tailed opossum (*Monodelphis domestica)* (Butcher and Ullmann 1996), secondary follicles from the Tammar wallaby (*Macropus eugenii*) (Richings *et al.* 2006) and preantral follicles from the stripe faced dunnart (*Sminthopsis macroura*) (Nation and Selwood 2005).

An advantage of preserving isolated follicles is the selection of those which contain immature oocytes. Smaller oocytes, arrested at the germinal vesicle stage are able to withstand sub zero temperatures more successfully than larger oocytes due to the large surface area to volume ratio and the arrested meiotic state which protects DNA and limits temperature induced cytoskeletal damage (Eroglu *et al.* 1998; Shaw *et al.* 2000). Slow freezing has been used to cryopreserve preantral follicles (Carroll *et al.* 1990; Jewgenow *et al.* 1998) but recent studies describe the benefits of vitrification of follicles and oocytes (Vajta *et al.* 1998; Vajta 2000; Yamada *et al.* 2007). Vitrification uses highly concentrated cryoprotectants and ultra-rapid freezing to create a glass like state and avoid ice crystal formation which can damage cell membranes (Vajta 2000).

In order to be used for ART following preservation, follicles need to be maintained in an *in vitro* culture system and in well studied laboratory and agricultural species successful *in vitro* oocyte maturation and fertilisation has been achieved (Eppig and Schroeder 1989; Hirao *et al.* 2004). The enzymatic dissociation protocol removes the basement membrane and thecal cells from isolated follicles and this results in the migration of granulosa cells away from the oocyte during *in vitro* culture. This is detrimental as continued growth and maturation requires communication between the granulosa cells and the oocyte (Anderson and Albertini 1976; Eppig 1979). In order to maintain this cellular contact and restrict the migration of granulosa cells away from the oocyte, follicles must be grown on a substrate (Eppig and Schroeder 1989). There has been little study into the development of such techniques in marsupials and no oocytes from marsupial preantral follicles have been cultured to the polar body stage (Butcher and Ullmann 1996; Nation and Selwood 2005; Richings *et al.* 2006).

This study examined the potential for cryostorage of the female gamete from native Australian dasyurid marsupials. We aimed to determine the optimal transport conditions for dasyurid ovarian tissue and establish a high yield follicle isolation protocol assessing survival with the aid of an *in vitro* follicle classification system. Subsequently the feasibility of these follicles to be used in further ART was examined by the investigation of vitrification and the potential for follicle repair following a short period of *in vitro* culture.

6.2 METHODS

6.2.1 Husbandry

The fat tailed dunnart *(Sminthopsis crassicaudata*) and the eastern quoll (*Dasyurus viverrinus*) were housed as described in Czarny *et al.* (2008). At random points in their reproductive cycle *S. crassicaudata* were euthanised by CO_2 inhalation and *D. viverrinus* were euthanised by >1 mL overdose of intra-cardiac sodium pentobarbitone (Lethabarb, Laser Animal Health, Salisbury, SA, Australia) whilst anesthetised with 4% isolfuorane (Virbac Animal Health, Peakhurst, NSW, Australia). Immediately after death the reproductive tract was dissected into pre-warmed (35 ºC) phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) and ovaries were removed and washed in fresh warm PBS. Opportunistically collected ovaries were available from seven *D. hallucatus* supplied by Territory Wildlife Park (Berry Springs, NT, Australia). Females were greater than two years of age and had either recently weaned their young (n=5) or were non-breeding (n=2). Euthanasia was performed as for *D. viverrinus* and ovarian tissue was collected onsite as described above before either being transported at 35 ºC and processed within four hours or transported on ice and processed within 48 hours of collection. Opportunistically collected ovaries were also available from four reproductively active *S. harrisii* supplied by The Department of Primary Industries and Water, Wildlife Management Branch (Prospect, Tasmania, Australia). Females were either carrying pouch young or had recently weaned their young and were suffering the effects of Tasmanian Devil Facial Tumour Disease, which led to their euthanasia. Euthanasia was performed as for *D. viverrinus* and ovarian tissue was collected onsite, transported on ice and processed within 48 hours.

The export and use of protected native species was licensed by New South Wales (NSW) National Parks and Wildlife Service and the Tasmanian Department of Primary Industries and Water (Australia). All experiments were approved by Newcastle University Animal Care and Ethics Committee which abides by the NSW Animal Research Act, NSW Animal Research Regulation, and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

6.2.2 Ovarian Dissociation

Ovarian tissue was sliced into 3 to 5 mm^2 pieces and incubated at 35 ºC in dissociation medium comprised of 0.1% collagenase and 0.02% DNAse in HEPES buffered high glucose (4.5 g L^{-1}) Dulbecco's Modified Eagle's Medium (DMEM, pH 7.4, all purchased from Sigma-Aldrich, St Louis, MO, USA). After 30 minutes (min) the solution was neutralised with an equal volume of bench medium containing high glucose HEPES buffered DMEM supplemented with 10% (v/v) fetal calf serum (FCS, Trace Biosciences, Castle Hill, NSW, Australia), 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Invitrogen). Tissue was then manually agitated with a pipette and individual follicles were then transferred into fresh bench medium for viability assessment as described in section 6.2.7. In this study we will refer to the isolated follicles as granulosa cell-oocyte complexes (GOC), not the more commonly used cumulus cell-oocyte complexes, as marsupial oocytes do not experience cumulus expansion and are instead ovulated naked (Breed and Leigh 1992; Mate 1996).

An alternative protocol was required for *D. hallucatus* as incubation periods of at least 15 min caused the loss of the zona pellucida. A pilot study examining GOC release from 2 to 3 slices of ovarian tissue at different dilutions of dissociation medium and incubation times determined that a 15 to 30 min incubation of ovarian tissue in a 1:1 dilution of dissociation medium with DMEM was the most successful protocol. Shorter time periods or more dilute dissociation medium resulted in a requirement to mechanically dissociate the tissue and reduced GOC survival (data not shown).

6.2.3 GOC Classification

Primordial GOC were identified by their association with flat granulosa cells and secondary GOC were multilayered (Falconnier and Kress 1992; Kress *et al.* 2001). To distinguish between early and late primary GOC the range of sizes at which the zona pellucida became obvious was examined. Because the zona pellucida was not able to be visualised due to the tight network of granulosa cells the oocyte needed to be extruded. GOC were measured then mounted onto glass slides with the coverslip supported by a 9:1 mixture of Vaseline and candle wax. Pressure was carefully exerted on the coverslip causing the oocyte to be extruded enabling visualisation of the zona. At least 50 GOC with and without the zona were assessed from at least three individuals from each species.

6.2.4 The Effect of Overnight Incubation

In order to use ovarian tissue from animals located externally or in the wild, optimal transport conditions needed to be established. The effect of refrigerated or room temperature storage was assessed in at least 100 primordial, early and late primary *S. crassicaudata* GOC (n=3). Secondary GOC were not assessed as earlier experiments indicated they were not high quality post-dissociation. Ovaries were placed in PBS and refrigerated or left at room temperature. After 24 hours the ovaries were dissociated and viability was compared as described in section 6.2.7.

6.2.5 Vitrification of GOC

To examine the ability of primary GOC to withstand cold shock and potentially toxic cryoprotectants, GOC from *S. crassicaudata* ($n \geq 3$) were either incubated in an ice slurry (0.5 to 1 ºC) for 20 min or taken through the complete set of vitrification and thawing media without exposure to liquid nitrogen (LN). The viability of at least 100 GOC was examined as described in 6.2.7 and subsequently GOC were stripped and the viability of at least 100 oocytes was assessed as described in section 6.2.7.

Vitrification of *S. crassicaudata* GOC (n=5) was carried out using a modified method from Vajta *et al.* (1998). Briefly enzymatically isolated GOC were washed twice in pre-vitrification medium (bench medium with 20% FCS) before being transferred to a 7.5% dimethyl sulphoxide (DMSO) and ethylene glycol (both Sigma-Aldrich) solution in pre-vitrification medium for 3 min. They were subsequently transferred into a small drop of vitrification medium- 1 M sucrose (Astral Scientific, Gymea, NSW, Australia; prepared in FCS) containing 18% DMSO and ethylene glycol for 30 seconds (sec) and loaded into cryostraws (IVM, L'Agile, France) which had been stretched over a hot plate and cut in half to create a narrow walled vessel. The loaded straw was then suspended above LN for 15 sec and immersed in LN.

To thaw samples, straws were dipped in thaw solution containing 60% pre-vitrification medium in 1 M sucrose. GOC were expelled and incubated for 1 min before being transferred to fresh thaw solution for 5 min. GOC were then transferred to equilibrating medium containing 80% pre-vitrification medium in 1 M sucrose for 5 min before being transferred to 100% pre-vitrification medium. The viability of at least 100 GOC was examined as described in section 6.2.7 and subsequently GOC were stripped and the viability of at least 100 oocytes was assessed as described in section 6.2.7.

6.2.6 *In Vitro* Culture of GOC

Post-vitrification GOC from *S. crassicaudata* (n=5) were cultured for up to two days on a layer of agar to determine if their damaged granulosa cells could repair. A pilot study presented in Addendum 2 at the end of this chapter, indicated that an agar layer was the most appropriate substrate for short term culture as GOC would adhere to the surface of uncoated culture vessels and collagen impregnated cell culture inserts.

