Optimisation and Validation of an *in vitro* bioassay as a tool for measuring luteinising hormone in several species of mammals

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Declaration

I declare that this thesis is my own account of my research and contains work that has not previously been submitted for a degree at any other tertiary institution.

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Penny Nice

ABSTRACT

Understanding the underlying reproductive physiology of threatened and native species is crucial when developing conservation management strategies, especially in situations where *ex-situ* captive breeding programs are crucial to a species short-term survival. However, research into the reproductive biology of threatened species is hampered by the fact that it is difficult to measure the gonadotrophins, luteinising hormone (LH); and follicle-stimulating hormone (FSH), the chief controllers of reproduction in vertebrates. This project, therefore, aims to develop and validate a simple, rapid LH bioassay which is capable of measuring LH concentrations from a diverse range of mammalian species. The bioassay uses a clonal murine Leydig tumour cell line as an indirect method for measuring LH in mammals. The cell line responds to LH stimulation *in vitro* by producing the steroid progesterone, which is much easier to measure across species. It is envisioned that this assay would be a useful tool in situations where a high volume of samples from various species require analysis, such as in a zoo or wildlife breeding facility.

Firstly an optimisation of the conditions of the assay was conducted; specifically cell density/well and incubation time required for cells to produce progesterone was optimised to improve the sensitivity of the assay. An optimal cell density of 9,375 cells/well was discovered to have greatest sensitivity to physiological levels of LH after an optimal incubation period of 120 minutes. Using these optimised conditions, pituitary LH preparations from eight species of mammals (human, porcine, monkey, rabbit, bovine, canine, equine and, rat) produced good dose-response curves demonstrating the practicality of this assay to measure LH from diverse species. The sensitivity of the bioassay differed for each species, although there was no obvious phylogenetic relationship between species and sensitivity. The sensitivity ranged from 0.0625 – 1 ng/100 µl (NB: 100 µl of

sample in each well) for most of the species tested, well within the normal physiological range, with only the bovine being slightly higher at 4 ng/100 µl, but still within the physiological range. There was no cross-reactivity with FSH in the bioassay. Therefore, it was concluded that the assay might be suitable for the analysis of samples from diverse mammalian species.

As standard available immunoassays are also often unsuitable for measurement of LH in marsupial species, this precludes assessment of the stage of their oestrous cycle. The application of the LH bioassay was investigated for its capability to act as an indirect method for measuring LH in marsupials, which has not been conducted previously as marsupial LH has often been described to be structurally and antigenically different from LH produced by mammals. The bioassay detected an increased circulating LH pre- and 25 minutes post- exogenous GnRH challenge in western grey kangaroos ($P<0.05$) and black-flanked rock wallabies ($P<0.05$), and also detected decreased LH levels in black-flanked rock wallabies treated with a contraceptive prior to GnRH challenge ($P<0.01$). Results obtained by this bioassay method were validated by comparison to those derived from an adapted enzyme immunoassay (EIA). There was no significant difference in change in LH concentrations as detected by either method in the western grey kangaroo or the control group of black-flanked rock wallabies, however, there was a significant difference in change in LH concentration between the two methods when assessing black-flanked rock wallabies on a contraceptive ($P<0.05$). It was concluded that the bioassay could be used to successfully measure changes in circulating levels of LH in two species of Macropodid marsupials, though it appears to be most effective at measuring LH concentrations at the mid to high end of the physiological range, and less reliable for ascertaining low physiological LH levels.

The aim of the next part of the study was to extend the species range of the bioassay to include elephants, which are currently the focus of a worldwide captive breeding program. The unique

physiology and the presence of different hormone metabolites in the Asian elephant results in many commercially available hormone detection kits lacking sensitivity and/or suitability for application in elephants. Application of the bioassay to measure LH concentrations during the anovulatory (anLH) and ovulatory (ovLH) surges of the Asian elephant oestrous cycle was investigated with particular interest in its ability to distinguish between basal and surge LH concentrations. These results were validated by comparing them to those obtained by an established EIA. The bioassay successfully detected both anovulatory and ovulatory surges of LH during three consecutive cycles, with surge concentrations determined as those values which exceed the previous nadir by a minimum of three standard deviations (SD). In addition to this, the present study calculated the time taken from sample acquisition to progesterone determination as four hours suggesting the practicality of this method for obtaining rapid results making it more applicable to 'real world' situations where timing of the ovulatory surge is critical for artificial insemination to be successful.

