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

This thesis was submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (VETERINARY STUDIES (D1015)), Murdoch University by:

Penelope Angela Nice

BSc (Conservation Biology) Hons.

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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.

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.
Acknowledgements

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,
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all this time (I will leave eventually I promise!) while spending all my money on overseas holidays,
and because you probably copped the worst of my unsavoury moods, but you have supported me
with every decision I make and I love you both. To my sister for our chats and hugs and exchange
of clothes, love you loads.

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my rock these past three years. Your support has been enormous and sometimes I wonder what I
would do without you? For allowing me to rant and rave and then helping me piece everything
together and put it all into perspective. I love you so much.

Finally I thank Dory for reminding me to just keep swimming!
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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<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>17β-HSD</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABP</td>
<td>Androgen Binding Protein</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial Insemination</td>
</tr>
<tr>
<td>anLH</td>
<td>Anovulatory Luteinising Hormone (surge)</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ART</td>
<td>Artificial Reproductive Technologies</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood-testis barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CG</td>
<td>Chorionic Gonadotrophin</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum (Corpora lutea)</td>
</tr>
<tr>
<td>CMF-PBS</td>
<td>Calcium and Magnesium Free – Phosphate Buffer Saline</td>
</tr>
<tr>
<td>DHT</td>
<td>(5α-)Dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>eCG</td>
<td>Equine Chorionic Gonadotrophin (see also PMSG)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno Sorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating Hormone</td>
</tr>
<tr>
<td>FSH-R</td>
<td>Follicle-stimulating Hormone Receptor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>gpGnRH</td>
<td>Guineapig Gonadotrophin-releasing Hormone</td>
</tr>
<tr>
<td>GnIH</td>
<td>Gonadotrophin-Inhibiting Hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotrophin-Releasing Hormone</td>
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<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotrophin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>----------------</td>
<td>----------------------------------------------------------------------------</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hFSH</td>
<td>Human Follicle-Stimulating Hormone</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamus-Pituitary-Gonadal</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinising Hormone</td>
</tr>
<tr>
<td>LH-CG-R</td>
<td>Luteinising Hormone - Chorionic Gonadotrophin Receptor</td>
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<tr>
<td>LH-R</td>
<td>Luteinising Hormone Receptor</td>
</tr>
<tr>
<td>mGnRH</td>
<td>Mammalian Gonadotrophin-releasing Hormone</td>
</tr>
<tr>
<td>MLTC-1</td>
<td>Mouse Leydig Tumour Cell Line</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>oFSH</td>
<td>Ovine Follicle-Stimulating Hormone</td>
</tr>
<tr>
<td>oLH</td>
<td>Ovine Luteinising Hormone</td>
</tr>
<tr>
<td>ovLH</td>
<td>Ovulatory Luteinising Hormone (surge)</td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant Mare Serum Gonadotrophin (see also eCG)</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio immunoassay</td>
</tr>
<tr>
<td>RPMI - 1640</td>
<td>Roswell Park Memorial Institute -1640</td>
</tr>
<tr>
<td>RRA</td>
<td>Radio receptorassay</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic Acute Regulatory (protein)</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotrophin-Releasing Hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating Hormone</td>
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Submitted papers and conference proceedings

Conference Proceedings:


Other publications arising from the period of candidature:

1. GENERAL INTRODUCTION

1.1 Introduction

As the human population increases, continued habitat destruction forces many native species to exist in small fragmented populations with little room to expand (Conway, 1995, Wildt et al., 1995). Conservation of genetic information and hence biodiversity of these threatened species is paramount for them to survive into the future. Of the 4,500 species of mammals in existence today, little is known about the basic reproductive processes for most of these species (Wildt et al., 1995, Wildt and Wemmer, 1999). Understanding the underlying reproductive physiology of native animals is crucial when assessing the reproductive status of individual animals, diagnosing fertility problems and for using assisted reproductive technology (ART) when undertaking assisted or captive breeding programs to ensure their survival (Graham et al., 2001). However, monitoring reproductive hormones in native and exotic wildlife is challenging as there are not suitable assays to assess them in many native species. Knowledge of the changing pattern of important anterior pituitary reproductive hormones, known as gonadotrophins, is critical to understanding their reproductive physiology. However, the gonadotrophins have a complex chemical structure which can vary according to the species and stage of reproductive life. Accordingly the measurement of gonadotrophins in different species can be difficult if the peptide structure of the hormone is not recognised by the standard immunoassays often designed for humans, rodents and some common domestic species. Practical, reliable methods for monitoring reproductive hormones can establish the onset of puberty and oestrous cyclicity, determine the optimum time to pair animals for breeding, diagnose pregnancy and predict parturition (Graham et al., 2001).
This literature review will cover the basics of both reproduction and endocrinology focusing chiefly on mammals and highlighting the similarities and differences associated with different species, and with each gender. It will also highlight the importance of one of the gonadotrophic hormones, luteinising hormone (LH) and its role in reproduction, with particular attention to the various methods in practice for detecting this hormone.

1.2 The importance of endocrinology

In order for an organism to maintain homeostasis, signals from the external and internal environment must be transmitted to the cells of the body so a response can be produced. The endocrine system and its secretions, hormones, act with the nervous system to maintain homeostasis and regulate almost all complex physiological processes of the body including; growth, metabolism, development, osmoregulation and reproduction (Bentley, 1998). Hormones are the messenger system of the endocrine system and include; protein hormones (prolactin; PRL, growth hormone; GH) glycoprotein hormones (thyroid-stimulating hormone; TSH, the gonadotrophins LH and follicle-stimulating hormone; FSH) peptide hormones (insulin, insulin-like growth factor-1; IGF-1, adrenocorticotrophic hormone; ACTH) amino acid derivatives (catecholamines such as adrenaline and noradrenaline) and lipids (steroids such as progestogens, androgens, oestrogens and glucocorticoids) (Bentley, 1998).

1.2.1 The hypothalamic-pituitary-gonadal axis

Reproduction in all vertebrates is controlled by the hypothalamic-pituitary-gonadal (HPG) axis, which is responsible for translating environmental stimuli into neuroendocrine signals (Maruska and Fernald, 2011, Tsutsui et al., 2012). The HPG axis begins with the hypothalamic region of the brain, situated just below the third cerebral ventricle (Bentley, 1998). The hypothalamus regulates hormone secretion of the pituitary gland via nerve impulses and hormone secretion (Bentley,
The axons of these nerves converge on the ventral peripheral region of the tissue and make up the median eminence (Bentley, 1998). The hypothalamus contains parvicellular neurons that produce such peptides as gonadotrophin-releasing hormone (GnRH) and thyrotrophin-releasing hormone (TRH) (Bentley, 1998). These peptides are released from the nerve terminals in the median eminence into the blood vessels of the hypothalamo-hypophyseal portal system, in which they are transported to the anterior pituitary gland (Bentley, 1998, Maruska and Fernald, 2011).

The hormones produced by the anterior pituitary gland help regulate the activities of the thyroid, the adrenal cortex and the gonads, thus regulating water and salt metabolism, growth, thermoregulation, pelage (coat), reproduction, lactation, parturition and pigmentation of the skin (Bentley, 1998). In the mammalian brain, the pituitary gland is located immediately ventral to the hypothalamus, and is connected to it via the infundibular stalk (Bentley, 1998). The pituitary gland is further divided into the posterior pituitary gland (neurohypophysis) developed as an outgrowth of the brain, and the anterior pituitary gland (adenohypophysis) which is derived from non-neural ectoderm (Bentley, 1998). The anterior pituitary gland is further divided into the *pars distalis*, the *pars intermedia* and the *pars tuberalis*. The *pars distalis* contains five phenotypically different types of cells; the corticotrophs which secrete ACTH, gonadotrophs which secrete LH and FSH, somatotrophs which secrete GH, lactotrophs which secrete PRL and thyrotrophs which secrete TSH (Bentley, 1998). Under trophic stimulation from the hypothalamus, the anterior pituitary gland releases both LH and FSH into the general circulation where they are transported to the gonads (ovary or testis) (Maruska and Fernald, 2011). The gonads contain the receptors for LH and FSH and binding with the respective hormone stimulates steroidogenesis and gametogenesis (Maruska and Fernald, 2011).
1.2.2 Hypothalamic and pituitary hormones

Gonadotrophin-releasing hormone & gonadotrophin-inhibitory hormone

The hypothalamus controls hormone secretion of the anterior pituitary gland through the production of releasing hormones and release-inhibiting hormones (Bentley, 1998). Gonadotrophin-releasing hormone, a decapeptide glycoprotein hormone, is responsible for stimulating secretion of LH and FSH from the anterior pituitary gland (Bentley, 1998). Thirteen variants of GnRH have been identified throughout the vertebrates, from fish to mammals (with an additional two being identified in tunicates; urochordata) (Bentley, 1998, Grove-Strawser et al., 2002). Each GnRH molecule was named according to the taxa in which it was originally discovered, although many have a much wider distribution amongst vertebrates (Bentley, 1998). Eutherian mammals appear to only possess one form of GnRH; mammalian GnRH (mGnRH), while other species including marsupials possess two or even three variants (Bentley, 1998). The Guinea pig appears to be the exception to this rule, expressing both mGnRH and guinea pig GnRH (gpGnRH) (Grove-Strawser et al., 2002). In addition to this, it is now clear that a second molecule, gonadotrophin-inhibitory hormone (GnIH), acts to suppress LH and FSH secretion across several mammalian species (Tsutsui et al., 2000, Tsutsui et al., 2010, Tsutsui et al., 2012). Although originally identified in quail brain, orthologs of this peptide have now been identified in other vertebrate species from fish to humans (Tsutsui et al., 2012).

Kisspeptin

In addition and subsequent to the discovery of GnIH, kisspeptin which is encoded by the KiSS-1 gene was discovered in mammals (Tsutsui et al., 2010). Kisspeptin has a stimulatory effect on GnRH neurons via its receptor which results in an up-regulation of the HPG axis, and it is considered an essential part of mammalian puberty and subsequent fertility (Clarkson and
In addition to mammals, the KiSS-1 gene has also been identified in amphibians and fish (Tsutsui et al., 2010).

**Luteinising hormone, follicle-stimulating hormone & chorionic gonadotrophin**

The gonadotrophic hormones of the anterior pituitary gland, (LH and FSH) control the activity of the testes and ovaries and are categorised according to their chemical structure; LH and FSH are glycoprotein hormones consisting of two polypeptide chains, the α- and β-subunits (Bentley, 1998, Kalia et al., 2004). The α-subunit is common to the glycoprotein hormone family which also includes TSH and chorionic gonadotrophin (CG; secreted by the placenta in primates, equines and humans; (Rahman and Rao, 2009), whereas the β-subunit has a differing structure which determines the type of activity the intact hormone will elicit and thus also confers biological specificity (Bentley, 1998, Kalia et al., 2004). The carbohydrate content of each of these molecules may vary which contributes to the presence of isoforms, each of which may differ in their activities (Bentley, 1998).

**1.2.3 Steroid hormones**

Steroids are chemical compounds derived from cholesterol, which is primarily synthesised in the liver (Bentley, 1998). The steroid hormones of the gonads and adrenal cortex are synthesised from cholesterol via a series of metabolic pathways, usually involving several hydrolase enzymes (Bentley, 1998). Several different types of steroids function as “sex hormones” in vertebrates, and these sex hormones show remarkable uniformity (Bentley, 1998). Cholesterol is comprised of carbohydrates or triglycerols and is catabolised to a reduced number of carbons to produce the five classes of steroid hormones (Bentley, 1998). Those based on pregnane, the progestogens, contain 21 carbon atoms (C_{21}) and serve as precursors for corticosteroids, androgens and oestrogens (Bentley, 1998). Progesterone functions as a sex hormone, especially during pregnancy in most mammals (Bentley, 1998). Corticosteroids are also C_{21} and can be further divided into
glucocorticoids (e.g. cortisol and corticosterone), and mineralocorticoids (e.g. aldosterone) (Bentley, 1998). Progestogens give rise to steroids based on androstane, the androgens, C\textsubscript{19} compounds which include the male sex hormones such as androstenedione and testosterone and its more active metabolite 5α-dihydrotestosterone (5α-DHT) (Bentley, 1998). Androgens themselves give rise to steroids based on oestrone, the oestrogens, C\textsubscript{18} compounds known as the female sex hormones which include oestradiol 17-β, oestrone and oestriol (Bentley, 1998) (Figure 1.1).