GOC were washed in culture medium containing high glucose DMEM supplemented with 10% (v/v) FCS, 10 µL mL^{-1} Insulin-Transferrin-Selenium (ITS, Sigma-Aldrich), 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin (Invitrogen) and 2 mM fresh glutamine (Sigma-Aldrich). GOC were cultured in groups of up to 20 in 5% CO_2 in air at 35 ºC on a 250 µL layer of 0.625% agar (Sigma-Aldrich) prepared in DMEM within a 1 mL non-treated multidish well (Nunc, Roskilde, Denmark). The viability of at least 100 primary GOC was examined after 48 hours as described in section 6.2.7.

6.2.7 Viability of the GOC and Oocyte

The membrane integrity of granulosa cells and oocytes was assessed using the Fertilight live/dead staining kit (Molecular Probes, Carlsbad, CA, USA) with reduced working concentrations optimised for use with dasyurid GOC. Samples were stained with 10 nM SYBR14 and 240 nM propidium iodide (PI) for 15 min at 35 ºC then assessed on an inverted microscope (Zeiss, Jena, Germany) with a heated stage using filter set 09 (excitation 450 to 490 nm, beam splitter 510 nm, emission 520 nm). Cells which stained green were viable and those which stained red had damaged cell membranes. Although designed for use in spermatozoa this stain has also been used to examine viability in granulosa cells and oocytes (Comizzoli *et al.* 2008). GOC viability was categorically assessed as class I, II, III or IV based upon the proportions of viable granulosa cells as described in Figure 6-1.

For vitrification experiments the recovery of viable granulosa cells was not sufficient to suggest that the oocyte was also viable, so the tightly adherent granulosa cells were stripped from the oocyte. GOC were washed two times in warm PBS before being incubated for 5 min in 2.5 g mL^{-1} trypsin (Invitrogen). The treated GOC were then transferred through two washes of bench medium and stripped using a fine pulled glass pipette. These oocytes were stained with Fertilight as described above. The validity of this protocol was determined in each species by assessing fresh GOC, which maintained high viability, and heat treated GOC (70 ºC) which showed membrane damage and non-viable staining.

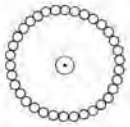
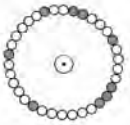
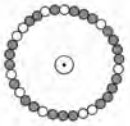
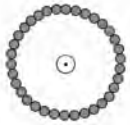
Class	Description	
I	100% viable	
II	99 to 50% viable	
III	49 to 1% viable	
IV	0% viable	

Figure 6-1: Schematic representation of the classification system used to determine viability classes I to IV for the assessment of dasyurid granulosa cell-oocyte complexes stained with SYBR14 (unfilled cells indicate viable granulosa cells) and propidium iodide (filled cells indicate non-viable granulosa cells).

6.2.8 Statistical Analysis

All values are presented as mean ± standard error of the mean (SEM). Significant differences were assessed using chi squared contingency tables with follow up stepwise partitioning (Agresti 2002) in JMP. Threshold values for the size of follicle at which the zona pellucida is observed were determined for each species using a receiving operator characteristic (ROC) curve considering the sensitivity and specificity of the value with SPSS (Chicago, IL, USA).

6.3 RESULTS

In order to differentiate between the early and late primary GOC we examined the presence of the zona pellucida. The size of GOC at which the zona became apparent, hence the size at which GOC became classified as late primary GOC, differed for all species and is described in Table 6-1.

Table 6-1: The size of granulosa cell-oocyte complex (GOC) at which the zona pellucida appears, and the transition between early to late primary GOC occurs.

Species	Threshold size (≥ µm)	Sensitivity	Specificity
Fat tailed dunnart (*Sminthopsis crassicaudata*)	155	0.922	0.824
Eastern quoll (*Dasyurus viverrinus*)	225	0.941	0.824
Northern quoll (*Dasyurus hallucatus*)	145	0.922	0.900
Tasmanian devil (*Sarcophilus harrisii*)	265	0.932	0.849

6.3.1 Ovarian Dissociation

Following dissociation of fresh *S. crassicaudata* ovarian tissue, all but the most mature cell type maintained a high proportion of viable cells (Figure 6-2A). There was no significant difference between the quality of primordial and early primary GOC, but late primary GOC were less viable (χ^2=36.93, P<0.0001). However loss of viability was most strongly observed in secondary GOC (χ^2=357.59, P<0.0001).

In *D. viverrinus* Figure 6-2B demonstrates a similar pattern of high survival in younger GOC. There was no significant difference between primordial and early primary GOC but late primary GOC were less viable (χ^2=34.06, P<0.0001). However loss of viability was most strongly observed in secondary GOC (χ^2=220.44, P<0.0001).

The pattern of high survival in primordial GOC with less in secondary follicles was again reflected in *D. hallucatus* (Figure 6-2C). However primary GOC were in general more damaged by the amended protocol than in other species as demonstrated by the reduced viability in early primary (χ^2=14.56, P<0.001) and late primary (χ^2=11.49, P<0.01) GOC. Nonetheless loss of viability was most strongly observed in secondary GOC (χ^2=172.67, P<0.0001). Interestingly there were less primordial GOC in these females and assessment of all animals did not reach the 100 cell target.

S. harrisii ovaries were always shipped overnight and the pattern of high survival of primordial GOC was again observed but the survival of the more mature GOC was lower (Figure 6-2D). Declines were apparent between primordial and early primary GOC (χ^2=35.72, P<0.0001) and late primary (χ^2=167.30, P<0.0001) but the largest loss of viability was observed in secondary GOC (χ^2=249.20, P<0.0001).

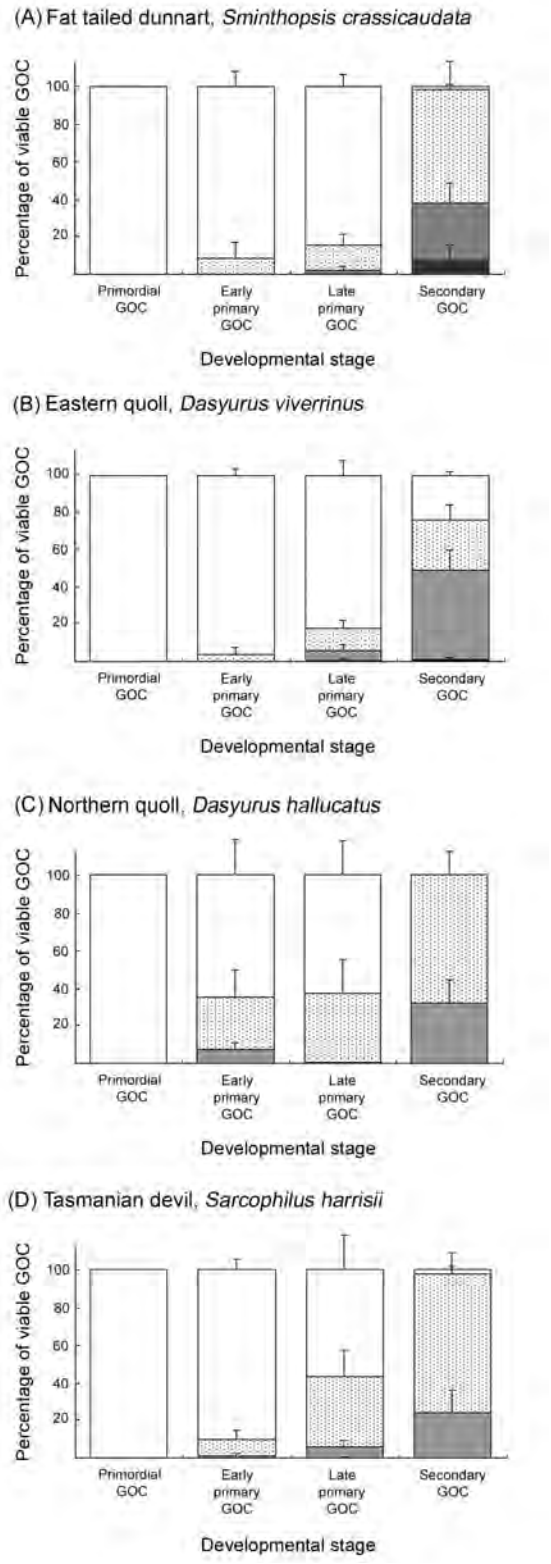


Figure 6-2: The percentage of class I (unfilled- 100% viable), II (stippled- 99 to 50% viable), III (grey- 49 to 1% viable) and IV (black- non viable) granulosa cell-oocyte complexes (GOC) at various developmental stages following ovarian dissociation in (A) fat tailed dunnart (*Sminthopsis crassicaudata*) (B) eastern quoll (*Dasyurus viverrinus*) (C) northern quoll (*Dasyurus hallucatus*) (D) Tasmanian devil (*Sarcophilus harrisii*).

6.3.2 The Effect of Overnight Incubation

S. crassicaudata ovarian tissue which was refrigerated showed better post-dissociation viability than ovarian tissue which was left at room temperature (Figure 6-3) and this was true at all developmental stages (χ^2=50.12 to 202.48, P<0.0001). However cooled tissue was not as healthy as fresh tissue (examined in section 6.3.1) which showed comparatively better viability in the early primary (χ^2=22.24, P<0.0001) and late primary (χ^2=56.88, P<0.0001) developmental stages.