The final part of the study used a unique animal model, the horse, to verify the LH bioassay against a definite LH surge-induced ovulation event. This is possible because the ovaries of the horse can be routinely assessed for signs of ovulation (presence of a corpus luteum) using rectal ultrasound. This is routinely done in large equine breeding facilities to assess the start of the breeding season. Horses are long day breeders, and the decreased daylight hours in winter are associated with decreased gonadotrophin secretion and hence decreased ovarian activity. The control of seasonal reproduction in the mare is primarily driven by photoperiod, yet other factors such as nutrition and body condition, environmental temperature, age and reproductive state can also have an effect. The aim of this part of the study was to investigate whether the bioassay could be used, firstly, to detect endogenous LH surges in the mare, and hence determine ovulation and the resumption of oestrous cyclicity; and secondly, to use the bioassay results to investigate whether

other factors apart from photoperiod influence the resumption of oestrous cyclicity. The bioassay accurately detected a recent or imminent ovulation in 85% of cases suggesting that it is a highly reliable method, and could be adopted by equine reproductive specialists. It was also found that while photoperiod was the strongest influence on resumption of oestrous cyclicity, seasonal changes in pelage (hair loss and coat condition) also had a strong influence, as did a higher body condition or an increase in body condition.

Evidence presented in this thesis demonstrated that a clonal mouse Leydig tumour cell line could be developed and used as an *in vitro* LH bioassay, to accurately detect LH concentrations from blood plasma samples from diverse mammalian species. Hence, this bioassay method could be adopted by researchers working in mammalian reproductive endocrinology, especially those working on wildlife species for which commercial gonadotrophin immunoassays are not readily available.

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Firstly I will begin by thanking my supervisors; Dr. David Miller, Dr. Phillip Matson and Dr. Mick Rae, for their insight, dedication and commitment to this project. Their support and encouragement has been invaluable and the project would not have been completed without them. Dave I really appreciate that you were willing to come to Murdoch very late at night when we had kangaroo testicles to process, and that you even finished the task alone due to my exhaustion! Frustratingly none of that hard work will be presented here, yet I know that not every supervisor would go that extra step and I am grateful for the commitment. Phill your contacts at Perth Zoo and with students at UWA have contributed enormously to the work presented in this project. In addition to this I was able to experience an automated progesterone analyser, and be blown away by a result calculated in 15 minutes! Mick while we only got to work side-by-side for a month (and not even every day as I was so busy that month) your knowledge of cell culture has been very valuable and I'm glad you got a "holiday" down under plus the ability to learn about the strange things that marsupials do as a break from the mundane (sheep).

I would also like to thank Dr. Serena Finlayson for accepting my offer of being a lab minion in the endocrine lab of the Perth Zoo. I learnt a number of things working with you and got to experience running ELISAs without a plate washer and how to develop RSI from scraping sand off dibbler poo samples! I also need to thank Dr Helen Robertson, Dr Cree Monaghan and Dr Simone Vitali of Perth Zoo as well as several of the Vet nursing staff (Kate & Mikaylie) for not only employing me in the endocrine lab but allowing me access to a range of animal samples. This was by far my favourite part of the project (sorry Anne!). Thanks also go to Dr. Anne Barnes and Dr. Patrick Brogan for collaborations on the horse part of my project. Both of you were eager to assist in data collection (rectal ultrasound) and coped with my complete lack of experience working with horses!

I'm glad that by the end of the sampling I was able to catch and halter a mare, though mostly the nicer and slightly smaller ladies.