1.2.4 Hormones: Stimulation, Production, Action & Termination

*Hormone Stimulation*

External cues such as photoperiod, nutrition, stress and social environment can influence reproductive functions. In many species, physiological processes, including reproduction, are synchronised to occur at times of the year when conditions enable survival of the young, and this is regulated by circannual rhythms (Notter *et al.*, 2011, Menassol *et al.*, 2012). In mammals, photic information such as day length is received by photoreceptors in the eyes and transmitted to the pineal gland in the brain. This in turn regulates the circadian pattern of secretion of a hormone produced by the pineal gland, melatonin, that correlates to day length (Yasuo and Yoshimura, 2009). These photoperiodic cues are reliable external cues that adequately reflect the changes of seasons and help regulate circannual cycles (Notter *et al.*, 2011). The extent to which photoperiodic cues impact on an animal’s reproductive processes appear to be related to the latitude of the animals’ habitat, with those species occurring within 10° of the equator demonstrating little to no response to photoperiodic stimuli (Nunes *et al.*, 2002). However, other factors such as rainfall, food quality and quantity can also influence the reproductive physiology of temperate and tropical mammals (Medger *et al.*, 2012).
Figure 1: Schematic metabolism of cholesterol

Schematic representation of the metabolism of cholesterol into the five classes of steroid hormones; progestogens (yellow), mineralocorticoids (purple), glucocorticoids (orange), androgens (blue) and oestrogens (pink) and the enzymes responsible for this breakdown. Adapted from (Bentley, 1998).
Nutrition is considered a secondary environmental stimulus that interacts with photoperiod (the primary stimulus) to regulate reproduction (Menassol et al., 2012). Menassol and colleagues found that the main effect that nutrition has on the ewe (a short-day, seasonally breeding species), was during the seasonal transitions into and out of the breeding season. Inadequate nutrition in other species of livestock has been noted to result in delayed onset of puberty, induction of anoestrous and prolonged postpartum anoestrous (Day et al., 1986, Schillo, 1992, Rhodes et al., 1996).

Many species of mammals have developed social systems, whereby reproduction is not partitioned equally amongst group members (Young et al., 2006, Clutton-Brock et al., 2008). In the naked mole-rat, for example, reproduction is restricted to a single dominant female and up to three males and subordinates rarely or never breed (Jarvis, 1981). In meerkats, subordinates are capable of breeding but do so less frequently or are less successful than dominant animals (Young et al., 2006). Finally, in some social species such as lions, most females breed at intervals and differences in breeding success between females are low (Packer et al., 2001). The method by which subordinate animals are prevented from breeding varies too. In meerkats, aggression levels tend to be low outside the breeding season but increase prior to the dominant female giving birth, whereby her attacks towards subordinate females increase (Young et al., 2006). In the African painted dog, corticosterone levels are higher in dominant animals than in subordinates, thus a decreased reproduction rate in subordinate animals does not appear to be mediated by social stress levels (Creel et al., 1997). Conversely, reproduction by the dominant animals is not inhibited by their increased corticosterone levels (Creel et al., 1997). This is interesting to note because it has long been reported that chronic stress levels that cause prolonged activation of the hypothalmo-pituitary-adrenal axis has a negative impact on reproductive performance in mammals (Boonstra and Singleton, 1993, Ferin, 1999, Sheriff et al., 2009). Sheriff and colleagues (2009) found that female hares with extremely high concentrations of faecal glucocorticoid
metabolites frequently produced non-viable offspring (i.e. abortions and stillbirths). The challenge hypothesis, developed by Wingfield et al (1990), explores the relationship between season, aggression and testosterone concentrations in seasonal breeders. Its major prediction is that as the breeding season commences, testosterone concentrations increase from a non-breeding nadir to a slightly higher breeding baseline, stimulated by environmental factors such as photoperiod, and any increases in testosterone beyond the breeding baseline are related to aggressive behaviours such as territory defence and mate guarding (Wingfield et al., 1990). While this hypothesis was developed primarily from data collected from birds, it has also been applied to mammals; for example Wied’s marmoset (Nunes et al., 2000) and chimpanzees (Muller and Wrangham, 2004). However, it is also worth noting that for some species including dwarf mongooses (Creel et al., 1993), and Ethiopian wolves (van Kesteren et al., 2012) data do not conform to the predications of the challenge hypothesis.

**Hormone synthesis & release**

Synthesis of hormones commences with transcription of DNA in the nucleus to produce RNA, which is followed by translation in the rough endoplasmic reticulum, into a primary protein (Andrews et al., 1987). Cleavage of the signal peptide in the endoplasmic reticulum results in the formation of the prohormone, which itself may be further processed by endopeptidases and carboxypeptidases (Andrews et al., 1987). A newly synthesised prohormone is released into the cisternae of the endoplasmic reticulum where it is transported to the Golgi complex (Bentley, 1998). Here, many proteins are packaged into secretory vesicles and a single prohormone may result in production of only a single hormone, multiple copies of the same hormone or, multiple types of hormones (Bentley, 1998). These granules move towards the peripheral regions of the cell, and in response to a stimulus, combine with the plasma membrane and discharge their contents into the circulation (Bentley, 1998).
In addition to this, some neurons form hormones in a process termed neurosecretion (Bentley, 1998). In this situation, the axon of a nerve cell, instead of terminating at another neuron or an effector tissue, lies near a capillary into which it can discharge its contents (Bentley, 1998). Hypothalamic hormones that regulate hormone secretion of the anterior pituitary gland are formed by neurosecretion (Bentley, 1998). For the gonadal steroid hormones, synthesis is the same in all species of vertebrates (Sandor, 1969). Synthesis of each hormone from the ovaries and testes involves several enzymatically mediated changes which occur in the endoplasmic reticulum and mitochondria of the cell (Bentley, 1998).

Secretion of a hormone is regulated as an appropriate stimulus is received by the endocrine gland, which will indicate whether an increase or decrease of the hormone is required (Bentley, 1998). This stimulus may be perceived either from the external environment (including; changes in light and temperature, acquisition of food and water), or from within the body itself (including; changes in salt concentrations, hydrostatic pressure of the blood vascular system and fluctuations in nutrient levels) (Bentley, 1998). The secretion of many hormones from cells is not continuous, but rather a single large surge or a series of pulses succeeding each other at regular intervals (Brabant et al., 1992). This mechanism of hormone release provides a more detailed message to the target organ rather than a continuous stimulation, which can avoid down-regulation of hormone receptors (Bentley, 1998). The pulsatile release of hormones results in a stable basal concentration in plasma (Brabant et al., 1992). When a hormone is first released into the circulation, it becomes diluted due to its redistribution in the body fluids, and it will then be removed from the circulation (Bentley, 1998). The length of time that this process entails is known as its clearance rate (Bentley, 1998). Removal from circulation occurs as a result of uptake by tissues, metabolic breakdown and excretion in urine or faeces (Bentley, 1998).
Hormone transport & action

When an endocrine gland receives a stimulus, it releases the hormones from the storage granules via exocytosis into the circulation (Bentley, 1998). Once released into the blood plasma, the hormone travels to its target cell, sometimes as a dissolved product in the plasma (bound to plasma albumin), other times while bound to a carrier or binding protein (e.g. steroids) in an equilibril (reversible) fashion (Bentley, 1998). The initiating event in the action of a hormone is its binding to a specific high affinity receptor (Bentley, 1998). Binding of the hormone to the receptor will initiate, enhance or inhibit a chemical event within the target cell (Bentley, 1998). When these two molecules combine, a cascade of biochemical events occurs culminating in the physiological effect of the hormone (Bentley, 1998). Hormone receptors may occur on the external surface of the cell membrane (e.g. gonadotrophin receptors), or inside the cell such as in the cytoplasm or nucleus (e.g. steroid receptors) (Bentley, 1998). Hormone receptors on the plasma membrane are coupled to a special protein (G protein) and initiate their effects by activating a second messenger system, which triggers the next series of biochemical events (Bentley, 1998). One such example of a second messenger is the nucleotide adenosine-3′,5′-monophosphate (cyclic AMP or cAMP) (Bentley, 1998). Derived from adenosine triphosphate (ATP) through the action of adenylate cyclase and degraded by phosphodiesterase, concentrations of cAMP will increase or decrease in response to such hormones as ACTH, FSH and LH (Bentley, 1998).

Hormone receptors in the nucleus produce their effects by initiating the transcription of target genes (Bentley, 1998). In this case, the receptor has functional domains, the ligand domain, the DNA-binding domain and the transcription activation domain (Bentley, 1998). The ligand domain binds with the hormone while the DNA-binding domain mediates the interaction of the receptor with the binding site on the target gene and, the transcription activation domain regulates post-binding biochemical events (Bentley, 1998). The affinity (or K-value) of a hormone is a
measurement of the tightness of binding of the hormone to the ligand domain (Bentley, 1998) An ideal fitting hormone would have a K-value of 1, while other molecules would have a K-value between 0 and 1 (Bentley, 1998).

**Hormone feedback & termination**

An important feature of the action of a hormone is a signal that informs the secretory cell that they have achieved their objective (i.e. produced an effect) (Bentley, 1998). This is known as feedback and the effect may be direct, or controlled by another hormone, a metabolite of the hormone’s action, or a physical factor, which tells the secretory cell that adequate hormone has been produced for the necessary changes required to maintain homeostasis, or changes in the level at which the target system operates (Bentley, 1998). The result of feedback control will either decrease hormone production (negative), or increase hormone production (positive) (Bentley, 1998). For example; high testosterone levels in the circulation will have a negative effect on GnRH release from the hypothalamus, and subsequently LH release from the anterior pituitary gland, which was required initially to stimulate production of testosterone (Bentley, 1998). While high levels of oestrogens will have a positive effect on GnRH release, and hence LH release which culminates in the pre-ovulatory surge of LH (Bentley, 1998).

While feedback will suppress hormone production after the desired effect has been accomplished, the remainder of the hormone left in circulation must be inactivated to prevent an overshoot of the response (Bentley, 1998). In order for a target cell not to be under constant stimulation from a hormone, complex biochemical pathways operate which metabolise and inactivate hormones (Bentley, 1998). The action of a hormone may be terminated in several ways: firstly for hormones secreted in short bursts, termination occurs as a result of the dilution and redistribution of the hormone in the body fluids (Bentley, 1998). Secondly hormone activity can be decreased due to the metabolism of hormones by enzymes particularly in the liver and kidneys (Bentley, 1998).
Protein hormones are metabolised by proteolytic enzymes, while steroids are conjugated to glucuronic and sulphuric acids resulting in increased water solubility, and hence excretion (Bentley, 1998). Both the inactive fragment and the active hormone can be excreted in urine and retain some biological activity (Bentley, 1998).

1.2.5 Species specificity of hormones: the marsupial example

The dual gonadotrophin hormone system occurs throughout all tetrapod classes, with the exception of the Squamate reptiles which appear to possess only a single gonadotrophin (Licht, 1983). Species specificity, which is the phenomenon whereby the hormonal sensitivity of a target organ depends on the species from which the hormone is derived, does not follow a clear phylogenetic pattern and cannot be used to reliably distinguish related hormones (Licht, 1983). Studies that have examined differences between marsupial and eutherian gonadotrophins have noted that their differences can affect their biological activity in different assay systems (Farmer et al., 1981). While the chemical differences between marsupial and eutherian gonadotrophins remain minor, in fact they share a lot of similarity in terms of their amino acid composition, N-terminal residue and carbohydrate composition (Licht, 1983), biological and binding assays have demonstrated important structural divergence between marsupial and eutherian hormones in terms of the active site on the hormone molecule (Gallo et al., 1978, Farmer et al., 1981).