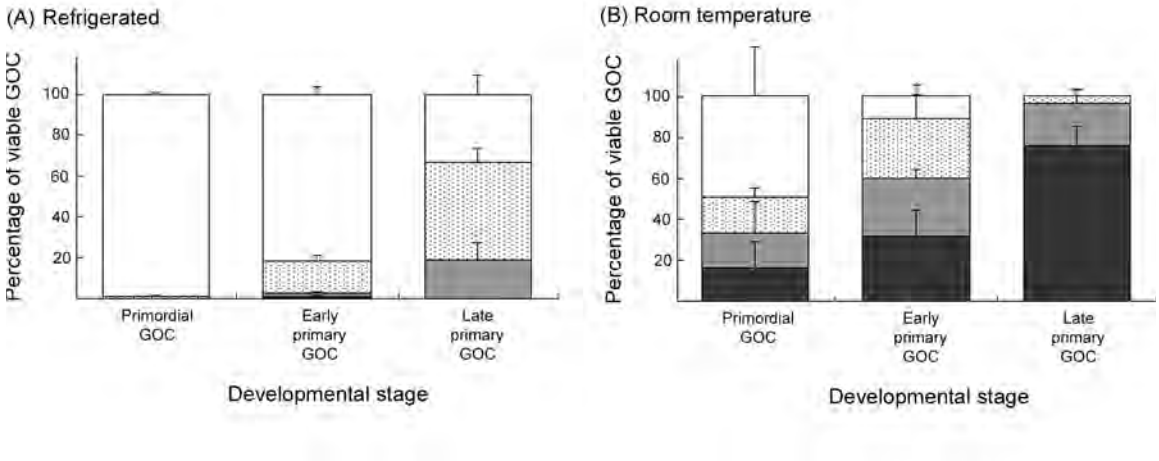


Figure 6-3: The percentage of class I (unfilled- 100% viable), II (stippled– 99 to 50% viable), III (grey- 49 to 1% viable) and IV (black- non viable) granulosa cell-oocyte complexes (GOC) at various developmental stages following 24 hours incubation when (A) refrigerated or (B) maintained at room temperature in the fat tailed dunnart (*Sminthopsis crassicaudata*).

6.3.3 Vitrification of GOC

Figure 6-2A demonstrated that fresh *S. crassicaudata* primary GOC were highly viable prior to preservation. Following vitrification GOC quality declined but improvements (χ^2=90.60, P<0.0001) were observed after 48 hours *in vitro* culture (Figure 6-4). But GOC quality was still reduced compared to fresh primary GOC (χ^2=117.30, P<0.0001).

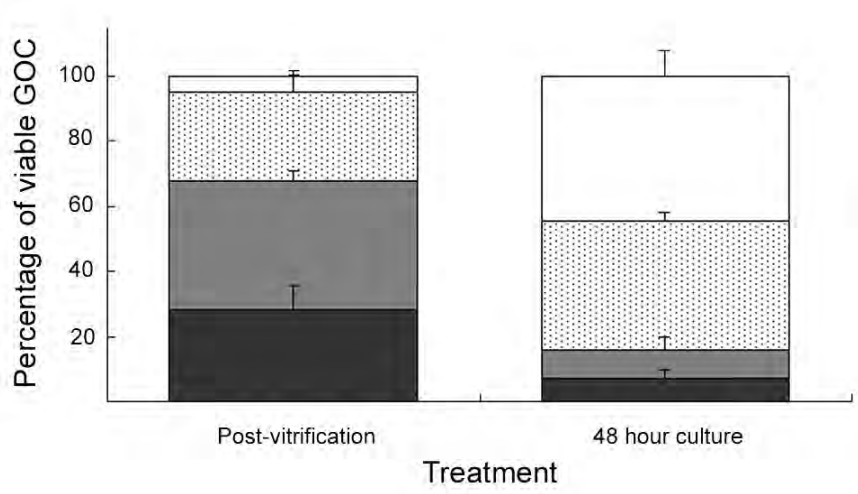


Figure 6-4: The percentage of class I (unfilled- 100% viable), II (stippled- 99 to 50% viable), III (grey- 49 to 1% viable) and IV (black- non viable) primary granulosa cell-oocyte complexes (GOC) after vitrification or following 48 hours *in vitro* culture in the fat tailed dunnart (*Sminthopsis crassicaudata*).

Fresh *S. crassicaudata* GOC, stripped with trypsin, had 80.25 ± 3.65% viable oocytes. This was not significantly reduced following cold shock, exposure to cryoprotectants or vitrification which resulted in 69.42 ± 2.44% viable oocytes (Figure 6-5).

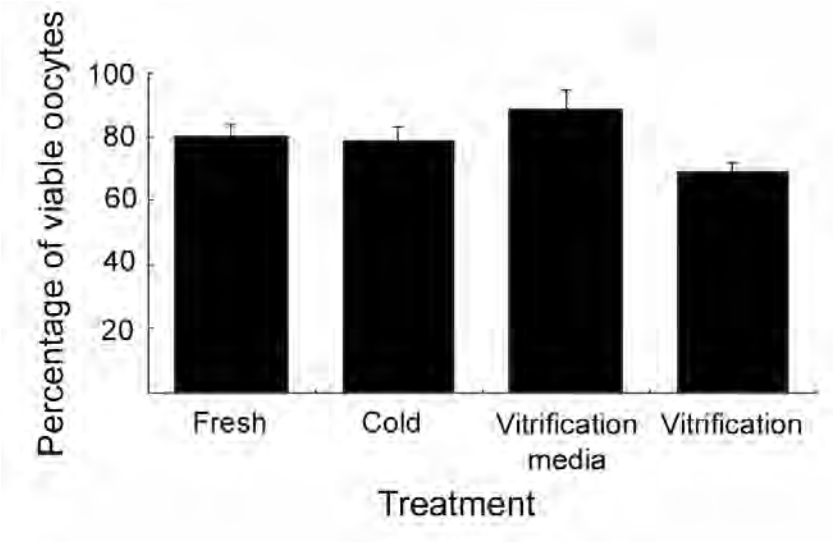


Figure 6-5: The percentage of viable oocytes assessed fresh and following exposure to low temperature, transfer through all vitrification media and vitrification in the fat tailed dunnart (*Sminthopsis crassicaudata*).

6.4 DISCUSSION

This study provides the first protocols for cryostorage of the immature female gamete from dasyurid marsupials including the well studied *S. crassicaudata* and the Endangered *D. hallucatus* and *S. harrisii*. We have devised a high yield, high viability follicle isolation protocol and provided a key for distinguishing developmental stages of the early follicle *in vitro*. In addition, low temperature short term storage was shown not to have highly detrimental effects on follicle viability and a vitrification protocol which results in a high proportion of viable post-thaw oocytes was developed. The reversible damage to the surrounding granulosa cells upon thawing suggests that vitrified GOC may be capable of continued growth and development. These protocols will play a fundamental role in the design and planning of genome resource banking systems for the conservation of dasyurid marsupials.

6.4.1 Collection of GOC

Post-isolation survival revealed that the smaller, less mature GOC survived the dissociation process better than larger GOC. Furthermore the smaller GOC also showed superior survival in the 24 hour incubation experiments. Although smaller follicles are relativity quiescent (Gosden *et al.* 1994; Shaw *et al.* 2000) and are reported to be more robust in suboptimal conditions (Snow *et al.* 2001; Richings *et al.* 2006) their survival post-dissociation was not guaranteed as Roy and Greenwald (1985) describe higher survival of larger hamster complexes post-enzymatic dissociation. However this was not the case in *S. crassicaudata* and we suggest that the damage observed in the larger GOC may relate to their multilayered morphology and the increasingly complex metabolic requirements in comparison to the relatively small quiescent primordial GOC.

The standard dissociation protocol required optimisation in *D. hallucatus* where a reduced concentration of dissociation medium was required to provide highly viable cells. It was important to find an equilibrium between the amount of time required to sufficiently dissociate the tissue whilst minimising exposure to the toxic dissociation medium. When dissociation was halted prematurely the tissue was more difficult to manually dissociate and viability was low, a likely effect of shearing damage as was observed in reduced incubations with human ovaries (Roy and Treacy 1993). The amended protocol was required as the standard protocol dissolved the zona- a likely action of collagenase. Previous reports describe dasyurid zona removal with pronase (Merry *et al.* 1995) and Bedford and Breed (1994) describe rapid dissolution of the zona from mature *S. crassicaudata* oocytes in the presence of just 0.1% trypsin.

The finding that low numbers of primordial GOC were observed in *D. hallucatus* species was interesting but most probably an indication of follicle depletion, an artifact of their advanced age. To examine this further, studying the effect of age on follicle prevalence in *D. hallucatus* is suggested. This could be carried out using histology or post-dissociation assessment as described by Figueiredo *et al.* (1993).

In addition to rapid collection of ovarian tissue post-mortem (Snow *et al.* 2001), the transport conditions of collected tissues are important. This study has demonstrated that a 24 hour incubation at low temperatures results in more healthy GOC than storage at room temperature most likely because the cooler temperatures limit detrimental apoptotic and enzymatic processes (Churchill 2004). The degeneration which is observed in the cow, pig, horse and dog following exposure to cool temperatures was not observed in dasyurid ovarian tissue (Didion *et al.* 1990; Wu *et al.* 1999; Love *et al.* 2003; Hanna *et al.* 2008). Our results were surprising as cold sensitivity can be associated with cytoplasmic lipid accumulation (Nagashima *et al.* 1999; Hanna *et al.* 2008) a feature of dasyurid oocytes (Breed and Leigh 1992). Cold sensitivity in marsupials has not previously been reported although cooled secondary follicles degenerated earlier than fresh follicles when cultured for four days in *M. eugenii* (Richings *et al.* 2006). Our study has also demonstrated that ovarian tissue from *D. hallucatus* and *S. harrisii* remain highly viable for up to 48 hours when transported in cold conditions.

