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

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Thesis submitted for the Degree of Doctor of Philosophy
School of Environmental and Life Sciences
The University of Newcastle, Australia

November 2009
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to the joint publications.

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Natasha Alexandra Czarny
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This project would not have been possible without my three supervisors Professor John Rodger, Dr Karen Mate and Dr Merrilee Harris. Their support and assistance throughout this project is appreciated. I also thank all the past and present members of the Marsupial Research Lab, especially the volunteers.

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One of the important aspects of this project was replication of the findings on threatened species and for this it is essential to thank the zoological parks which made the effort to send me samples or animals. This includes Australian Ecosystems Foundation, Remabi Park, Alice Springs Desert Park, Territory Wildlife Park, Featherdale Wildlife Park and the Tasmanian Government. I also acknowledge the contribution of all the animal house and veterinary staff, especially Jude Rodger and Dr Robyn Gentle, who cared for the dunnart and quoll colonies. Finally I am appreciative of the support of Woolworths Ltd. Jesmond store who generously donated meat to feed the eastern quolls.

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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 °C at 0.5 °C 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.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
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<td>ART</td>
<td>Assisted reproductive techniques</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
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<td>CEC</td>
<td>Cornified epithelial cells</td>
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<td>cm</td>
<td>Centimetres</td>
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<td>CO₂</td>
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<td>1,2-dioctanoyl-sn glycerol</td>
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<tr>
<td>dUTP</td>
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<td>eSG</td>
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<td>FSH</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
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<td>g</td>
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<td>Granulosa cell-oocyte complexes</td>
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<td>GV</td>
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<td>HEPES</td>
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<td>hCG</td>
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<td>Intracytoplasmic sperm injection</td>
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<td>i.p.</td>
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<td>LN</td>
<td>Liquid nitrogen</td>
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<td>Monobromobimane</td>
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<tr>
<td>PMSG</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<td>TUNEL</td>
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<td>µg</td>
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<td><em>Oryx tao</em></td>
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<td><em>Trichosurus vulpecula</em></td>
<td>Brush tailed possum</td>
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<tr>
<td><em>Vombatus ursinus</em></td>
<td>Common wombat</td>
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PUBLICATIONS AND PRESENTATIONS

Peer-Reviewed Publications


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, 23931.

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.


Presentations


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.