Farmer and colleagues (1981) extracted LH from two species of kangaroos; eastern and western greys. When examined in an ovine LH (oLH) radio immunoassay (RIA), the kangaroo LH were essentially equipotent to oLH (Farmer et al., 1981). Yet despite this apparent similarity between marsupial and eutherian immunological activity in a eutherian RIA, biological and binding assays (radio receptor assay) produced notable differences (Farmer et al., 1981). Using a rat Leydig cell bioassay (measuring testosterone production), the eastern grey kangaroo LH was 3.2% and the western grey kangaroo was 2.9% the activity of oLH (Farmer et al., 1981). Results were improved
upon when using a receptor binding assay with kangaroo testis as the receptor source, as the kangaroo LH was 33% as potent as oLH (Farmer et al., 1981). The structural differences between marsupial and eutherian LH is denoted by the species specificity in biological activity (Gallo et al., 1978). Gallo and colleagues (1978) found low potency of tammar wallaby LH when compared to oLH using a rat Leydig cell bioassay yet this was in contrast when the testes were sourced from opossums. In a more recent study, brush-tailed possum LH was found to be 4% that of oLH in a receptor binding assay using bovine corpora lutea cells as the receptor source, but 20% that of oLH when using possum testicular receptors (Moore et al., 1997). These findings demonstrate a high degree of species specificity between assay systems based on either marsupial or eutherian receptor sources (Gallo et al., 1978, Farmer et al., 1981). The differences recorded between marsupial LH and eutherian LH in their ability to bind marsupial or eutherian receptors suggests that there has been some degree of structural divergence at the active site of hormone molecules between marsupial and eutherian gonadotrophin hormones (Moore et al., 1997).

1.3 Reproductive Endocrinology in Mammals

The basic pattern of hormonal control of reproduction is remarkably similar across the vertebrate species (fish, amphibians, reptiles, birds and mammals), as it involves the endocrine secretions of the anterior pituitary gland and the gonads (Bentley, 1998). As has been stated previously, the hypothalamic region of the brain influences gonadotrophin release (LH and FSH) from the anterior pituitary gland which is vital in successful steroidogenesis and gametogenesis in most vertebrates (Bentley, 1998). Vertebrate gonads serve a dual purpose, both as the producer of gametes (ova and sperm) and as the producer of some of the hormones controlling the reproductive process (Bentley, 1998). The testis of males provide the environment for production and maturation of spermatozoa and also produce androgens, mainly testosterone, while the ovary of females produce follicles which contain maturing ova as well as producing the sex steroids, oestrogens and
progestogens (Bentley, 1998). It is worth noting here that the gonadotrophic hormones are chemically analogous, but have undergone evolutionary changes in their structures, yet by comparison, the gonadal steroids (androgens, oestrogens and progestogens) are almost identical throughout vertebrate species (Bentley, 1998).

As sperm and ova mature, the changing hormonal milieu contributes to other physiological changes required for successful fertilisation and development of the resulting embryo (Bentley, 1998). In males, mature sperm that are capable of ovum fertilisation may be produced during a particular season or may be available at any time of year (Bentley, 1998). In females, however, a mature ova is produced cyclically and if not fertilised is likely to survive for less than a day (Bentley, 1998). In order to advertise her receptivity to the male, the female may display various external signals or even actively seek out a mate (Bentley, 1998). In mammals, the period of sexual receptivity in females is termed oestrus, and the oestrous cycle refers to the recurrent set of physiological and behavioural changes that take place from one period of oestrus to another (Bentley, 1998). Many mammals experience only a single oestrous cycle in a year (monestrous), while others may experience several cycles confined to a specific time of year (seasonally polyoestrous) or several cycles year-round (continually polyoestrous) (Bentley, 1998). However, classification into these categories is not always clear cut. For example, monestrous cycles and spontaneous ovulation appears to be the general rule for canid species including dogs, wolves, jackals and coyotes (Concannon et al., 2009). Domestic cats can be seasonally or non-seasonally polyoestrous induced ovulators (Concannon et al., 2009).
1.3.1 Steroidogenesis & gametogenesis in males

**Steroidogenesis**

The two functions of the testis, spermatogenesis and steroidogenesis, depend on stimulation by the pituitary gonadotrophins FSH and LH, which in turn are stimulated by GnRH from the hypothalamus (McLachlan et al., 1995). In male mammals, the testes are organised into distinct seminiferous tubules surrounded by interstitial tissue which is comprised of Leydig cells, macrophages, and mesenchymal cells, in addition to networks of capillaries and nerves (Saez, 1994, McLachlan et al., 1995, Oatley and Brinster, 2012). Within the seminiferous tubules, somatic Sertoli cells associate closely with developing germ cells (spermatogonia) and form tight junctions with each other that compartmentalises the seminiferous epithelium into basal and adluminal compartments thus forming the blood-testis barrier (BTB) (Oatley and Brinster, 2012). The male sex steroid testosterone is secreted by the Leydig cells of the interstitial tissue, and this is under control of LH stimulation, as Leydig cells contain the LH receptor (LH-R) (McLachlan et al., 1995, Preston et al., 2012). Testosterone plays a key role in the development of secondary sexual characteristics and in promoting spermatogenesis (McLachlan et al., 1995). The action of FSH on spermatogenesis is mediated through Sertoli cells which contain the FSH receptor (FSH-R) (McLachlan et al., 1995).

In the testes, most progesterone is converted firstly to androstenedione (a weak androgen) which acts as the precursor for production of testosterone, the chief vertebrate androgen (Bentley, 1998). Testosterone itself can then be converted into DHT, a more potent androgen through the action of the enzyme 5α-reductase (Grover et al., 2005). The receptor for testosterone (androgen receptor; AR) are abundant on both Sertoli and Leydig cells, but more so on the former (McLachlan et al., 1995). The AR binds testosterone with an affinity lower than that for DHT, however, because
the relative abundance of testosterone in normal testis is greater than that of DHT, testosterone is
the predominant ligand for the AR (McLachlan et al., 1995) (Figure 1.2).

**Spermatogenesis**

The process by which spermatogonia develop into mature spermatozoa is termed spermatogenesis, and is a complex process of mitosis, meiosis and cell differentiation (Dadhich et al., 2010, Oatley and Brinster, 2012). Within the basal compartment of the seminiferous epithelium resides the population of spermatogonia which have the capacity to replicate themselves to maintain a pool of spermatogonia, and also to provide cells that will go on to differentiate into spermatozoa (Kula et al., 2001, Oatley and Brinster, 2012). Only Sertoli cells possess receptors for testosterone and FSH, thus these cells are the major targets of the hormones that regulate spermatogenesis; the germ cells themselves do not appear to possess a functional AR (De Gendt et al., 2004, Walker and Cheng, 2005). Androgens are essential for the maintenance of spermatogenesis, and although 5α-DHT is crucial for the development of the male reproductive tract, testosterone is the androgen in the male testis that regulates spermatogenesis (Walker and Cheng, 2005).

Due to the close association of the Leydig cells with the seminiferous tubules, high concentrations of testosterone produced under LH stimulation is delivered to the tubules (Saez, 1994). The BTB formed by the Sertoli-to-Sertoli tight junctions provides a specialised, testosterone-enriched microenvironment within the seminiferous tubules of the testis for successful spermatogenesis (Munell et al., 2002, Walker and Cheng, 2005). In addition to this, Sertoli cells are crucial to normal spermatogenesis as they also provide support, nourishment and anchorage to developing
Figure 1: 2 Hormonal control of male reproductive function

Schematic representation of the hormonal regulation of male reproductive function. Red arrows indicate an inhibitory effect, while blue arrows indicate a stimulatory effect. Adapted from Ross and Pawlina (2006).
germinal cells in the seminiferous epithelium, phagocytose the residual bodies of elongating spermatids, and also produce androgen binding protein (ABP), which assists in the maintenance of the high levels of testosterone required in the tubular compartments of the mammalian testes (Gunsalus et al., 1978, Munell et al., 2002, Grover et al., 2005, Walker and Cheng, 2005). Androgen binding protein, a testicular glycoprotein binds androgens with high affinity and transports them into the epididymis (Munell et al., 2002, Grover et al., 2004, Grover et al., 2005). Once the spermatogonia have completed differentiation, the elongated spermatid is released from the Sertoli cell, moves out of the testis along with the tubular fluid and into the epididymis (Sairam and Krishnamurthy, 2001, Grover et al., 2004). The epididymis depends upon high levels of testosterone for maintaining its structure and functions in relation to sperm motility and fertility (Grover et al., 2005). The epididymis is a single, highly convoluted tubule linking the efferent ducts of the testis to the vas deferens, and can be divided into three sections; caput, corpus, and caudal (Robaire and Viger, 1995).

The epididymis is comprised of two major compartments: the epithelium and the lumen (Robaire and Viger, 1995). The major functions carried out by the epididymis are sperm transport, maturation (acquisition of fertilizing potential) and storage (Robaire and Viger, 1995). As sperm mature they acquire motility which is an essential but not sufficient condition for their potential to fertilise an ova and produce viable offspring (Robaire and Viger, 1995). The caudal region of the epididymis is the major site for the storage of sperm in the male reproductive tract (Robaire and Viger, 1995). In the epididymal lumen, the ABP produced by Sertoli cells is responsible for maintaining high levels of testosterone for the epithelial cells where testosterone is converted to 5α-DHT by the enzyme 5α-reductase which is required to maintain epididymal function (Robaire and Viger, 1995, Grover et al., 2005). One important difference in gametogenesis between males and females is that the ovary contains a fixed number of non-renewable reserves of primordial
follicles endowed during embryonic development, whereas the male contains spermatogonia that can continually renew themselves (Sairam and Krishnamurthy, 2001) (Figure 1.3). The most recent evidence on this topic now suggests that putative stem cells which exhibit germline characteristics are present in adult mouse ovaries challenging the widely held belief that mammalian females lose the ability to produce new oocytes at or shortly after birth (Tilly et al., 2009).

In males, seasonal breeding is accompanied by periodic maturation of the sperm and associated sex glands and secondary sexual characteristics required to ensure its delivery on an appropriate occasion (Bentley, 1998). In these species, the testes undergo circannual cycles of activation and involution (Dadhich et al., 2010). Low concentrations of plasma gonadotrophins coincide with low testosterone production in the non-breeding season (Dadhich et al., 2010). Return to breeding status prompts reactivation of testosterone production and spermatogenesis in males and is also often accompanied by increases in testes size (Preston et al., 2012).

1.3.2 Steroidogenesis & gametogenesis in females

Steroidogenesis

As per the testis of the male, the ovary of the female preforms two functions; steroidogenesis and gametogenesis (Conti, 2011). The principle cell types involved in follicular steroidogenesis in female mammals are of two basic types; i) LH-responsive theca cells of the follicular envelope and the interstitial cells of the ovarian stroma, and ii) FSH-responsive granulosa cells which later in follicular maturation also develop the ability to respond to LH (Gore-Langton and Armstrong, 1988). There is a two-cell two-gonadotrophin concept for steroidogenesis in the ovaries (Magoffin, 2005) (Figure 1.4). Theca cells, which are capable of de novo production of androgens but have limited or no capacity to convert androgens into oestrogens, cooperate with the granulosa cells which do not express the enzymes required for androgen production but do contain abundant
Figure 1: Schematic diagram of seminiferous epithelium

Schematic diagram of seminiferous epithelium showing Sertoli cells and germ cells in different stages of spermatogenesis. Adapted from Ross and Pawlina (2006). Ap; type A pale spermatogonium, Ad; type A dark spermatogonium, B; type B pale spermatogonium, P; primary spermatocyte, SC; Sertoli cell, ES; early spermatid, LS; late spermatid, RB; residual body.
Figure 1: 4 Hormonal control of female reproductive function

Schematic representation of the hormonal regulation of female reproductive function. Red arrows indicate an inhibitory effect, while blue arrows indicate a stimulatory effect. Adapted from Gore-Langton and Armstrong (1988).
aromatase and 17β-HSD which produce oestrogens from androgens (Magoffin, 2005). In the ovary, the LH-R is present on the theca cells and stimulation results in androgen production (androstenedione, testosterone and 5α-DHT) (Tarumi et al., 2012). The production of androgens in theca cells stimulates proliferation of granulosa cells and development of early follicles as well as promoting oocyte maturation (Tarumi et al., 2012). In addition, LH increases expression of the LH-R, and the steroidogenic acute regulatory protein (StAR), a protein that facilitates transport of cholesterol to the inner leaflet of the mitochondria and together these enzymes enable the theca cells to synthesise androgens from cholesterol (Magoffin, 2005). The principal steroid product of the theca cells in most species is androstenedione, which diffuses across the basal lamina where it is metabolised to oestradiol by the granulosa cells (Magoffin, 2005). The mid-cycle surge of LH that triggers ovulation causes a down regulation of the enzymes in theca cells (androgen producing enzymes), transforming them from androgen-producing to progesterone-producing cells (Magoffin, 2005).