6.4.2 Preservation of GOC

Younger follicles are more likely to survive sub zero preservation (Eroglu *et al.* 1998; Shaw *et al.* 2000) and this study chose to vitrify primary GOC as they are large enough to be seen when transferring through the several vitrification and thawing solutions yet have the potential benefits of a small immature oocyte. Our study demonstrated no significant reductions in oocyte viability following vitrification. This is the first study which specifically examines the vitrification of isolated GOC from marsupial species and reports almost 70% survival of oocytes from primary GOC. This is comparable to immature oocyte survival following vitrification in mice (dela Peña *et al.* 2002) and the cow (Yamada *et al.* 2007) and improved on post-cryopreservation survival of immature oocytes in the pig and cat (Didion *et al.* 1990; Jewgenow *et al.* 1998). We were also able to demonstrate that immature dasyurid oocytes were not damaged by exposure to the cold. Low temperatures are detrimental to mature oocytes where temperature induced cytoskeletal damage can cause chromosomal abnormalities but in immature oocytes, where the meiotic spindle is yet to be formed, damage is less likely (Eroglu *et al.* 1998). Nonetheless it was important to establish the ability of dasyurid oocytes to

withstand low temperatures as other species, with a similarly high content of cytoplasmic yolk content, suffer degeneration or reduced developmental competence following exposure to the cold (Didion *et al.* 1990; Wu *et al.* 1999; Love *et al.* 2003; Hanna *et al.* 2008). We also demonstrated that dasyurid GOC were not damaged by the potentially toxic cryoprotectants used for vitrification. Previous studies on embryo preservation in *S. crassicaudata* demonstrated resilience to DMSO (Breed *et al.* 1994b) and ovarian slices have been successfully freeze thawed in cryoprotective media containing either DMSO or ethylene glycol (Shaw *et al.* 1996). Interestingly a pilot study in *S. crassicaudata* which examined vitrification in sucrose, DMSO and glycerol produced non-viable GOC (data not shown) indicating potential glycerol toxicity as was also seen in a proportion of oocytes from domestic cats (Jewgenow *et al.* 1998).

Despite the high quality oocytes observed post preservation there was significant damage to the surrounding granulosa cells with very few GOC assessed as Class I. Reduced granulosa cell function may affect post-thaw follicle culture as the oocyte must maintain communication between the granulosa cells and the oocyte via gap junctions for continued growth and development (Anderson and Albertini 1976; Eppig 1979). The findings that significant improvements to GOC quality are made following a short term culture on an agar layer are encouraging for the ongoing growth and maturation of preserved GOC.

Successful fertilisation of *in vitro* grown preantral follicles has been achieved in mice, rats and cows (Daniel *et al.* 1989; Eppig and Schroeder 1989; Hirao *et al.* 2004). Similar success is yet to be achieved in marsupials. The follicles of *M. domestica* increase in size but have no antrum development (Butcher and Ullmann 1996), preantral follicles from S. *macroura* survive culture but do not form an antral space (Nation and Selwood 2005) and secondary follicles from *M. eugenii* increase in size but do not form an antrum and degenerate after four days in culture (Richings *et al.* 2006). Although it is known that early antral follicles from *S. crassicaudata* have a diameter of approximately 375 µm (Chapter 5) without separate assessment in all the dasyurid species an understanding of the required size to achieve antral follicles *in vitro* is difficult. This is further demonstrated by the varied size range for secretion of the zona pellucida between species indicated in Table 6-1.

6.4.3 Identification of Success

In the present study the tightly bound granulosa cells precluded visualisation of the germinal vesicle and they were removed with trypsin. Their removal was important because the survival of the small and robust granulosa cells does not indicate survival of the large fluid filled oocyte (Didion *et al.* 1990; Jewgenow *et al.* 1998) however this process is most likely detrimental and resulted in a wrinkled zona pellucida and misshapen oocytes. Prior studies indicated that lower trypsin concentrations dissolved the zona of mature *S. crassicaudata* in less than 10 seconds (Bedford and Breed 1994) however, despite the assault, in the present study the oolemma remained intact allowing viability assessment. Nonetheless further *in vitro* culture of trypsin stripped oocytes is not recommended. Interestingly Merry *et al.* (1995) described removal of adherent granulosa cells with hyraluronidase in *S. macroura*. This was not able to be achieved in the present study (data not shown), assumedly because marsupials do not secrete hyaluronic acids into a surrounding cumulus cell layer (Bedford and Breed 1994).

6.4.4 Conclusion

This study has developed protocols for the collection, transport, vitrification and culture of immature ovarian follicles in dasyurid marsupials. We present an enzymatic protocol for the collection of highly viable GOC from ovaries and demonstrate that this technique is a feasible option for genome resource banking in endangered species by showing that GOC remain highly viable following a 24 to 48 hour transportation in refrigerated conditions. The oocytes from primary GOC show no cold induced damage, cryoprotectant toxicity or loss of viability following vitrification. Although the quality of GOC was reduced post-vitrification, *in vitro* culture for 48 hours resulted in significant improvements. This study is a significant step towards banking gametes from female dasyurids and we suggest these protocols are integrated into conservation programs for the two species of endangered dasyurids- the northern quoll and the Tasmanian devil.

ADDENDUM 1 CHAPTER 6: Enzymatic dissociation of ovarian follicles from the spotted tailed quoll (*Dasyurus maculatus*) and Tammar wallaby (*Macropus eugenii*)

1 INTRODUCTION

The high survival of enzymatically dissociated ovarian follicles has been described for *S. crassicaudata*, *D. viverrinus*, *D. hallucatus* and *S. harrisii* in the body of this chapter. Additional data has also been collected regarding preliminary findings for two other species, the spotted tailed quoll (*Dasyurus maculatus*) and a macropod, the Tammar wallaby (*Macropus eugenii*). These samples were both sourced opportunistically and provide the basis upon which the studies into ovarian dissociation in *S. crassicaudata* were established. The findings suggest that enzymatic ovarian dissociation is able to be achieved in *D. maculatus* and *M. eugenii*, and the latter is especially encouraging as it indicates the applicability of this technique in other non-dasyurid species.

2 METHODS

Opportunistically collected ovarian tissue was sourced from one geriatric female *D. maculatus* housed at Featherdale Wildlife Park (Blacktown, New South Wales, Australia). Ovarian tissue was collected into PBS immediately after death and tissue was transported overnight on ice. The ovaries of two reproductively active *M. eugenii* housed at The University of Newcastle Animal House and euthanised due to injury were also available for study. *M. eugenii* tissues were collected as described above and examined immediately.

Ovarian dissociation was carried out as described in section 6.2.2. Briefly, ovarian tissue was sliced into small pieces and incubated for 30 min at 35 ºC in dissociation medium which was comprised of 0.1% collagenase and 0.02% DNAse in HEPES buffered high glucose DMEM. The enzymatic reaction was neutralised with bench medium containing HEPES buffered DMEM supplemented with 10% (v/v) FCS and antibiotics. Isolated follicles were moved to fresh bench medium for assessment.

The viability of isolated GOC was determined using the Fertilight live/dead staining kit as described in the body of this chapter (6.2.7). GOC viability was categorically assessed as class I, II, III or IV based upon the proportions of viable granulosa cells as previously described in Figure 6-1. Due to the low sample size and the role that these tissues played in protocol development, the survival of GOC in separate age classes was not examined. Instead the viability class of 117 *D. maculatus* GOC and 114 *M. eugenii* GOC were recorded to establish if enzymatic dissociation was a potential tool for isolation of marsupial preantral ovarian follicles.

3 RESULTS

An assessment of the health of GOC following the enzymatic dissociation protocol is shown in Table 1. In *D. maculatus* the GOC harvested were between 50 and 500 µm and over 70% of GOC were highly viable being allocated to class I or class II. Greater than 85% of GOC collected from *M. eugenii* were also highly viable following enzymatic dissociation and this was observed in a range of GOC from the smallest 5 µm GOC, likely to represent primordial GOC, to large 500 µm GOC, which are likely to be multilayered secondary GOC.

Table 1: The post-dissociation viability of granulosa cell-oocyte complexes (GOC) from the spotted tailed quoll (*Dasyurus maculatus*) and Tammar wallaby (*Macropus eugenii*).

	Spotted tailed quoll		Tammar wallaby	
Viability class	Number of GOC	Percentage of total GOC (%)	Number of GOC	Percentage of total GOC (%)
class I	50	42.74	98	85.96
class II	35	29.91	12	10.53
class III	28	23.93	4	3.51
class IV	4	3.42	0	0

4 DISCUSSION

This small study has shown that ovarian dissociation is not only successful in *S. crassicaudata*, *D. viverrinus*, *D. hallucatus* and *S. harrisii* but can also be used on another dasyurid, *D. maculatus*. It has further demonstrated that enzymatic dissociation is an effective tool for the collection of preantral follicles from the ovaries of a macropod- *M. eugenii*. Secondary follicles have previously been mechanically dissected from *M. eugenii* ovarian tissue (Richings *et al.* 2006), however the current protocol provides a method for collection of primordial GOC as small as 5 µm.

There was some evidence that the *D. maculatus* GOC did not survive the dissociation process as well as GOC from *M. eugenii* however it is important to consider that *D. maculatus* tissues were processed with a 24 hour delay, as they were collected offsite. Previous findings reported in this chapter have suggested that an overnight storage period can slightly reduce GOC quality, even when tissues are refrigerated. Another aspect to consider is that the *D. maculatus* sample was from a geriatric female and enzymatic dissociation can be more difficult to achieve in older animals (Telfer 1996).

This study has reiterated the value of enzymatic ovarian dissociation as a high yield tool for the collection of immature ovarian follicles. It is encouraging that in addition to being successful in another dasyurid, this protocol is also applicable to another marsupial family. This suggests that the oocyte preservation and *in vitro* culture techniques developed in this chapter may also be relevant to gamete resource banking in other marsupial species.

