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

Czarny, N. A., Mate, K. E. and Rodger, J. C. (2008). Acrosome stability in the sperm of dasyurid marsupials. *Reproduction Fertility and Development* **20,** 295-302.

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.

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Czarny, N. A., Harris, M. S. & Rodger, J. C. (2007) Preservation of spermatozoa from dasyurid marsupials. Society of Reproductive Biologists Annual Scientific Meeting, Christchurch, New Zealand.

Czarny, N. A., Harris, M. S. & Rodger, J. C. (2009) Enzymatic dissociation and vitrification of preantral follicles from threatened carnivorous marsupials. International Embryo Transfer Society, San Diego, USA.

Czarny, N. A., Harris, M. S. and Rodger, J. C. (2009). Oocyte vitrification as a tool for genome resource banking in a dasyurid marsupial, the fat tailed dunnart. Australian Mammal Society 55th Conference, Perth, Australia.

Czarny, N. A. and Rodger, J. C. (2009). The first evidence of high susceptibility to cold shock by the spermatozoa of a marsupial, the fat tailed dunnart (*Sminthopsis crassicaudata*). Proceedings of the Society of Reproductive Biologists Annual Scientific Meeting, Adelaide, Australia.