The role of FSH in the ovary is primarily associated with its effect on follicular growth (Sirard et al., 2007). The FSH-R located on granulosa cells triggers cell proliferation in the early stages of follicle development, prevents atresia, and induces the synthesis of LH-R and steroid hormone expression (Sirard et al., 2007). Thus, FSH is considered the most important factor in the follicle selection process by controlling steroidogenesis through signalling cascades downstream of FSH (Sirard et al., 2007). Follicle-stimulating hormone appears to be the chief regulator of ovarian aromatase activity in granulosa cells (Gore-Langton and Armstrong, 1988). The production of progesterone and its metabolites is a major biosynthetic activity of the granulosa cells in large antral and pre-ovulatory follicles (Gore-Langton and Armstrong, 1988). Initially, progesterone biosynthesis occurs in granulosa cells as a response to stimulation by FSH, but this process is later enhanced by LH after its receptors have differentiated (Gore-Langton and Armstrong, 1988). Although
androstenedione is the major ovarian androgen (in most species), the action of 17β-HSD favours the production of oestradiol-17β as the dominant ovarian oestrogen (Gore-Langton and Armstrong, 1988).

**Oogenesis and Folliculogenesis**

The growth and development of the ovarian follicle is regulated by circulating gonadotrophins under primary control of the hypothalamus and anterior pituitary gland (Conti, 2011). FSH is required for the growth phase of the follicle from primordial to Graafian, as well as inducing oestrogen production and massive replication of granulosa cells (Conti, 2011). LH regulates the terminal differentiation of the follicle and induces ovulation and the subsequent development and progesterone-secreting function of the corpus luteum (CL) while suppressing oestrogen production and inducing exit from the cell cycle (Conti, 2011). The female gamete or oocyte is contained within a follicle comprised of somatic cells, and the process of maturation and development of the female gamete is termed oogenesis, while the development of the follicle is termed folliculogenesis (Magoffin, 2005, Conti, 2011, Canipari et al., 2012, Tarumi et al., 2012). At the early stages of development, a primordial follicle is a simple spherical structure comprised of an oocyte surrounded by a single layer of squamous (flattened) granulosa cells, and the majority of these follicles exist in a quiescent state (Magoffin, 2005, Conti, 2011, Canipari et al., 2012). Periodically, under hormonal stimulation, primordial follicles are recruited to the growing pool where the number of layers of granulosa cells increases and the primordial follicle becomes a primary follicle (Magoffin, 2005, Conti, 2011, Canipari et al., 2012). As the primordial follicle develops into a primary follicle granulosa cells change their morphology from squamous to cuboidal (Canipari et al., 2012) (Figure 1.5). Development then progresses to the secondary follicle stage when the second layer of cuboidal granulosa cells begins to appear and the first theca cells begin to differentiate into the theca interna.
Figure 1: Schematic diagram of an ovary

Schematic diagram of an ovary indicating the different stages of folliculogenesis, ovulation, and development of the corpus luteum. Adapted from Ross and Pawlina, (2006).
(Albertini et al., 2001, Magoffin, 2005, Canipari et al., 2012). As the follicle becomes bilaminar, granulosa cells undergo differentiation and start to express FSH-R (Canipari et al., 2012).

At the time the theca cells become morphologically distinguishable adjacent to secondary follicles, they have the capacity to secrete steroid hormones and respond to LH (Magoffin, 2005). The theca interna is comprised of highly vascularised steroidogenic cells adjacent to the basal lamina, while the theca externa is a loosely organised band of non-steroidogenic cells situated between the theca interna and the interfollicular stroma (Magoffin, 2005). This layer of thecal cells is separated from the epithelial granulosa cells by a basal lamina (Albertini et al., 2001, Conti, 2011). In addition to this, the oocyte secretes an extracellular matrix known as the zona pellucida, which separates it from the somatic granulosa cells (Albertini et al., 2001, Conti, 2011). Follicular development continues to progress and during this time small fluid-filled cavities become apparent (Canipari et al., 2012). The Graafian (pre-ovulatory) follicle is formed when these cavities coalesce into a single antrum (Canipari et al., 2012). When this happens, two distinct populations of granulosa cells can be identified; those lining the follicle wall, and the cumulus cells surrounding the oocyte (Canipari et al., 2012). The important difference between these two types of granulosa cells are the presence of the LH-R, which is restricted largely to the granulosa cells lining the follicle wall (Canipari et al., 2012).

Throughout the follicular growth phase, oocyte development is arrested at prophase I of meiosis, and meiosis does not resume until the time of the preovulatory LH surge (Conti, 2011, Canipari et al., 2012, Tarumi et al., 2012). At the time of the LH surge and resulting ovulation, functional changes occur in several ovarian cell types both within the follicle (granulosa and theca cells) and the stroma (fibroblasts and endothelial cells), as well as to the ovarian surface epithelium (Robker et al., 2000). Subsequently, structural reorganisation and remodelling of the follicle occurs as the
granulosa cells and theca cells luteinise to form the CL (Robker et al., 2000). The theca luteal cells produce steroid hormones (mainly progesterone) until luteolysis, unless pregnancy occurs, in which case progesterone production by the CL is either maintained by chorionic gonadotrophin (CG) stimulation, or replaced by placental progesterone production (Magoffin, 2005). In the event of luteolysis, the theca luteal cells lose their steroidogenic capacity and the CL regresses into a structure called the corpus albicans (Magoffin, 2005).

The oestrous cycle & ovulation
At the onset of puberty the female develops a cyclical pattern of ovarian activity which facilitates the release of a fertilisable egg (or eggs), and prepares for establishment and maintenance of pregnancy (Forde et al., 2011). Known as the oestrous cycle, it is described by the behavioural and physiological changes associated with ovulation, development and full attainment of functional activity of the CL, and the associated behavioural changes of the female. The oestrous cycle is divided into two basic phases, the follicular and the luteal phase and its duration varies between species and within individuals of the same species for example; sow 18 – 24 days (Soede et al., 2011), cow 18 – 24 days (Forde et al., 2011), bitch 60 – 115 days (Concannon, 2011), mare 22 days (Aurich, 2011).

During the follicular phase, the largest antral follicles in the pool are recruited and commence their development (Knox, 2005, Soede et al., 2011). Follicular recruitment occurs when pulsatile GnRH and hence LH release shifts from a lesser frequency/greater amplitude pattern to a greater frequency/lesser amplitude pattern (Flowers et al., 1991, Forde et al., 2011, Soede et al., 2011). The follicular phase is characterised by low levels of circulating progesterone due to luteolysis of the CL (when applicable) and high concentrations of oestradiol derived from rapid proliferation of the emerging dominant follicle (Forde et al., 2011). Approximately 6 days prior to ovulation in the sow, peripheral concentrations of progesterone have decreased to basal levels and large sized
follicles (6mm diameter) develop in the ovaries (Noguchi et al., 2010, Soede et al., 2011). These follicles contribute to increased production of 17β-oestradiol, which has a negative feedback effect on the hypothalamus and increased inhibin secretion which specifically targets FSH production (Noguchi et al., 2010, Soede et al., 2011). As gonadotrophin hormone secretion is disrupted, and smaller follicles have insufficient LH-R, they are therefore dependent on FSH and when peripheral concentrations of FSH decrease they undergo atresia (Lucy et al., 2001, Soede et al., 2011). The remainder of the follicular phase is characterised by an increased development of LH-dependent larger sized follicles which have escaped from regression and where they develop to the preovulatory stage (Soede et al., 2011). Each species will demonstrate differences in the number of follicles that attain the pre-ovulatory stage and the sow can ovulate 15 – 30 oocytes in a single oestrous cycle (Soede et al., 2011).

As follicles approach an ovulatory diameter the pulsatile secretion of LH and FSH decrease to barely detectable concentrations approximately 2 to 3 days prior to ovulation (Prunier et al., 1987). Oestrogen production from the preovulatory follicle(s) reaches its maximum causing the pre-ovulatory LH surge, and a small surge of FSH, via positive feedback (Prunier et al., 1987, Noguchi et al., 2010, Soede et al., 2011). The LH surge also initiates the follicular changes resulting in ovulation and luteinisation of the follicle wall, resulting in progesterone being the dominant steroid produced (Soede et al., 2011). In the sow, ovulation occurs approximately 30 hours post LH-surge (Soede et al., 1994). The luteal phase of the oestrous cycle commences immediately post-ovulation, when progesterone concentrations are minimal (Soede et al., 2011). After the Graafian follicle ruptures at ovulation releasing the ova, extensive reorganisation of the tissue takes place, culminating in formation of the fully functional CL by the mid-luteal phase (Soede et al., 2011). In the sow, the developing CL begins producing increased levels of progesterone that reach peak concentrations approximately 8 to 9 days post-ovulation (Soede et al., 2011). Secretion
of LH during the luteal phase is characterised by a lower frequency/greater amplitude of pulses (Langendijk et al., 2007). In a non-pregnant cycle in the sow, luteolysis occurs around day 15 post-ovulation and is caused by prostaglandins secreted by the uterus (Soede et al., 2011). In a pregnant cycle, however, the gravid uterus also secretes prostaglandins, but oestrogen signalling from the developing embryos causes prostaglandins to be secreted into the uterine lumen rather than into the circulation (Soede et al., 2011).

The oestrous cycle of domestic animals is further divided into five stages (cf the basic follicular and luteal phases), though they are often difficult to distinguish. Proestrus is the stage when external indicators of impending oestrus can be detected in the female; such as vulval swelling, vaginal discharge, vaginal epithelial proliferation and, cornification of cells and oedema (vaginal cytology) (Concannon, 2011). In the bitch it lasts on average nine days and concludes when the female becomes sexually receptive to the male (Concannon, 2011). Behavioural oestrus is characterised by receptivity to mounting by the male and often increased male-seeking behaviour (Concannon, 2011). Oestrus is used as the central point of the cycle (day zero), but will vary in duration according to species from several hours to several days and can also vary between individuals of the same species. In the bitch, oestrus averages nine days and occurs in response to the decline in oestradiol concentrations that commences shortly before the LH surge, and continues throughout oestrus (Concannon, 2011).

Metestrus follows on from oestrus, commencing when the female refuses to accept the advances of the male (day one of the cycle) and is considered to last until evidence of the ongoing luteal phase becomes minimal (Concannon, 2011). Metestrus in the cow is described as lasting 3 – 4 days and is characterised by formation of the CL from the collapsed ovulated follicle (Forde et al., 2011). Progesterone production by the newly formed CL increases in readiness for establishment
and maintenance of pregnancy, and/or resumption of the oestrous cycle (Forde et al., 2011). Dioestrous is often also used to describe this phase, commencing when the CL is fully developed, and the reproductive organs are under the dominant influence of progesterone (Forde et al., 2011). In cows it follows on from metestrus and is characterised by recurrent waves of follicular development, however, dominant follicles in these waves fail to ovulate as the high levels of progesterone act through negative feedback resulting in the secretion of greater amplitude but lesser frequency LH pulses that are inadequate for ovulation of the dominant follicle to occur (Forde et al., 2011). Depending on the source of information, some authors claim dioestrous to be synonymous with metestrus and omit it as a term when describing the oestrous cycle, while other authors claim that metestrus is only of academic significance and that dioestrous is the longest phase of the cycle in the non-pregnant animal (Concannon, 2011, Forde et al., 2011).

For most species, a period of acyclicity, termed anoestrous, is common whether an obligate phase as in the dog, during the non-breeding season of seasonally polyoestrous species (e.g. horse and sheep), and during periods of lactation (Irvine et al., 2000, Concannon, 2011, Soede et al., 2011). During lactational anoestrous, concentrations of LH and LH-pulsatility are suppressed due to the sucking-induced inhibition of GnRH secretion (Soede et al., 2011). The influence that lactation has on FSH is primarily due to negative feedback of inhibin produced by limited growth of antral follicles (Soede et al., 2011). During seasonal anoestrous, melatonin is primarily responsible for contributing to the decreased hypothalamic GnRH content and release (Aurich, 2011).