ADDENDUM 2 CHAPTER 6: The *in vitro* culture of enzymatically dissociated ovarian follicles from the fat tailed dunnart (*Sminthopsis crassicaudata*)

1 INTRODUCTION

The mammalian ovary contains many thousands of small immature follicles which can be harvested in dasyurids using the protocols described in this chapter. Collection of these immature granulosa cell-oocyte complexes (GOC) is beneficial as they are prevalent throughout the cycle and are, in general, more robust than larger follicles (Shaw *et al.* 2000). Hence they provide a good source of female gametes for genome resource banking. However in order to grow and eventually mature these follicles, *in vitro* culture methods must be developed. One of the most important factors in designing *in vitro* culture systems is to ensure that the granulosa cells do not migrate away from the oocyte. This can be difficult in enzymatically harvested follicles as the collection process removes both the basement membrane and thecal cells, leaving granulosa cell-oocyte complexes instead of intact follicles (Eppig 1994). The contact between these surrounding granulosa cells and the oocyte must be maintained as they allow fundamental communication to the oocyte via gap junctions (Anderson and Albertini 1976) and these are essential for continued growth and maturation (Eppig 1979).

In this chapter, the *in vitro* culture of post-vitrification primary follicles on an agar pillow was described. In order to develop this protocol three *in vitro* culture systems were examined in a pilot study- GOC were cultured on a cell culture plate with no additional substrate, on an agar pillow or on a collagen impregnated membrane insert (Eppig and Schroeder 1989; Jewgenow and Göritz 1995). This pilot study describes the rates of granulosa cell loss and attachment to the substrate in the three *in vitro* culture systems over a period of ten days.

2 METHODS

2.1 GOC Collection

Primary GOC were collected from *S. crassicaudata* as described in the body of this chapter (section 6.2.2). Briefly, ovarian tissue was sliced into small pieces and incubated for 30 min at 35 ºC in dissociation medium which was comprised of 0.1% collagenase and 0.02% DNAse in HEPES buffered high glucose DMEM. The enzymatic reaction was neutralised with bench medium containing HEPES buffered DMEM supplemented with 10% (v/v) FCS and antibiotics. Primary GOC were then washed in culture medium containing high glucose DMEM supplemented with 10% (v/v) FCS, 10 µL mL^{-1} ITS, antibiotics and 2 mM fresh glutamine and transferred to one of three culture systems.

2.2 *In Vitro* Culture

Groups of up to 20 primary GOC were cultured together in 5% CO_2 in air at 35 ºC either on culture plates containing no additional substrate (non-treated multiwell dish, Nunc, Roskilde, Denmark), on a pillow of 0.625% agar made in high glucose DMEM or on a pre-incubated collagen impregnated cell culture insert (Transwell-COL, Corning Life Sciences, Lowell, MA, USA). All groups were grown in culture medium which had been pre-equilibrated for several hours and had half its volume replaced with fresh pre-equilibrated medium every three days. The attachment of GOC to the substrate and loss of granulosa cells in at least 100 GOC from at least three females was assessed at 0, 1, 2 and 10 days at x40 on an Axiovert 35 inverted microscope fitted with a heated stage set at 35 ºC.

2.3 Statistical Analysis

All values are presented as mean ± SEM. Significant differences were assessed in transformed data within individual treatment groups at 0 and 1 day using T-tests in JMP. Differences between the three treatment groups at each time point was determined using ANOVA followed by post-hoc Tukey's test in JMP.

3 RESULTS

Enzymatically harvested follicles were difficult to grow for longer than 48 hours *in vitro*. Figure 1 shows that the percentage of oocytes which remained surrounded by granulosa cells was high in all groups when GOC were collected but after 24 hours in culture all groups had declined (culture plate $P<0.01$, agar $P<0.001$, collagen treated membrane $P<0.05$). After 48 hours *in vitro* there were less surrounded oocytes in the culture plate group compared to the agar or the collagen treated membrane group ($P<0.01$), with only 2% of oocytes in the culture plate group still surrounded by granulosa cells. Instead the granulosa cells had become attached to the surface of the culture plate forming a monolayer and only maintained a distal association with an otherwise naked or semi-naked oocyte, which was often misshapen (Figure 2A). After losing their association with the granulosa cells, the oocytes underwent cytoplasmic degradation, shrinkage and vacuolation. In contrast GOC cultured on agar appeared normal at 48 hours (Figure 2B). The deficiencies of the culture system were apparent at ten days when less than 15% of GOC were surrounded in all groups.

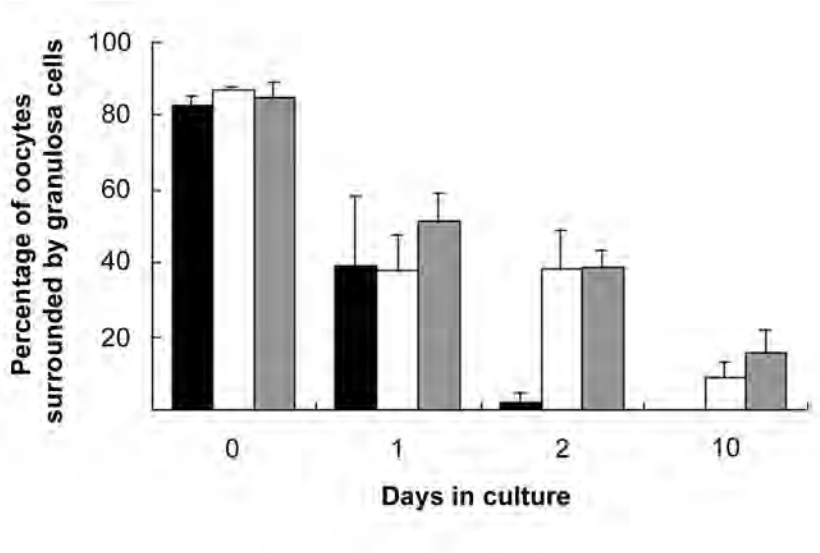


Figure 1: The percentage of fat tailed dunnart (*Sminthopsis crassicaudata*) oocytes surrounded by granulosa cells following culture for up to ten days on the culture plate (black), an agar pillow (unfilled) or a collagen treated membrane (grey).

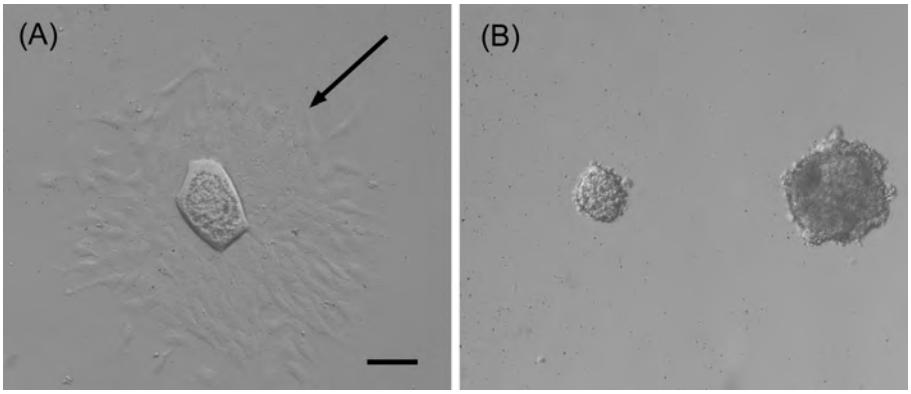


Figure 2: Dissociated granulosa cell-oocyte complexes from the fat tailed dunnart (*Sminthopsis crassicaudata*) following 48 hours *in vitro* culture (A) on the culture plate- note the surrounding layer of granulosa cells adhered to the plate (arrow) and the abnormal shape of the oocyte (B) on an agar pillow- oocytes remain surrounded by the granulosa cell layers and are free floating in the medium. Bar=40 µm.

Figure 3 indicates that immediately after the culture period was initiated greater than 95% of GOC in both the culture plate and collagen treated membrane groups had adhered to the substrate. This was different to the agar group initially ($P<0.001$) and after 48 hours ($P<0.05$), and throughout the culture period the proportion of GOC in the agar group which were free floating in the medium never decreased below 60%. However as the culture period progressed the deficiencies in the culture system became apparent and semi-naked oocytes were released into the medium. Thus the proportion of free floating partially surrounded GOC from the culture plate and collagen treated membrane groups increased when assessed at ten days.

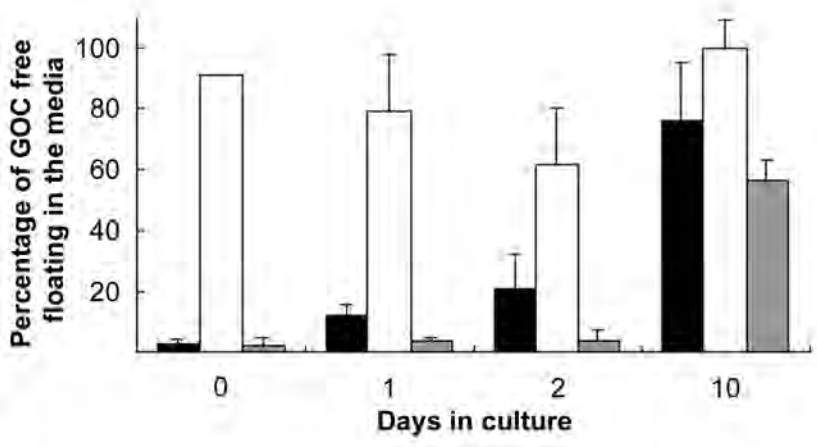


Figure 3: The percentage of fat tailed dunnart (*Sminthopsis crassicaudata*) oocytes free floating in the medium following culture for up to ten days on the culture plate (black), an agar pillow (unfilled) or a collagen treated membrane (grey).