Next I will thank my colleagues in the Dungeon and Bat Cave for putting up with me for the last four years (longer if you want to count Honours!). Special mentions go to Kathryn, Tracey, Shannon, Narelle, Heather, Jesse, Will, Ivan, John, Bryony, Chelsea, & Dennis who had to endure my crazy outbursts, my breakdowns and my occasional lack of civilised conversation. Sorry to everyone I scared off doing a PhD! To "Aunty" Kelly for being a mentor, a shoulder to cry on, and the queen of task management! It has helped immensely and I'm very grateful.

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Finally I thank Dory for reminding me to just keep swimming!

Dedication

This thesis is dedicated to my nonna, Angela Rosa Ierace and the women of *La Rocca*. In recognition of the triumphs over adversity that she endured when immigrating to Australia, and for having the determination to make the best of her situation. Your ability to persist under trying circumstances has thankfully been passed onto me!

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List of Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
Ab	Antibody
ABP	Androgen Binding Protein
AChE	Acetylcholinesterase
ACTH	Adrenocorticotrophic Hormone
Ag	Antigen
AI	Artificial Insemination
anLH	Anovulatory Luteinising Hormone (surge)
AR	Androgen receptor
ART	Artificial Reproductive Technologies
ATP	Adenosine triphosphate
BTB	Blood-testis barrier
cAMP	Cyclic Adenosine Monophosphate
CG	Chorionic Gonadotrophin
CL	Corpus luteum (Corpora lutea)
CMF-PBS	Calcium and Magnesium Free – Phosphate Buffer Saline
DHT	(5α-)Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
eCG	Equine Chorionic Gonadotrophin (see also PMSG)
EDTA	Ethylene Diamine Tetra-acetic Acid
EIA	Enzyme immunoassay
ELISA	Enzyme-Linked Immuno Sorbent Assay
FBS	Foetal Bovine Serum
FSH	Follicle-stimulating Hormone
FSH-R	Follicle-stimulating Hormone Receptor
GH	Growth Hormone
gpGnRH	Guineapig Gonadotrophin-releasing Hormone
GnIH	Gonadotrophin-Inhibiting Hormone
GnRH	Gonadotrophin-Releasing Hormone
hCG	Human Chorionic Gonadotrophin

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFSH	Human Follicle-Stimulating Hormone
HPG	Hypothalamus-Pituitary-Gonadal
IGF-1	Insulin-like Growth Factor 1
LH	Luteinising Hormone
LH-CG-R	Luteinising Hormone - Chorionic Gonadotrophin Receptor
LH-R	Luteinising Hormone Receptor
mGnRH	Mammalian Gonadotrophin-releasing Hormone
MLTC-1	Mouse Leydig Tumour Cell Line
OD	Optical Density
oFSH	Ovine Follicle-Stimulating Hormone
oLH	Ovine Luteinising Hormone
ovLH	Ovulatory Luteinising Hormone (surge)
PMSG	Pregnant Mare Serum Gonadotrophin (see also eCG)
PRL	Prolactin
RIA	Radio immunoassay
RPMI - 1640	Roswell Park Memorial Institute -1640
RRA	Radio receptorassay
StAR	Steroidogenic Acute Regulatory (protein)
TRH	Thyrotrophin-Releasing Hormone
TSH	Thyroid-stimulating Hormone

Submitted papers and conference proceedings

Conference Proceedings:

Nice, P.A., Rae, M.T., Matson, P.L., & Miller D.W. (2012) Validation of a bioassay for measuring luteinising hormone (LH) in two species of marsupial. *Abstracts of the 7th Asia and Oceania Society for Comparative Endocrinology Conference,* pp 46.

Nice, P.A., Robertson, H., Monaghan, C., Matson, P.L., & Miller D.W. (2012) Verification of a bioassay for measuring luteinising hormone (LH) in the Asian elephant (*Elephas maximus*). *Reproduction in Domestic Animals*, **47,** Suppl 4, pp 611.

Other publications arising from the period of candidature:

Nice, P.A., Fleming, P.A., Bennett, N.C., Bateman, P.W., & Miller, D.W. (2010) Exposure to non-kin females rapidly affects testicular morphology in non-reproductive male Damaraland mole-rats. *Journal of Zoology, London* **282** 84-90.