1.3.3 Marsupials: alternative strategies in reproduction

Marsupials have evolved a successful reproductive strategy that is remarkably different to that expressed in eutherian mammals (Renfree, 1981). The young of all marsupials are born at a very early stage of development and the remainder of their growth occurs after birth (Behringer et al., 2006, Renfree, 2010). In most marsupial species postnatal development occurs with the offspring
attached to a teat in a marsupium (pouch) located on the ventral side of the mother’s body (Behringer et al., 2006, Renfree, 2010). Interestingly, though not all marsupial species possess a pouch, and for the species that do not, their offspring are exposed to the external environment while attached to the teat (Behringer et al., 2006, Renfree, 2010). Marsupials invest more energy in lactation than they do in gestation, with the milk they consume changing in composition throughout their postnatal development (Renfree, 1981, 2010). One of the theories for the birth of such altricial offspring is that it is thought to be advantageous in the ecological conditions of Australia as mating and birth in marsupials may take place under less than ideal conditions, but as development continues in the pouch, the young do not emerge until conditions are favourable (Renfree, 1981, Tyndale-Biscoe, 2001). For example in the brown antechinus (Antechinus stuartii), birth occurs in winter but young are weaned in spring some three months later when insect prey levels are increased (Tyndale-Biscoe, 2005). The tammar wallaby (Macropus eugenii), as another example, spends eight months developing in the pouch so despite being born in mid summer at the hottest and driest time of year when females are often in poor body condition, emergence of the young occurs in spring when there is abundant new plant growth (Tyndale-Biscoe, 2005). Eutherian mammals are often referred to as ‘placental’ mammals; however, this is a misnomer as marsupials also have a placenta (Behringer et al., 2006). In marsupials, the yolk sac forms the definitive chorio-vitelline placenta, with only a few species such as bandicoots, quolls and wombats developing a chorio-allantoic placenta (as seen in eutherians) later in gestation which supplements the yolk sac placenta (Freyer and Renfree, 2009, Renfree, 2010).

The basic hormonal control of reproduction in marsupials is similar for that described in placental mammals. The secretion of both LH and FSH from the anterior pituitary gland is controlled by GnRH secretion from the hypothalamus (Tyndale-Biscoe, 2005). In female marsupials secretion of FSH is primarily associated with follicle growth and oestrogen secretion by the follicle cells prior to
ovulation, while LH is responsible for the induction of ovulation and the formation of luteal cells which produce progesterone (Tyndale-Biscoe, 2005). The marsupial oestrous cycle is divided into two phases (similar to that of eutherian mammals), the pro-oestrus phase during which follicles grow to the Graafian stage leading to oestrus and ovulation, and the luteal phase controlled by the newly formed CL (Tyndale-Biscoe, 2005). In most marsupials, pregnancy is accommodated in the luteal phase of the cycle which comprises approximately ⅔ of the length of the oestrous cycle (Tyndale-Biscoe, 2005). After the birth of the young, suckling suppresses the next pro-oestrus phase and ovulation (Tyndale-Biscoe, 2005). In contrast to this in many species of Macropodid marsupials, the length of pregnancy is the same as the length of the oestrous cycle such that oestrus can occur a few hours after birth (post-partum oestrus) and the female is able to conceive at this time (Tyndale-Biscoe, 2005). Assuming the newly born young begins suckling on a teat, development of the newly formed CL and the embryo is arrested until the end of lactation in a process known as embryonic diapause (Tyndale-Biscoe, 2005). It is interesting to note that the phenomena of embryonic diapause is not just restricted to marsupials, being also used as a reproductive strategy by approximately 100 different mammals in seven different orders (reviewed in Renfree and Shaw, 2000). Embryonic diapause, the delayed implantation of a conceptus, is noted in the following orders of mammals: Marsupialia, Insectivora, Chiroptera, Edentata, Carnivora, Rodentia, and Artiodactyla (Renfree and Shaw, 2000). The ecological benefit of this widespread phenomena is that the lengthening of the gestation period allows for young to be born at a time of year that is most favourable to their survival (Renfree and Shaw, 2000).

1.4 Luteinising Hormone

The gonadotrophins (LH and FSH) work synergistically to stimulate gametogenesis and steroidogenesis in the gonads of mammals (Bentley, 1998). Determining LH concentrations in animals or humans, as a predictor of impending ovulation, is crucial when assessing the
reproductive status of individual animals, diagnosing fertility problems and for using ART when undertaking captive breeding programs to ensure the survival of threatened species (Graham et al., 2001, Matson et al., 2009). Many different assay techniques have been developed to determine LH concentrations in biological fluids, and these are classified as either bioassays or immunoassays. LH exists in multiple forms in the pituitary gland and in circulation throughout the body (Chappel, 1990, Jeffcoate, 1993). These variants include peptide variants and glycosylation isoforms (glycoforms), that can be separated on the basis of their overall charge which is determined by the oligosaccharide chains incorporated into each isohormone (Chappel, 1990, Jeffcoate, 1993). These sugar chains affect not only the charge of the molecule but also its half-life in circulation, receptor binding activity and in vivo and in vitro biological activity (Chappel, 1990).

The source of LH, e.g. plasma, serum or urine, will influence the type and proportion of isoforms in the sample (Storring, 1992). This results from the fact that glycosylation of the polypeptide moiety is influenced by the species and tissue type of the cell in which the glycoprotein is synthesised, and the physiological conditions acting on this cell (Storring, 1992). Different isoforms of LH have different immunoreactivities and this is likely responsible for discrepancies noted between laboratories and between assay systems that are used to measure LH (Storring, 1992). Immunoassay selectivity depends (amongst other things) on the epitope specificity of the antibodies employed in the system (Jeffcoate, 1993). The in vivo bioavailability and bioactivity of LH is affected by the extent of sialylation and sulfation of the isoforms due to the fact that these terminal residues determine the rate at which LH is cleared from the circulation (Jeffcoate, 1993, Ulloa-Aguirre et al., 2001). For LH, epitope specificity is dependent mainly upon amino acid composition, and only to a lesser extent upon oligosaccharide structure (Jeffcoate, 1993).

Ratios comparing the biological and immunological activity of LH have been in use for some time (Chappel, 1990, Ding et al., 1991, Huhtaniemi et al., 1992). However, several studies have
demonstrated bias in these ratios as different studies use different methods for measuring both biological activity and immunological activity of LH (Ding et al., 1991). The target cell used in a bioassay appears to be the main cause of discrepancies, as the LH receptor is often sourced from rat, mouse or human (Ding et al., 1991). LH molecules from different species differ in their affinity to a LH receptor of a given species (Ding et al., 1991). In terms of immunological activity, conventional immunoassay methods tend to overestimate low levels of LH, and the type of antibody (mono- or polyclonal) can also impact on these measurements (Huhtaniemi et al., 1992). There is no such property as the ‘biological activity’ or ‘immunological activity’ of a molecule, rather this activity is defined by the system chosen to measure the molecule (Jeffcoate, 1993). In terms of the current research, the important thing is to establish a simpler and readily applicable method to measure LH from diverse mammalian species. Such a system will require the sensitivity to detect both basal and surge secretion of LH from animal samples (e.g. plasma), such that the practice can readily be adopted by zoos and other captive breeding facilities in an effort to improve endocrine monitoring of native and exotic wildlife and ultimately increase breeding success.

1.4.1 Methods for measuring Luteinising Hormone

Immunoassays

Immunoassays measure the amount of immunoreactive hormone molecules in a sample, and mainly vary according to the type of tracer employed in the system (e.g. radioactive, enzymatic, chemiluminescent or fluorescent) (Kalia et al., 2004). The basic components for developing an immunoassay are as follows: i) an antibody (Ab) that is extremely specific for the analyte that is to be measured (i.e. an antibody for LH), ii) a labelled form (tracer) of the analyte (in a competitive assay) or a labelled secondary antibody to the analyte (reagent excess assay) and, iii) a system that will separate antibody bound tracer from unbound tracer (Kalia et al., 2004). In a radio
immunoassay (RIA), the most common tracer is $^{125}$Iodine and the method is based on the competition between serum LH (unknown sample) and $^{125}$Iodine-labeled LH for binding to a LH specific antibody in the following equation:

$$\text{Ab} + \text{Ag} + \text{Ag}^* \leftrightarrow \text{Ab.Ag} + \text{Ab.Ag}^*$$

*Where* $^*$ is the labelled antigen.*

In this method, the concentration of labelled LH (antigen; Ag) and antibody remain constant, thus the amount of labelled LH (Ab.Ag*) is inversely proportional to the amount of endogenous antigen (Ab.Ag) in the sample (Kalia et al., 2004). Following an incubation period, the bound fraction must be separated from the unbound fraction by centrifugation and the radioactivity is measured by a gamma counter (Kalia et al., 2004). Radio immunoassays have been developed for measuring LH in a number of different species including; cow (Hejl et al., 1992), Sumatran rhinoceros (Roth et al., 2001), pig (Stewart et al., 2010), rat (Watanabe et al., 1990), sheep (Baird, 1978) brushtailed possum (Moore et al., 1997), tammar wallaby (McFarlane et al., 1997) and Asian elephant (Brown et al., 1999). However, RIAs have several disadvantages arising from the use of radioactive tracers such as health hazards, short shelf-life, radioactive waste disposal and expensive equipment, which limits applicability of the technique (Kalia et al., 2004).

Thus, to overcome the challenges of RIAs, many researchers utilise enzyme immunoassays (EIA). The enzyme-linked immunosorbent assays (ELISA) is a type of EIA which involves the use of a solid-phase which means the antibody is bound to a microtitre plate. In this method, there is an excess of reagents as the assay depends on occupancy of the binding sites as opposed to competition for the binding sites in the following equation (Wheeler, 2006).

$$\text{Ab}_1 + \text{Ag} \rightarrow \text{Ab}_1.\text{Ag} \quad \text{Ab}_1.\text{Ag} + \text{Ab}_2^* \rightarrow \text{Ab}_1.\text{Ag}.\text{Ab}_2^*$$
The development of the reagent excess assay (or sandwich assay) uses a capture antibody (Ab1) specific for a unique site on the β-LH molecule that is usually bound to a solid phase (e.g. a microtiter plate) (Kalia et al., 2004). A sample of endogenous LH is added to the well along with a secondary antibody raised against a different epitope of the antigen and conjugated to an enzyme (commonly horseradish peroxidase) (Kalia et al., 2004). After incubation, the substrate to the enzyme is added which produces a colour reaction, and the intensity of the colour is proportional to the concentration of LH in the sample (Kalia et al., 2004). Enzyme immunoassays can accurately detect LH from cell culture media, pituitary extracts and sera and are sensitive enough to detect the mid-cycle LH surge and establish the day of ovulation as well as removing the hazards of radioisotope usage, they are highly sensitive, simple, have low interassay variation and require less working time (Kalia et al., 2004).

**Bioassays**

Bioassays measure the biological activity of LH indirectly as the measured response is what occurs downstream of LH binding e.g. measuring cAMP, progesterone or testosterone production (Kalia et al., 2004). Early bioassay work used freshly isolated mouse or rat interstitial (Leydig) cells as an indirect method for measuring LH from several mammalian and non-mammalian species (Farmer et al., 1977, Yu and Wang, 1987). Farmer and colleagues (1977) used isolated rat interstitial cells which were exposed to different mammalian species of LH plus human chorionic gonadotrophin (hCG) and equine chorionic gonadotrophin (eCG) and found dose response curves from all samples tested. Five non-mammalian species (turkey, snapping turtle, alligator, bullfrog and sea turtle) were also tested in this assay system, but required a 1,000 fold dose increase in order to elicit a response thus demonstrating some taxa specificity of the rat cells for LH (Farmer et al., 1977). In the late 1980s, dispersed testicular interstitial cells from mice and roosters were used to measure LH from eleven different gonadotrophins on androgen production (Yu and Wang, 1987). The
mammalian gonadotrophins (ovine LH, hCG, and eCG) all produced dose-dependent androgen production (Yu and Wang, 1987). When using LH from chicken, frog and turtle the response was much less potent than ovine LH, and when using piscine gonadotrophins the response was very low and involved concentrations ranging from 500 – 200,000ng to elicit any response (Yu and Wang, 1987). Primary culture of cells (i.e. freshly isolated), rapidly undergo dedifferentiation and can cease testosterone production, hence for each batch of samples to be analysed fresh cells needed to be isolated (Ascoli, 1981, Finaz et al., 1987).