4 DISCUSSION

The ability to successfully culture enzymatically isolated ovarian follicles will be fundamental to realising the true potential of the follicle vitrification studies described earlier in this chapter. This study has clearly shown that *in vitro* culture on the surface of the culture plate is inappropriate, as was expected from the literature (Eppig 1979; Jewgenow and Göritz 1995; Telfer 1996). The finding that granulosa cells remained associated with the oocytes in the agar and collagen treated membrane group for up to 48 hours is encouraging and suggests that future studies focus on optimising the culture conditions required to maintain GOC *in vitro* for longer than 48 hours.

Despite the health of GOC cultured on the collagen treated membrane, GOC cultured on agar were selected to examine the repair of post-vitrification GOC *in vitro*. This selection was made because the GOC on the collagen treated membrane were physically attached, and their removal would be likely to have damaged the granulosa cells. This was prohibitive because it would have been impossible to differentiate between vitrification damage and damage incurred after removal from the membrane. Conversely, the GOC cultured on agar could be easily transferred into the staining solution without any additional mechanical damage.

This small study was useful in determining an appropriate *in vitro* culture system for post-vitrification *S. crassicaudata* GOC. However it is very clear that none of these systems, in their current form, are adequate for long term *in vitro* culture. A key element that could be improved is a reduction of the concentration of oxygen in the culture environment. Eppig and Wiggelsworth (1995) describe the importance of a 5% O_2 culture environment and demonstrate that 20% O_2 is detrimental to oocyte development. Low oxygen may be required because *in vivo* preantral follicles develop within a hypoxic environment- as the surrounding cumulus cells consume, and protect the oocyte from, free oxygen (Gosden and Byatt-Smith 1986).

Despite the need to further improve the culture protocol this study has demonstrated that primary GOC from *S. crassicaudata* can be successfully maintained *in vitro* for up to 48 hours without loss of granulosa cells or attachment to the culture substrate. These findings have played an important role in determining suitable conditions for post-vitrification repair *in vitro* and it is likely they will play a role in defining improved *in vitro* culture protocols in the future.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7: General discussion

7.1 INTRODUCTION

This project aimed to advance the assisted reproductive techniques (ART) available for use in model and threatened dasyurid marsupials, with particular emphasis on genome resource banking as a tool for their conservation. Prior to this thesis there had been numerous descriptive studies regarding the reproductive biology of dasyurids (Hill 1910; Harding *et al.* 1982; Selwood 1992; Breed 1996). But investigations into dasyurid ART were rare and limited to the study of sperm preservation (Taggart *et al.* 1996), ovarian stimulation (Rodger *et al.* 1992a; Menkhorst *et al.* 2007), embryo biology (Selwood 1987; Breed and Leigh 1996) and ovarian cryopreservation (Shaw *et al.* 1996).

The collection of studies presented in this thesis describe progress in dasyurid genome resource banking for both spermatozoa and oocytes, and the development of protocols for the production of large numbers of mature oocytes for use in fertilisation experiments. The major findings of the studies focused on dasyurid spermatozoa describe a relatively unstable acrosome, tolerance to high concentrations of glycerol and a susceptibility to rapid temperature declines below 4 ºC. This cold shock is likely to have contributed to the inability to successfully cryopreserve dasyurid spermatozoa which did, however, demonstrate normal morphology and nuclear integrity post-thaw. In female dasyurids a reliable tool for the production of mature oocytes was developed and protocols for the collection, vitrification and short term *in vitro* culture of immature ovarian follicles were described. These advances provide a solid and comprehensive framework for continued study of dasyurid ART, and have also increased the understanding of the reproductive biology of both the model species, the fat tailed dunnart (*Sminthopsis crassicaudata*), and more threatened dasyurids. This work is timely due to the urgent need for genome resource banking of the endangered northern quoll (*Dasyurus hallucatus*) and Tasmanian devil (*Sarcophilus harrisii*) but may also have application in any of the other 16 dasyurids listed as threatened by the Australian Government including the kowari (*Dasyuroides byrnei*), Julia Creek dunnart (*Sminthopsis dougalsi*) or the Carpenterian antechinus (*Pseudantechinus mimulus*) (Department of the Environment and Heritage 1999).

7.2 ACHIEVEMENTS IN MALE GENOME RESOURCE BANKING

7.2.1 Dasyurid Sperm Acrosome Biology

The sperm preservation studies began by examining the dasyurid acrosome. The demonstration of a relatively fragile dasyurid acrosome, due to the absence of disulphide stabilisation, contrasts markedly with observations of a stable acrosome with significant disulphide bonding in the brush tailed possum (*Trichosurus vulpecula*) and Tammar wallaby (*Macropus eugenii*) (Sistina *et al.* 1993a; Mate *et al.* 1994; Lin *et al.* 1995). This finding provided a tool for the assessment of post-freezing damage in dasyurid spermatozoa, similar to that used for identification of damage following freezing in eutherian spermatozoa (Molinia *et al.* 1994; O'Brien and Roth 2000; Isachenko *et al.* 2008). However because the dasyurid acrosome is not as sensitive to insult as the eutherian acrosome, this tool is less useful for predicting morphological damage in dasyurid spermatozoa than it would be for eutherian spermatozoa. The finding that the acrosome of dasyurid spermatozoa lacks disulphide stabilisation is unique in marsupials thus far examined. The implications of this remain unclear but may be associated with zona penetration, which also differs in dasyurids compared to other marsupials (Breed and Leigh 1988; Breed 1994b).

The absence of disulphide stabilisation resulting in a relatively fragile acrosome, which can be used as a morphological indicator, is a feature common to a broad range of dasyurids. This has been demonstrated in two species of quoll, the eastern quoll (*Dasyurus viverrinus*) and *D. hallucatus*, and in a preliminary examination of the red tailed phascogale (*Phascogale calura*). Although Harding *et al.* (1982) describe morphological similarities in the spermatozoa of dasyurids, it is exciting to acknowledge that physiological similarities also exist and that the biological tools developed in model species are likely to be applicable to more threatened dasyurids.

7.2.2 Preservation of Dasyurid Spermatozoa

Previous research showed that *S. crassicaudata* spermatozoa had 3% motility after being rapidly frozen with 8% glycerol and suggested sensitivity towards higher concentrations (Taggart *et al.* 1996). The current study used a glycerol toxicity trial to determine if cryoprotectant sensitivity existed. *S. crassicaudata* spermatozoa tolerated high concentrations of glycerol, as had been reported in *T. vulpecula* (Rodger *et al.* 1991), this provided evidence that intolerance to glycerol was not a limiting factor and justified subsequent investigations of alternative freezing protocols. But thawed spermatozoa were all immotile and non-viable. Hence ART with the current preservation protocols should focus on intracytoplasmic sperm injection (ICSI), instead of *in vitro* fertilisation (IVF) which requires motile spermatozoa.

Although ICSI can be carried out with spermatozoa that are non-viable, the nucleus must be intact (Wakayama and Yanagimachi 1998; Kwon *et al.* 2004; Martins *et al.* 2007). Thawed dasyurid spermatozoa had normal morphology, which has little functional significance for ICSI but is still a valuable indicator of overall damage. Thawed *S. crassicaudata* spermatozoa also had no evidence of DNA damage which is an extremely positive indication that preserved spermatozoa would be appropriate for ICSI, especially in light of the absence of nuclear stabilisation in most dasyurids which results in nuclear decondensation following physical insult (Cummins 1980; Retief *et al.* 1995a). The subsequent use of thawed *S. crassicaudata* spermatozoa in ICSI has been described in a small preliminary study which demonstrated that sperm heads did not decondense spontaneously when injected into an oocyte. This supports the concept that non-viable frozen dasyurid spermatozoa could be used to produce offspring if ICSI protocols can be developed.

The sperm freezing studies describe encouraging progress in the preservation of spermatozoa from *S. crassicaudata* and represent the first published report of sperm freezing in quolls. The normal morphology of post-thaw *D. viverrinus* and *D. hallucatus* spermatozoa suggest that the effects of exposure to cryoprotectants and the freezing protocols are similar across the dasyurid family, again reiterating that the biological tools developed in the model species are likely to be applicable to more threatened dasyurids.

Freeze drying was also investigated as an alternative preservation method for dasyurid spermatozoa. This technique has the advantage of avoiding the costs and dangers associated with the use of liquid nitrogen and the freeze dried spermatozoa can remain capable of fertilisation if used with ICSI (Wakayama and Yanagimachi 1998). Freeze dried *S. crassicaudata* spermatozoa had minimal acrosomal loss and no evidence of DNA damage. Thus the first study of freeze drying marsupial spermatozoa has yielded extremely encouraging results. This is perhaps surprising given the absence of disulphide stabilisation in the marsupial sperm nucleus (Retief *et al.* 1995b) and the reported inability of murine spermatozoa to withstand freeze drying without disulphide stabilised nuclei (Kaneko *et al.* 2003). In eutherians freeze drying provides a cost effective method of sperm storage which can be successfully used to produce live young via ICSI (Wakayama *et al.* 1998; Wakayama and Yanagimachi 1998). The findings in *S. crassicaudata* suggest that similar techniques may now be considered as an option for genome resource banking in dasyurids.