The development of testicular clonal Leydig cell lines saw further advances in the use of bioassays as a tool for measuring LH. The M5480 testicular Leydig tumour originating in the C57BL/6 strain of mouse was found to be hormone responsive and adaptable for growth in culture (Ascoli and Puett, 1978). This original tumour has given rise to two additional tumours known as M5480P and M5480A both of which possess the LH-CG receptor (LH-CG-R) and respond to stimulation with steroid production (Ascoli and Puett, 1978). Under basal conditions, the M5480P tumour produces mainly progesterone, while M5480A produces both progesterone and testosterone (Ascoli and Puett, 1978). When the LH-CG-R is stimulated with hCG the M5480P tumour produces increasing amounts of progesterone, while M5480A increases production of both progesterone and testosterone, though the former dominates (Ascoli and Puett, 1978). The M5480P tumour has given rise to a number of clonal cell lines (MA-10, MA-12, MLTC-1), all of which retain the LH-CG-R and respond to stimulation by increased progesterone production via the cAMP second messenger pathway (Ascoli, 1981, Rebois, 1982). The BLT-1 cell line was established from the gonadal tumour of transgenic (TG) mice containing a fragment of the mouse inhibin α-subunit promoter fused with the Simian virus 40 T-antigen (SV40 Tag) coding sequences (Kananen et al., 1996). This cell line responds to hCG stimulation with elevated cAMP and progesterone production, and while they do still produce low amounts of testosterone these concentrations gradually decline over prolonged
culture (Kananen et al., 1996). Other studies have used the MA-10 cell line and hybridised it with freshly isolated Leydig cells thus giving rise to a clonal cell line (K9) which, when stimulated with hCG was able to produce testosterone (Finaz et al., 1987). However in this case, testosterone production was not maintained after a six month culture period, thus the K9 cell line would be unsuitable for long term culturing and hormone detection (Finaz et al., 1987).

Despite differences in the steroidogenic pathway compared to normal Leydig cells, cultured Leydig tumour cell lines provide a relatively simple, rapid and sensitive measurement of LH bioactivity in vitro, and while the cells are of mouse origin they are not species specific thus allowing measurement of hormones in relatively diverse taxa (Dahl and Sarkissian, 1993). The advantages of this method are that it eliminates the use of live animals, allows unlimited cells to be grown which display similar LH responsiveness, reduces interassay variation and the techniques are simple and rapid allowing wider application of the assay (Dahl and Sarkissian, 1993). While the study by Dahl and Sarkissian (1993) was the first to demonstrate that LH sourced from different mammalian species could stimulate progesterone production of clonal Leydig cells, further application of this technique has not been considered until the current study.

1.5 The Importance of a Multi-species Bioassay

Determining LH concentrations in the circulation, as a predictor of impending ovulation, is crucial when assessing the reproductive status of animals (Graham et al., 2001, Matson et al., 2009). Due to the complex chemical structure of LH, measuring its concentration in various mammalian species (including native and exotic wildlife) can be difficult if the peptide structure of the hormone is not recognised by the standard immunoassays often designed for humans, rodents and some common domestic species (Graham et al., 2001). On the other hand, the downstream product of gonadotrophin action on the sex organs (gonadal steroids) can prove more reliable to
measure as they show remarkable uniformity between species (Bentley, 1998). Produced from the metabolism of cholesterol, in gonadal cells, steroids such as progesterone and testosterone can be stimulated by LH sourced from a number of different mammalian species (Dahl and Sarkissian, 1993).

To determine LH-stimulated steroid production, a bioassay method can be developed using a cell with a receptor for LH (e.g. Leydig cells of the testis or theca cells of the ovary). In this situation, a blood plasma sample with an unknown quantity of LH would produce high levels of progesterone if there was a high concentration of LH (more receptors occupied results in greater progesterone production), and low levels of progesterone if there was a low concentration of LH (fewer receptor sites occupied results in lower progesterone production). As demonstrated by Dahl and Sarkissian (1993), a clonal mouse cell line was not species specific to the source of LH used to stimulate progesterone production. This method then has great applicability for use by zoo and wildlife breeding facilities that may routinely monitor oestrous cycles of any number of different mammalian species, and would likely be investigating species less commonly studied.

1.6 General Aim and Hypothesis

The overall aim is to develop and validate a simple, rapid in vitro LH bioassay which is able to detect both basal and surge concentrations of LH from a diverse range of mammalian species. The general hypothesis is that by utilising a clonal cell line in a bioassay, we will be able to indirectly measure LH concentrations from blood plasma samples of several mammalian species, as the cells will be stimulated to produce progesterone regardless of the origin of the source of LH. Such a bioassay will prove to be a useful tool for increasing the understanding of the reproductive endocrinology of lesser studied wildlife species, and in applying assisted reproductive technologies to threatened species. We envisage that this research will benefit i) zoo and wildlife facilities that
are breeding endangered species, ii) native species (marsupial) research including work on fertility control and, iii) production and companion animal research.
2. GENERAL MATERIALS AND METHODS

2.1 Experiments involving animal samples

All experiments involving animals, or samples taken from animals, were approved by the Murdoch University Animal Ethics Committee.

2.2 Basic cell culture procedures

2.2.1 Preparation of cell culture medium.

Phenol red-free RPMI-1640 culture medium powder (Mediatech, Manassas, Virginia, U.S.A. #90-022-PC) was reconstituted as follows, according to manufacturer’s instructions: powdered media, 10.10g was added to 500mL of sterile water (Baxter Healthcare, Old Toongabbie, Australia; #88-09-02-005H) in a one litre beaker. Sodium bicarbonate (Sigma-Aldrich, Sydney, Australia; #S5761-500G) 2.00g, and between 4.5 – 5g of HEPES powder (Sigma-Aldrich, Sydney, Australia; #83264) was added to the beaker. Once all powders had completely dissolved, stirring was ceased as over mixing promotes the loss of bicarbonate through CO₂ disposition. More sterile water was added to the beaker to make up a final volume of 1L. The pH of the culture medium was adjusted to 7.2 to allow for an approximate increase of 0.2 units after filtration. A 0.45µm bottle-top vacuum filter (Nalgene, Rochester, New York, U.S.A. #295-4545) was used for filtration (Appendix 9.1).

After the powdered medium had been reconstituted, 10% foetal bovine serum (FBS; Invitrogen, Melbourne, Australia; #10099-141), 1% penicillin-streptomycin solution (Mediatech, Manassas, Virginia, U.S.A. #30-001-CI), and 1% L-glutamine (Invitrogen, Melbourne, Australia; #2530-081) were added to the solution. Medium prepared in this manner will henceforth be referred to as complete medium. In addition to this some experiments required serum-free medium, as FBS
would be expected to contain endogenous hormones which would interfere with our results and this was prepared as above minus the addition of 10% FBS and 1% penicillin-streptomycin solution.

2.2.2 Acquisition of cell line

The MLTC-1 clonal cell line was purchased from American Type Culture Collection (ATCC; Manassas, Virginia, U.S.A. CRL-2065). Cells were established from the M5480P transplantable Leydig cell tumour of the testis in the C57B1/6J strain of mouse (Mus musculus) (Rebois, 1982). Cells were shipped frozen and upon arrival the cryovial was rapidly defrosted and the cell suspension (1ml) transferred to a 25cm² culture flask. Ten millilitres of complete medium was added to the culture vessel, which was placed in a 5% CO₂ incubator at 37°C. Cultures were grown to a confluency of ~70 – 80% before splitting 1:3. Stock aliquots (1ml) of cell suspension were stored in liquid nitrogen as a reserve supply of cells at differing passages (Plate 1). Cells may differ in their responsiveness with continued passage, thus all experiments conducted herein used cells of <25 cycles (Rebois, 1982).

Splitting cells at confluence

An inverted microscope was used to determine the confluency of cells in their flask. Once the cell monolayer occupied approximately 80% of the available surface of the flask, the culture was split to prevent cell death. Old culture medium was aspirated and discarded, then the monolayer washed briefly with Ca²⁺ and Mg²⁺ free phosphate buffered saline (CMF-PBS; Mediatech, Manassas, Virginia, U.S.A. #21-040-CM). A 1x solution of trypsin with EDTA (Invitrogen, Melbourne, Australia; #15400-054) was added to the flask after the removal of CMF-PBS to detach the monolayer from the culture vessel. The flask was incubated at 37°C for approximately 10 minutes, or until complete cell detachment occurred. The cell suspension was then transferred to a 15ml centrifuge tube and cell clusters dispersed using a needle (25 gauge 1”) and syringe.
Following this, ⅓ of the cell suspension was transferred to each new 25 cm² flask and complete medium added prior to incubation at 37°C, 5% CO₂.

**Storing cells in liquid nitrogen**

Following detachment of cells as outlined above, cell suspensions were transferred to a 15ml centrifuge tube and 2mls of complete medium was added. The suspension was centrifuged at 452.79 g for 5 minutes. The supernatant was removed and the pellet was resuspended in 1 ml of 4°C freezing medium (95% complete medium plus 5% dimethyl sulfoxide) (DMSO; Mediatech, Manassas, Virginia, U.S.A. #25-950-CQC). A further 2 ml of freezing medium were added to the resuspended pellet. One millilitre aliquots of cell suspension were transferred to 2 ml cryovials. The cryovials were placed into a -80°C freezer overnight and transferred to liquid nitrogen storage the next day.

**Resuscitation of cell stock from liquid nitrogen**

Briefly, a cryovial of cells was carefully removed from the liquid nitrogen storage container. The cell suspension was rapidly (<2 mins) defrosted under hot water and then transferred to a 15ml centrifuge tube. Five millilitres of pre-warmed complete media was added to the tube slowly (approximately 1 ml per minute). The tube was centrifuged for 10 mins at 201.24 g. The supernatant was removed and the cell pellet was resuspended in 1 ml of complete medium. The new cell suspension was transferred to a T25 culture flask and an additional 8 ml of complete media was added to the flask. The flask was then sealed and returned to the incubator.

**Counting cells**

The number of cells contained within a cell suspension collected from a confluent flask was determined for experimental procedures. To do this a 10 µl sample of cell suspension was transferred to an eppendorf tube containing 90 µl of complete medium. After aspiration, a sample
of 50 µl was collected from the first eppendorf tube and transferred to a new eppendorf tube containing 50 µl of the staining dye Trypan Blue (Sigma-Aldrich, Sydney, Australia; T8154-20ML). A small droplet of the Trypan blue-stained cell suspension was transferred onto a Neubauer haemocytometer and viewed under a light microscope at 100X magnification. Live cells (those that appear a pale blue colour with a halo-like ring around them) were counted in the five primary squares of the haemocytometer (the four corner squares plus the central square) (Figure 2.1). Dead cells (those which are a dark blue colour due to absorption of the dye) were not counted. Cell number per ml was calculated based on the following formula:

\[
\text{No cells} = \bar{x} \times a \times 10^4
\]

Where \( \bar{x} \) is the mean number of cells counted from the five primary squares of the haemocytometer, \( a \) is the dilution factor, and \( 10^4 \) is the volume correction for the haemocytometer.

![Figure 2: 1 Schematic diagram of a haemocytometer](www.microbehunter.com)

Schematic diagram of a haemocytometer used to determine concentration of cells per ml. Cells in the four corner squares and the central square were counted and an average was calculated. Image was sourced from [www.microbehunter.com](http://www.microbehunter.com)

### 2.3 Standard competitive EIA protocol

The commercial progesterone EIA kit used (Cayman Chemical, Ann Arbor, Michigan, USA; #582601) was designed for measurement of either human or rodent samples sourced from either
plasma or culture medium. According to the manufacturer’s instructions, the assay is based on the competition between progesterone and a progesterone-acetylcholinesterase (AChE) conjugate (progesterone tracer) for a limited number of progesterone-specific rabbit antiserum binding sites. The cross reactivity of the progesterone antibody with several steroid hormones is summarised in Table 2.1. To determine the standard curve for progesterone, the progesterone EIA standard was serially diluted as stated in the manufacturer’s instructions. The rabbit antiserum-progesterone complex binds to the mouse monoclonal anti-rabbit IgG that has previously been attached to the well. The plate was washed to remove any unbound reagents then Ellman’s reagent (which contains the substrate to AChE) was added to the well producing a distinct yellow colour.

Each of the dilutions from the standard curve (50 µl) was dispensed (in duplicate) into the appropriate wells, and each sample (50 µl) was added to appropriate wells. The progesterone AChE tracer was added to each well (50 µl) followed by progesterone EIA antiserum (50 µl). The plate was covered with acetate plate sealer and incubated for one hour at room temperature on an orbital shaker. The contents of the wells were decanted out over a sink and the wells washed five times with wash buffer using an automated system set to 250 µl per well. The wells were decanted and blotted on absorbent paper to remove any residual liquid. Following this, 200 µl of Ellman’s reagent was added to each well. The plate was covered with acetate plate sealer and placed on an orbital shaker and covered with foil to allow reagents to develop in the dark. After one hour, absorbance was measured at 405 nm on a 96-well microplate reader. Inter- and intra-assay coefficients of variation were calculated based on data obtained when assaying LH preparations from eight species of mammals. Intra-assay coefficient of variation was determined from the 2 ng/ml of LH samples (which should fall within the reliably detectable area of the standard curve) and averaged across all eight species as 4.33 ± 4.3%. Inter-assay coefficient of variation was obtained from the different concentrations of LH analysed and averaged across all
eight species as 4.19 ± 2.4% respectively. The manufacturer lists the detection limit as 10 pg/ml, however, we considered anything under 15 pg/ml (or the equivalent 300 pg/ml for samples diluted 1:20) to be unreliable and hence these samples were recorded as 15 pg/ml (basal).