7.2.3 Cold Shock in Dasyurid Spermatozoa

The finding that dasyurid spermatozoa did not suffer from glycerol toxicity, and that freezing protocols as slow as 1 ºC minute^{-1} did not result in viable spermatozoa, justified the investigation of the long standing assumption that cold shock does not occur in marsupial spermatozoa (Rodger and White 1978; Taggart *et al.* 1996; Miller *et al.* 2004; MacCallum and Johnston 2005; McClean *et al.* 2006). This assumption has been embraced as part of the studies presented earlier regarding acrosome stability and sperm preservation. Thus the crucial finding that *S. crassicaudata* spermatozoa suffered cold shock when rapidly cooled to 0.5 ºC was surprising and extremely important. As demonstrated in other species (White 1993), the presence of egg yolk and slower cooling protocols was protective and *S. crassicaudata* spermatozoa cooled at 0.5 ºC minute^{-1} in the presence of egg yolk were viable at 0 ºC. These findings clearly show that the accepted paradigm that the spermatozoa of all marsupial species are resistant to cold shock is incorrect. This understanding may soon enable dasyurid spermatozoa to be successfully cryopreserved as studies can now focus on the protection of spermatozoa from cold shock and very slow cooling rates, rather than the toxicity of cryoprotectants. These findings also have repercussions for short term sperm cooling and the overnight transport of testicular tissue. Although this cold shock study only examined *S. crassicaudata*, the morphological similarities of dasyurid spermatozoa (Harding *et al.* 1982) and the physiological similarities already presented for the studies in acrosomal morphology and sperm freezing, suggest it is likely that cold shock sensitivity may be a feature common to the spermatozoa of the dasyurid family. Thus this insight may contribute to successful sperm cryopreservation and genome resource banking for the threatened dasyurids.

7.3 FUTURE STUDIES IN MALE GENOME RESOURCE BANKING

The present series of studies did not produce viable spermatozoa post-thaw, but the potential for cryopreservation of dasyurid spermatozoa is significant following the description of tolerance to glycerol and the ability to overcome cold shock with slow cooling and egg yolk. As a result the highest priority of future studies will be to utilise this knowledge in the design of protocols for the cryopreservation of dasyurid spermatozoa. A very limited pilot study has indicated that *S. crassicaudata* spermatozoa cooled in the presence of egg yolk and glycerol at 0.5 ºC minute^{-1} remain motile at -6 ºC before ice crystal formation, but when the solution froze the spermatozoa became non-viable. This suggests that although the spermatozoa survived cold shock they were still damaged by ice formation. Thus future studies could examine traditional methods of passive cooling (Jones 1973; Hammerstedt *et*

al. 1990) or utilise more regulated cooling protocols to control the formation of ice crystals whilst examining the effects of ice crystal formation using a cryomicroscope (Holt *et al.* 1992; Holt *et al.* 1999). Furthermore the use of more sophisticated compounds for membrane protection could be investigated including low density lipoprotein, cholesterol-loaded cyclodextrins or lipid containing vesicles (Watson 1976; Simpson *et al.* 1986; De Leeuw *et al.* 1993; Mocé and Graham 2006). The ability to produce viable spermatozoa post-thaw would enable the long term storage of spermatozoa which could be used for artificial insemination (AI), a less technical post-thaw fertilisation technique with a higher likelihood of success than ICSI (Rodger *et al.* 2009).

The examination of spermatozoa from *S. crassicaudata* has also raised several questions regarding the fundamental biology of dasyurid spermatozoa. The studies on acrosomal stability used bromobimane staining to detect the presence of disulphide bonds in the acrosomal membranes, but a more accurate method for visualising disulphide bonds in acrosomal membranes uses transmission electron microscopy (Lin *et al.* 1995). Another interesting question also relates to cold shock and the lipid profiles of marsupial spermatozoa. Miller *et al.* (2004) examined the spermatozoa of the eastern grey kangaroo (*Macropus giganteus*) and common wombat (*Vombatus ursinus*) following “cold shock”, which involved chilling to 4 ºC, and correlated the results to membrane lipid profiles which indicated that marsupial spermatozoa contained a high proportion of unsaturated fatty acids. As neither species demonstrated cold shock at 4 ºC the study concluded that marsupials do not conform to the phospholipid cold shock model established in eutherians, which associates high unsaturated fatty acids in sperm membranes with the susceptibility to cold shock (reviewed in White 1993). It may be necessary to revisit this idea in light of the evidence that cold shock occurs in *S. crassicaudata*, and may occur in other marsupials when tested more critically using the Mann (1964) protocol. In addition it will also be important to systematically examine the effect of low temperatures on the spermatozoa of other dasyurids to confirm that the susceptibility to cold shock is a feature shared by the spermatozoa of several dasyurid marsupials.

Finally it is important to acknowledge that although morphological aspects and DNA integrity are important factors in determining cellular health, the ultimate proof of healthy spermatozoa is a functional test indicating the potential for fertilisation. ICSI has been used to fertilise oocytes with spermatozoa that is traditionally considered non-viable (Wakayama *et al.* 1998) and ICSI has been developed in two marsupials- *M. eugenii* and *V. ursinus* (Magarey and Mate 2003; Magarey and Mate 2004; Richings *et al.* 2004; West *et al.* 2007). This study presents encouraging preliminary ICSI results in *S. crassicaudata*, and future attempts should examine the use of smaller injecting micropipettes to limit the injection of excess medium into the oocytes.

7.4 ACHIEVEMENTS IN OVARIAN STIMULATION

Previous studies reported ovarian stimulation in *S. crassicaudata* but the results were not sufficiently reliable for use in assisted reproduction programs (Rodger *et al.* 1992a; Anderson and Breed 1993; Breed and Leigh 1996). This study has developed the first robust and reliable protocol for ovarian stimulation in *S. crassicaudata* which enables the collection of up to 28 morphologically normal pre-ovulatory oocytes that are mature, or can be matured within 48 hours. The finding that *in vitro* maturation, to the first polar body stage, occurs in almost all oocytes represents a significant advancement in ovarian stimulation for dasyurids as *in vitro* maturation was unreliable in previous studies, with less than 60% of oocytes reaching the first polar body stage (Merry *et al.* 1995; Maleszewski and Selwood 2004). The ability to collect oocytes is essential for the progress of ART in dasyurids and will allow investigation of fertilisation techniques such as IVF and ICSI, furthermore the availability of mature oocytes is also important for the continuing fundamental studies regarding oocyte biology.

A surprising finding of this study was the difference in post-stimulation oocyte development timetables between *S. crassicaudata* of different age groups. This understanding may explain some of the variation observed in prior studies of ovarian stimulation in *S. crassicaudata* and also perhaps the stripe faced dunnart (*Sminthopsis macroura*) (Rodger *et al.* 1992a; Menkhorst *et al.* 2007). There is also a captive management advantage to this finding as it describes a robust protocol for the collection of healthy mature pre-ovulatory oocytes from post-breeding females which no longer contribute to the captive breeding colony under established husbandry practices. As a result the management of the colony can be more sustainable, without compromising the requirements of scientific research projects.

Although allocating females into age classes accounted for some variation, clearly avoidance of the corpus luteum (CL) increased the reliability of the ovarian stimulation protocol. The action of the CL in marsupials cannot be halted, as it can in eutherians, because it is not gonadotrophin dependant (Tyndale-Biscoe *et al.* 1974; Short *et al.* 1985; Gemmell 1995; Bó *et al.* 2002). To avoid the luteal phase of the reproductive cycle the pattern of vaginal cornified epithelial cells (CEC) was used as an indicator of oestrus, and stimulation was carried out after a period of time which encompassed the duration of the luteal phase. An interesting finding of the CEC study was that *S. crassicaudata* does not ovulate during the influx of leukocytes, as observed in *S. macroura* (Godfrey 1969b; Woolley 1990; Selwood and Woolley 1991), or when the CEC decline, as observed in the brown antechinus (*Antechinus stuartii*) (Selwood 1980). Instead ovulation is likely to occur when CEC are initially rising and this could be used to enable more effective captive breeding by timed introduction of genetically valuable males when females are sexually receptive.

7.5 FUTURE STUDIES IN OVARIAN STIMULATION

Although protocols for the collection of pre-ovulatory *S. crassicaudata* oocytes were developed, oocytes left to ovulate were of poor quality. Thus the current protocol is not suitable for the development of AI programs or timed natural mating. The reason for the low quality oocytes is probably related to an inadequate endogenous luteinising hormone (LH) surge for the initiation of ovulation. A priority for future studies in this field should examine the supplementation of the equine serum gonadotrophin (eSG) stimulation protocol with a single dose of exogenous LH to induce ovulation followed by monitoring of the female's endocrine status with steroid assays, behavioural observations or CEC monitoring.

Once again it is important to be aware that morphologically normal polar body stage oocytes may not be capable of fertilisation or further development (Smith and Godfrey 1970). Thus it will be essential to examine the fertility of the mature pre-ovulatory *S. crassicaudata* oocytes obtained using the eSG stimulation protocol. However reports of embryos harvested and live young born following previous 1 IU eSG protocols in *S. crassicaudata* suggest that the hormone treatment from such studies, although unreliable, is not highly detrimental to developmental capacity (Rodger *et al.* 1992a; Anderson and Breed 1993; Breed and Leigh 1996). Furthermore the ICSI pilot study, reported in this thesis, has shown that oocytes collected from *S. crassicaudata* following ovarian stimulation and *in vitro* maturation have the competency to cause sperm head decondensation within 20 hours of sperm injection.