Table 2: 1 Antibody specificity

Cross reactivity of the progesterone antibody with several steroid hormones, according to manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross Reactivity</th>
<th>Compound</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>100%</td>
<td>5β-pregnan-3α,20α-diol glucuronide</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>7.2%</td>
<td>5β-pregnan-3α,20α-diol</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>5β-pregnan-3α-ol-20-one</td>
<td>6.7%</td>
<td>17α-estradiol</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>2.5%</td>
<td>Estriol</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>17-hydroxy progesterone</td>
<td>0.5%</td>
<td>Estrone</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;0.05%</td>
<td>Promegestone</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>5α-pregnan-3α,20α-diol</td>
<td>&lt;0.01%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4 Progesterone Standards

Due to the nature of this project commercial progesterone kits were purchased in bulk, usually four boxes at a time with each box capable of running five plates, or 480 wells. However, one problem encountered was the degradation of the progesterone standard, as evident by a gradient shift in the standard curve over time, despite the kit being stored at -20°C (according to manufacturer’s instructions) and not exceeding the expiry date of the kit. It was decided to make our own progesterone standard for use in the commercial assay kit.

Progesterone standards were prepared as follows. First, 1 mg (±0.0001) of progesterone (Sigma Aldrich, Sydney, Australia #P0130-25G) was added to 10 ml of absolute ethanol giving a 100 µg/ml stock solution (stock #1) which was stored in aliquots at -80°C. Secondly, 50 µl of stock #1 was added to 5 ml of ethanol giving a 1 µg/ml stock solution (stock #2) which was stored in aliquots at -
80°C. Finally, to create the working stock, 200 µL of stock #2 was diluted in 10 ml of either EIA buffer or 10 ml of serum-free medium, depending on which samples would be run through the assay. Serum-free medium was used in the standards when assaying cell culture supernates while EIA buffer was used for assaying blood samples directly. The working stock had a concentration of 1,000 pg/50 µl as 50 µl is required per well in the assay. As 1,000 pg is the top standard in the commercial kit, serial dilutions were prepared from this standard to a final (most dilute) concentration of 7.8 pg/50 µl. Stock aliquots of working stock were also stored at -80°C until required for assay.

2.5 Dilution of cell culture supernates

After analysis of samples for optimisation experiments, it became apparent that some samples, in particular those from longer incubation times and those with higher doses of hCG, read above the maximum detectability of the standard curve on the commercial progesterone kit (1,000 pg/ml). In order to dilute these samples down to a level that could be detected by the commercial kit an appropriate dilution factor had to be determined. To do this, cells were grown to confluency and transferred to a 96-well plate as described above. After a day of serum starving, these cells were exposed to 64 ng/well of bovine LH (AFP-9070B), to generate a pool of culture medium that could be diluted (1:1 to 1:1024) and analysed in the commercial progesterone assay (Figure 2.2, Table 2.2). The optimal dilution factor for cell culture supernates was determined to be 1 in 20.

2.6 Endogenous progesterone determination

As all blood samples collected for use in this study would naturally be expected to contain endogenous progesterone, and the cell based bioassay’s steroidogenic pathway produces mainly progesterone, each blood sample used also required measurement of endogenous progesterone
Table 2: 2 Dilution factors of cell culture supernates

Dilution factors and mean optical density (OD) of samples run in triplicate. A dilution factor of 1:20 was selected as optimal.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean OD</th>
<th>Dilution</th>
<th>Mean OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.202</td>
<td>1:64</td>
<td>0.505</td>
</tr>
<tr>
<td>1:2</td>
<td>0.197</td>
<td>1:128</td>
<td>0.531</td>
</tr>
<tr>
<td>1:4</td>
<td>0.258</td>
<td>1:256</td>
<td>0.529</td>
</tr>
<tr>
<td>1:8</td>
<td>0.335</td>
<td>1:512</td>
<td>0.550</td>
</tr>
<tr>
<td>1:16</td>
<td>0.422</td>
<td>1:1024</td>
<td>0.623</td>
</tr>
<tr>
<td>1:32</td>
<td>0.448</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: 2 Serial dilutions of cell culture supernates

Progesterone standards analysed in triplicate for determining serial dilutions of cell culture supernates.
already present in the sample, in order for the bioassay values to more accurately reflect buffer supplied in the kit as opposed to serum-free medium according to manufacturer’s instructions.

2.7 Statistical Analysis

All data was analysed using SPSS version 19.0 (IBM SPSS Statistics, United States of America, 2010). Statistical significance was accepted when $\alpha < 0.05$. Optical density (OD) values generated by the progesterone EIA were used in the logarithmic regression formula generated by the standard curve to calculate a concentration for each sample (in duplicate). For example:

$$y = a \ln(x) + b$$

Where $a$ is the slope, $b$ is the intercept and $x$ is the OD value. The concentration of a sample as measured in progesterone units is therefore an index of the amount of LH (positive relationship).
Plate 2: Photograph of MLTC-1 cells

Representative photograph of non-confluent MLTC-1 cells growing in a 25cm² flask.
3. OPTIMISATION AND VALIDATION OF AN IN VITRO BIOASSAY TO MEASURE LUTEINISING HORMONE (LH) IN DIVERSE MAMMALIAN SPECIES

3.1 Introduction

It is estimated that there are some 4,500 species of mammals on the planet, and little is known about the basic reproductive processes for most of these species (Wildt et al., 1995). As the human population continues to grow, and destruction of wilderness continues unabated, many wildlife species are forced to exist in fragmented habitats as small populations with no room to grow (Conway, 1995). Ex-situ breeding programs for threatened species are hampered by the inability to accurately predict or identify ovulation due to the fact that commercial gonadotrophin immunoassays are only available for a limited number of species, e.g. laboratory or domestic animals and humans, and these assays usually fail to recognise other species variants of the gonadotrophin peptides (Dahl and Sarkissian, 1993).

The original work that utilised in vitro bioassays for measuring LH levels in different species mainly used freshly isolated Leydig cells from rats or mice (Farmer et al., 1977, Yu and Wang, 1987). While these assays could successfully bind LH sourced from mammals, results from animals of different vertebrate classes were not as robust (Farmer et al., 1977, Yu and Wang, 1987). Other drawbacks to these primary culture methods were that there needed to be continual sacrifice of animals as a source of cells, and dedifferentiation of isolated cells resulting in no steroid production after a few days (Farmer et al., 1977, Finaz et al., 1987, Yu and Wang, 1987, Dahl and Sarkissian, 1993). Ascoli and Puett (1978) found that cells from a Leydig tumour of the C57BL/6 strain of mice designated M5480, were found to be hormone responsive and easily transplantable and adapted for growth
in tissue culture. This original tumour gave rise to two other tumours known as M5480A and M5480P (Ascoli and Puett, 1978). Under basal conditions, the M5480P tumour cells produce mainly progesterone, while M5480A produces both progesterone and testosterone (Ascoli and Puett, 1978). Based on these findings, several immortalised clonal cell lines (MA-10, MA-12 and MLTC-1) originating from the M5480P tumour were developed and found to show retention of the LH-CG-R, by responding to LH stimulation with increased progesterone production (Ascoli and Puett, 1978, Rebois, 1982). Of these cell lines, previous research used the MA-10 cells to develop a simple, rapid in vitro bioassay for measuring LH from diverse species (Dahl and Sarkissian, 1993). The findings of this work indicated that the bioassay could successfully detect LH from several species of mammals, yet samples from avian and reptilian species were unable to be detected, and the sensitivity of the assay for several of the species that they tested was above the normal physiological range (Dahl and Sarkissian, 1993). In the current study we sought to develop a sensitive in vitro LH bioassay using the commercially available MLTC-1 cell line. The hypotheses were that the optimised bioassay: (1) would successfully detect LH pituitary preparations from a range of mammalian species; (2) would successfully detect LH within the normal physiological range; (3) would be specific to LH and show no cross reactivity with FSH in the bioassay.

3.2 Materials and Methods

3.2.1 Optimisation of assay conditions using hCG

We chose human CG (hCG) to optimise the assay because of its structural similarity to LH, and that both hormones bind to the same receptor (LH-CG-R) located on testicular Leydig cells (McFarland et al., 1989, Rahman and Rao, 2009). Confluent cells were detached as detailed in Chapter 2, then transferred to 96-well microplates with different cell concentrations per column (six cell densities tested ranging from 300,000 to 9,375 per well) and incubated overnight in complete medium.
Medium was then removed and cells washed twice with CMF-PBS to remove all traces of serum from the wells. The cells were then incubated in serum-free medium (prepared as described in Chapter 2) overnight. On the following day, spent serum-free medium was removed and replaced with fresh serum-free medium plus graded doses of urinary-derived hCG (Pregnyl®; Schering-Plough New South Wales, Australia) ranging from 250 – 1.95 ng/100 µl. Cells were incubated for varying time periods (15, 30, 60, 120 or 240 minutes) with hCG, with each 96-well plate representing one discreet time period (Appendix 3.1). After specified incubation times, spent medium was collected and stored at -20°C until required for progesterone analysis (Appendix 9.2).

3.2.2 Dose response curves using LH Preparations from eight species of mammals

Pituitary preparations of LH were purchased from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) through National Hormones and Pituitary Program (NHPP; Baltimore, Maryland, USA) from the following eight species; human (NIDDK-hLH-I-SIAFP-2), rat (NIDDK-rLH-RP-3), monkey (rec-moLH-RP-1), canine (AFP-5216B), equine (AFP-5130A), rabbit (AFP-7818C), bovine (AFP-9070B), and porcine (AFP-3881A).

Based on the same procedure described above for the optimisation of culture conditions, two 96-well plates were prepared with a concentration of approximately 9,375 cells/well (this optimal cell density, and the optimal incubation time, were determined in the previous experiment 3.2.1). Different amounts of lyophilised LH preparations from each species were reconstituted with PBS according to manufacturer’s instructions. All preparations were stored at a working stock of 64 ng/100 µl (well) which was the top (most concentrated) standard tested. Serial dilutions were prepared from the top standard to a final concentration of 0.0625 ng/100 µl (well), plus a blank sample (serum-free medium only); with four species of LH used per plate allowing replicates of
each sample (Appendix 9.3 and 9.4). Cells were incubated for two hours at 37°C / 5% CO₂ before media was collected and stored at -20°C until required for progesterone analysis.

3.2.3 Determination of cross reactivity with recombinant human FSH

To determine if there was any cross reactivity with recombinant human FSH (hFSH; Gonal-f®, Merck Serono, Frenchs Forest, New South Wales, Australia) in the culture bioassay hFSH was serially diluted in CMF-PBS at dilutions ranging from 250 – 31.25 IU/ml. Diluted samples of recombinant hFSH were run through the bioassay at the optimised conditions of approximately 9,375 cells/well for two hours, in duplicate before media was collected and stored at -20°C until progesterone analysis.