7.6 ACHIEVEMENTS IN FEMALE GENOME RESOURCE BANKING

In order to establish ART for occasions when an animal is already deceased, and to include female tissues in a genome resource bank, oocyte preservation was examined. This series of experiments reported high survival protocols for the collection, vitrification and post-thaw *in vitro* culture of immature ovarian follicles from dasyurids, and limited data regarding immature follicle collection from a macropod. The high yield collection of good quality immature follicles suggested that the enzymatic dissociation medium was not toxic to follicles but was of sufficient strength to dissociate the ovarian connective tissue. This protocol allowed the collection of small 2 µm primordial to large 650 µm secondary follicles, all of which can either be used for fundamental studies on follicle development, *in vitro* culture or genome resource banking. This is the first time enzymatic ovarian dissociation has been described in a marsupial and the potential conservation value of these findings is significant given the ability to harvest immature ovarian follicles from the model species, threatened dasyurids and a macropod. Such high rates of survival following vitrification of enzymatically harvested primary follicles were surprising and primary follicles survived all of the potentially damaging processes related to cold shock and media toxicity. Thus this study provided the first evidence that marsupial oocytes survive vitrification. Combined with preliminary evidence regarding the ability for *in vitro* cultured follicles to survive for 48 hours, this is the first realistic description of a promising technique for genome resource banking for female dasyurid gametes.

The development of a practical follicle classification system was essential to the success of these studies as it allowed rapid *in vitro* differentiation of early and late primary follicles. The literature described several histological studies, but they were of little value when examining live tissues *in vitro* (Hill 1910; Kress *et al.* 2001). The follicle classification system has not just been developed in *S. crassicaudata*, but has also been established in *S. harrisii* and two quoll species, *D. viverrinus* and *D. hallucatus*. The ability to differentiate early and late primary follicles from these species *in vitro* is likely to have continual use in future studies assessing the development of dasyurid follicles grown in culture.

Another important, and practical, aspect of this study was the determination of optimal transport conditions for the transport of tissues between field sites and interstate laboratories. High quality oocytes were obtained following cold storage with no evidence of the cold induced damage previously described for the lipid rich oocytes of pigs and dogs (Nagashima *et al.* 1999; Hanna *et al.* 2008). This simple assessment supports the collection of dasyurid ovarian tissues from zoological institutions or field stations which can be transported overnight to scientific laboratories for preservation. These findings further support the notion that follicle vitrification could realistically become standard practice for preserving ovarian tissues collected from threatened female dasyurids.

7.7 FUTURE STUDIES IN FEMALE GENOME RESOURCE BANKING

These studies have demonstrated that ovarian follicles can be collected from the model species, three target species and a macropod. However a systematic assessment of vitrification and post-thaw *in vitro* culture is needed for all species and this would be a priority for further studies. This study only examined the *in vitro* culture of post-preservation *S. crassicaudata* follicles for 48 hours but the growth, development and maturation of enzymatically isolated follicles is expected to take at least three weeks (O'Brien *et al.* 2003). Such long term *in vitro* culture will require significant optimisation of the media and gaseous environment and require endocrine support in the final stages (Eppig and Telfer 1993; O'Brien *et al.* 1993). However the ability of granulosa cells to proliferate and maintain a cellular association with the oocytes cultured on an agar pillow, suggests that the most significant difficulty with the *in vitro* culture of enzymatically isolated follicles has been eliminated.

7.8 SUMMATION

This thesis has made significant contributions to the study of dasyurid sperm preservation, oocyte preservation and the collection of mature oocytes for future ART. As such it has significantly advanced the ART available for the conservation of dasyurid marsupials, as well as increasing the understanding of the reproductive biology of both the model and the target species. In the introductory chapter several unresolved issues regarding dasyurid reproductive biology and the application of ART were described. In order to conclude, a brief summary of these issues and the advances made towards their resolution is presented.

1. *Is the dasyurid acrosome robust, as in other marsupials? If not can it be used as a morphological indicator as it can in eutherians?*

The acrosome of dasyurid marsupials is not stabilised by disulphide bonds. This was established by chemical and physical insult and morphological staining with bromobimane to examine the membrane distribution of disulphide bonds. However it was not as fragile as the eutherian acrosome, and although it can be used as an indicator of post-preservation damage it was not observed to be highly sensitive.

2. *Can successful freezing of dasyurid spermatozoa be achieved with a systematic examination of cryoprotectants and freezing protocols?*

S. crassicaudata spermatozoa do not suffer from glycerol toxicity, even when exposed to concentrations as high as 40%. Nevertheless no spermatozoa were viable following freezing with cooling rates ranging from rapid pellet freezing to 1 ºC minute^{-1} protocols. However none of these protocols considered the most recent finding that *S. crassicaudata* spermatozoa were susceptible to cold shock.

3. *If dasyurid sperm nuclei lack disulphide stabilisation, will they remain intact following freeze drying?*

The spermatozoa from *S. crassicaudata* are not viable following freeze drying, but the sperm heads do not show any indication of nuclear decondensation or DNA fragmentation when assessed using TUNEL.

4. *No marsupial spermatozoa have yet been shown to be sensitive to cold shock, are dasyurid spermatozoa sensitive to cold shock?*

Marsupials were believed to be tolerant of cold shock for the past 30 years. But *S. crassicaudata* spermatozoa rapidly cooled to 0.5 ºC were immotile upon warming, suggesting that *S. crassicaudata* are highly sensitive to cold shock. This susceptibility could be overcome, allowing spermatozoa to remain viable at 0 ºC, when cooling occurred at 0.5 ºC minute^{-1} in the presence of 10 or 20% egg yolk.

5. *What is the relationship between increased vaginal cornified epithelial cells, vaginal leukocytes and ovulation in S. crassicaudata?*

When vaginal leukocytes were present in urine samples *S. crassicaudata* had already ovulated, indicating that leukocytes appear after ovulation. If the CEC had been elevated for a short period of time before the leukocytes appeared then the ovulated oocytes were morphologically normal. But if the CEC had been elevated for several days before the leukocytes appeared they showed signs of parthenogenesis-an indicating that ovulation had occurred several days prior. This suggests that in *S. crassicaudata* ovulation occurs when CEC initially rise and not when CEC are maximal or when leukocytes appear, as had been reported for other small dasyurids.

6. *Can avoiding the progesterone secreting corpus luteum decrease the variation in S. crassicaudata ovarian stimulation protocols?*

Avoidance of the corpus luteum, by monitoring the patterns of CEC and stimulating females after the luteal phase, resulted in a highly reliable ovarian stimulation protocol. However, age provided an additional influence. Post-breeding retired females (>12 months of age) indicated a lower and slower response to ovarian stimulation than breeding age females (≤12 months of age).

7. *Can pre-ovulatory S. crassicaudata oocytes collected following ovarian stimulation reach the first polar body stage in vitro?*

Pre-ovulatory oocytes from younger breeding *S. crassicaudata* were at the first polar body stage upon collection, four days following ovarian stimulation. However pre-ovulatory oocytes collected from retired females, four days following stimulation, were able to be matured to the first polar body stage with almost 100% efficiency.

8. *Can individual preantral follicles be harvested by enzymatic ovarian dissociation in dasyurids?*

Enzymatic dissociation is an effective method of harvesting large numbers of high quality primordial, early primary, late primary and secondary granulosa cell-oocyte complexes from the ovaries of *S. crassicaudata*, *D. viverrinus*, *D. hallucatus* and *S. harrisii*. Vital staining of the granulosa cell-oocyte complex revealed that the smaller and less mature complexes were of better quality than the larger secondary granulosa cell-oocyte complexes which are likely to require an alternative, more gentle, collection protocol.

9. *Can isolated immature S. crassicaudata oocytes survive vitrification?*

Vital staining indicated that oocytes contained within enzymatically dissociated primary granulosa cell-oocyte complexes from *S. crassicaudata* showed no significant loss of viability following vitrification. However, upon thawing, there was a decline in the quality of the surrounding granulosa cells.

10. *Can thawed primary granulosa cell-oocyte complexes from S. crassicaudata be cultured in vitro?*

Primary granulosa cell-oocyte complexes from *S. crassicaudata* which had been thawed remained intact when cultured for 48 hours on an agar pillow without the formation of a granulosa cell monolayer. This short culture period was sufficient for the vitrification-induced damage of granulosa cells to be repaired, probably due to granulosa cell proliferation.

7.9 CONCLUSION

This project recognised that 18 species of Australian dasyurid marsupials were listed as threatened (Department of the Environment and Heritage 1999) with several species, such as the northern quoll (*D. hallucatus*) and Tasmanian devil (*S. harrisii*), threatened by processes which cannot currently be mitigated by *in situ* conservation. In these species *ex situ* captive breeding populations have been established. However significant loss of genetic diversity is still occurring, hence it is important to explore genome resource banking for these species, before additional genetic diversity is lost.

This thesis is the first to have focused entirely on ART in dasyurids and has advanced the techniques available for use in dasyurid marsupials by providing promising sperm and oocyte preservation protocols and describing reliable ovarian stimulation. In addition several unique findings regarding fundamental dasyurid reproductive biology have been described including the absence of acrosomal stabilisation and the susceptibility of spermatozoa to cold shock. Nevertheless the findings presented in this thesis require further extrapolation before they can become practical tools which can be used for gamete resource banking and conservation of dasyurid marsupials.

Recent developments in the internationally renowned efforts to conserve the black footed ferret (*Mustela nigripes*) have highlighted the importance of ART in wildlife conservation as 10 year old cryopreserved spermatozoa were used to inseminate a female who successfully gave birth to genetically important individuals, maintaining a viable genetically varied population (Howard and Wildt 2009). This demonstrates the potential of ART in wildlife conservation and the protocols developed in the present study provide a solid framework from which similar goals will ultimately be achieved in the conservation of dasyurid marsupials.

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