3.2.4 Statistical Analysis

All statistical analysis was carried out using SPSS version 19.0 with data being log-transformed. Dose response curves were fitted with a 3 parameter sigmoid curve using Sigma Plot version 11.0, according to the following equation:

\[ y = \frac{a}{1+e^{-\frac{(x-x_0)}{b}}} \]

In 64% of cases a 3 parameter sigmoid plot fit the data significantly better than a 4 parameter plot as determined in Sigma Plot using the F-test. In select cases, however, a 3 parameter plot did not fit the data due to assumptions being violated and in these cases a 4 parameter plot was used instead. To determine the potency of each LH species in the bioassay, a one way analysis of variance (ANOVA) was carried out with a LSD post-hoc test to determine the concentration of LH that caused the first significant increase in progesterone production by MLTC-1 cells.
3.3 Results

3.3.1 Optimisation of cell density and incubation time

After an incubation period of fifteen minutes, the three greatest concentrations of cells (300,000, 150,000 and 75,000) were beginning to show a dose-response in progesterone production at the higher concentrations of hCG (Figure 3.1). The three lower concentrations of cells (37,500, 18,750 and 9,375), however, failed to produce dose-response curves indicating no or very low hCG stimulated progesterone production (Figure 3.1). All cell densities cultured for thirty minutes, in the presence of all hCG concentrations tested, failed to produce a dose-response to hCG stimulation (Figure 3.2). In this case there was no obvious relationship between hCG stimulation and progesterone production by the MLTC-1 cells. At sixty minutes incubation there was a more obvious dose-response in the four highest densities of cells (300,000; 150,000; 75,000; and 37,500) examined in this study (Figure 3.3). However, the two lower cell densities (18,750; and 9,375) still failed to produce a dose-response to hCG stimulation (Figure 3.3). An incubation period of 120 minutes resulted in all concentrations of cells producing a dose-response in hCG-stimulated progesterone production (Figure 3.4). However, the lower densities of cells (18,750; and 9,375) showed greater sensitivity over the lower concentrations of hCG (Figure 3.4). An incubation period of 240 minutes resulted in dose-response progesterone production, however, all densities of cells showed saturation in the amount of progesterone produced from a concentration of 32 ng/100 µl (well) onwards (Figure 3.5). From these results the optimal cell density per well was judged to be approximately 9,375 cells and the optimal incubation time was 120 minutes for the M-LTC1 cells to produce a dose-response of progesterone in response to physiological levels of hCG with good sensitivity over all concentrations tested.
15 minutes incubation of different doses of hCG (ng/100 µl) with different densities of cells per well. Data has been log-transformed. These samples were not diluted as they all read on the standard curve <1,000 pg/ml. Data was fit with either a 3- or 4-parameter sigmoid plot.
Figure 3: 2 Incubation for 30 minutes

30 minutes incubation of different doses of hCG (ng/100 µl) with different densities of cells per well. Samples have been diluted 1:20 and data has been log-transformed. Data was fit with either a 3- or 4-parameter sigmoid plot.
Figure 3: 3 Incubation for 60 minutes

60 minutes incubation of different doses of hCG (ng/100 µl) with different densities of cells per well. Samples have been diluted 1:20 and data has been log-transformed. Data was fit with a 3- parameter sigmoid plot.
Figure 3: 4 Incubation for 120 minutes

120 minutes incubation of different doses of hCG (ng/100 µl) with different densities of cells per well. Samples have been diluted 1:20 and data has been log-transformed. Data was fit with a 3-parameter sigmoid plot.
Figure 3: 5 Incubation for 240 minutes

240 minutes incubation of different doses of hCG (ng/100 µl) with different densities of cells per well. Samples have been diluted 1:20 and data has been log-transformed. Data was fit with a 3-parameter sigmoid plot.
3.3.2 Progesterone Production by Pituitary LH Preparations from Eight Species of Mammals

Pituitary LH preparations from all eight species were able to produce dose-response curves of LH-stimulated progesterone production of the M-LTC1 cells under the optimised assay conditions outlined above (Figure 3.6). The sensitivity of response differed according to species, ranging from as low as 0.0625 ng/100 µl (well) to as high as 4 ng/100 µl (well), although there was no obvious phylogenetic relationship between species and response (Table 3.1) (Figure 3.7). For example, of the two primate species tested, the monkey LH produced significant increases in progesterone at 0.0625 ng/100 µl (well), while the human LH did not produce significant increases in progesterone production until a concentration of 1 ng/100 µl (well). For the two species of rodent tested in the assay, the rat LH preparation produced significant increases of progesterone production at 0.125 ng/100 µl (well), while the rabbit LH produced significant levels of progesterone production at 0.25 ng/100 µl (well).

Table 3: 1 Potency of mammalian LH

First significant increase (potency) in progesterone production by MLTC-1 cells when incubated with LH sourced from eight different species of mammals.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration of LH (ng/100µl) required to produce a significant increase in progesterone</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>1</td>
<td>0.001</td>
</tr>
<tr>
<td>Porcine</td>
<td>0.0625</td>
<td>0.026</td>
</tr>
<tr>
<td>Monkey</td>
<td>0.0625</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bovine</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Canine</td>
<td>0.0625</td>
<td>0.005</td>
</tr>
<tr>
<td>Equine</td>
<td>0.125</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rat</td>
<td>0.125</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Dose response curves of pituitary preparations of LH sourced from eight species of mammals. Concentrations of LH (ng/100 µl) were incubated with 9,375 cells/well for two hours. Data has been log-transformed and fit with a 3-parameter sigmoid plot, but was not diluted prior to analysis.
Figure 3: 7 Dose-response curve: linear

Dose response curves of pituitary preparations of LH sourced from eight species of mammals. Concentrations of LH (ng/100 µl) were incubated with 9,375 cells/well for two hours.
3.3.3 Comparison of the sensitivities of the MA-10 and MLTC-1 bioassays, with peak LH endogenous concentrations

In terms of assay sensitivity, we compared the sensitivity of the eight species tested in the MLTC-1 bioassay with the results of the MA-10 bioassay as performed by Dahl & Sarkissian (1993), and with peak endogenous concentrations of LH as determined by other methods (Table 3.2). In most cases where the same species were investigated by both the current study and by Dahl & Sarkissian (1993) using the MA-10 bioassay, the MLTC-1 bioassay demonstrated greater sensitivity. For example, the MA-10 bioassay did not detect porcine LH until a concentration of 32 ng/well, while the current study detected significant progesterone production at 0.0625 ng/100 µl (well). The only species for which the MA-10 bioassay showed greater sensitivity was the bovine which was detected at a concentration of 1 ng/well in the MA-10 bioassay, but only at 4 ng/100 µl (well) in the current study. The peak endogenous concentrations outlined in Table 3.2 imply that the MA-10 bioassay would not be able to detect peak endogenous LH concentrations in porcine, bovine and equine samples, while the MLTC-1 bioassay would be able to detect peak LH concentrations in all samples except bovine. The MLTC-1 bioassay would also be able to detect basal endogenous LH concentrations in all samples except bovine (Table 3.2), whereas the MA-10 bioassay would not be able to detect any basal LH concentrations in the species depicted.

3.3.4 Determination of cross-reactivity with recombinant human FSH

When recombinant hFSH was tested in the bioassay it produced minimal to no progesterone when incubated with MLTC-1 cells for two hours (Figure 3.8). Most samples read at or below the minimum detectable level of 300 pg/ml, though samples from the 250 IU/ml dose showed slight progesterone production at around 600 pg/ml (Figure 3.8).
Table 3: Comparison of sensitivities

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>Basal LH endogenous concentration</th>
<th>Peak LH endogenous concentration</th>
<th>Sensitivity of the MA-10 Bioassay (Dahl and Sarkissian, 1993) (concentration per ml in brackets)</th>
<th>Sensitivity of the MLTC-1 Bioassay (this study) (Concentration per ml in brackets)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>(review)</td>
<td>~1 U/L</td>
<td>35 U/L</td>
<td>n/a</td>
<td>1 ng/100µl (10 ng/ml)</td>
<td>(Buffet et al., 1998)</td>
</tr>
<tr>
<td>Porcine</td>
<td>TR-FIA</td>
<td>~1 ng/ml</td>
<td>9.0 ng/ml</td>
<td>32 ng/100 µl (320 ng/ml)</td>
<td>0.0625 ng/100µl (0.625 ng/ml)</td>
<td>(Noguchi et al., 2007)</td>
</tr>
<tr>
<td>Monkey</td>
<td>Bioassay*</td>
<td>232 ± 44 ng/ml</td>
<td>4 ng/100 µl (40 ng/ml)</td>
<td>0.0625 ng/100µl (0.625 ng/ml)</td>
<td>(Duffy and Stouffer, 2002)</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>EIA</td>
<td>35.1 ± 6.1 ng·mL⁻¹</td>
<td>n/a</td>
<td>0.25 ng/100µl (2.5 ng/ml)</td>
<td>(Quintela et al., 2004)</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>RIA</td>
<td>21.6 ± 4.2 ng/ml</td>
<td>1 ng/100 µl (10 ng/ml)</td>
<td>4 ng/100µl (40 ng/ml)</td>
<td>(Randel et al., 1982)</td>
<td></td>
</tr>
<tr>
<td>Canine</td>
<td>RIA</td>
<td>3.54 ng/ml</td>
<td>n/a</td>
<td>0.0625 ng/100µl (0.625 ng/ml)</td>
<td>(Wright, 1991)</td>
<td></td>
</tr>
<tr>
<td>Equine</td>
<td>Bioassay*</td>
<td>~82 ng/ml</td>
<td>8 ng/100 µl (80 ng/ml)</td>
<td>0.125 ng/100µl (1.25 ng/ml)</td>
<td>(Alexander and Irvine, 1982)</td>
<td></td>
</tr>
<tr>
<td>Equine</td>
<td>RIA</td>
<td>~86 ng/ml</td>
<td>8 ng/100 µl (80 ng/ml)</td>
<td>0.125 ng/100µl (1.25 ng/ml)</td>
<td>(Alexander and Irvine, 1982)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>RIA</td>
<td>1080.1 ± 226.7 ng/ml</td>
<td>0.5 ng/100 µl (5 ng/ml)</td>
<td>0.125 ng/100µl (1.25 ng/ml)</td>
<td>(Watanabe et al., 1990)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3: 8 Cross-reactivity of hFSH

Determining cross-reactivity of recombinant hFSH in the bioassay. Four different dilutions of hFSH were tested (250 – 31.25 IU/ml) in quadruplicate and progesterone production of MLTC-1 cells was determined after two hour incubation with 9,375 cells/well. Samples were diluted 1:20 prior to analysis. The minimum amount of progesterone that can be determined by the EIA (300 pg/ml) is indicated by a solid line.
3.4 Discussion

The *in vitro* bioassay was capable of detecting LH in several different mammalian species, using the clonal cell line MLTC-1, therefore supporting the first hypothesis. In order to achieve this, the parameters of the assay were optimised; namely cell density per well and incubation time. Six different cell densities (range 300,000 to 9,375) over five time periods (240 – 15 minutes) using eight concentrations of hCG (250 – 1.9 ng/100µl) were tested. The results indicated greatest sensitivity of the assay over the lower concentrations of hCG when a lower cell density was used. Therefore, 9,375 cells per well was deemed optimal. It was found that two hours was sufficient time for cells to produce progesterone in a dose-dependent manner. These findings are comparable to earlier research by Dahl & Sarkissian (1993), who used a similar clonal cell line (designated MA-10) as an LH bioassay and found the optimal cell density was 5,000 cells per well and the optimum incubation time was two hours.

The second hypothesis, that the bioassay would successfully detect LH from different mammalian species, within the normal physiological range was supported. To test the ability of the MLTC-1 cell line to respond to different mammalian species LH preparations, eight different mammalian LH pituitary preparations were tested using the optimised conditions of the bioassay, and all were able to produce dose-dependent production of progesterone over a range of LH concentrations (64 – 0.0625 ng/100 µl) with sensitivities ranging from 0.0625 to 1 ng/100 µl 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. The different species of mammals gave different responses; however, there did not appear to be any phylogenetic relationship to this response. Dahl & Sarkissian (1993) also tested LH preparations from different mammalian species in their bioassay using MA-10 cells and they also found that the seven different mammals they tested all produced dose-dependent progesterone production. When comparing sensitivities
between these two cell lines, in almost all cases the MLTC-1 bioassay had greater sensitivity than the MA-10 bioassay. The exception to this was bovine LH which demonstrated a sensitivity of 1 ng/well in the MA-10 bioassay, but 4 ng/100 µl (well) in the MLTC-1 bioassay (Dahl and Sarkissian, 1993). Given the peak endogenous concentrations described in Table 3.2, and the sensitivity of the MA-10 bioassay, the MA-10 bioassay would not be able to detect peak endogenous LH concentrations in porcine, bovine, and equine samples, while the MLTC-1 bioassay would be able to detect peak LH concentrations in all samples with the exception of bovine. Indeed, the MLTC-1 bioassay would be able to detect basal endogenous LH concentrations in all samples except bovine (Table 3.2), whereas the MA-10 bioassay would not be able to detect any basal LH concentrations in the species depicted. Successful determination of LH concentrations in animals, as a predictor of impending ovulation, is crucial in all animal breeding situations (e.g. production animals, companion animals and threatened wildlife species). During the luteal phase of the oestrous cycle, LH concentrations remain at a basal level until declining progesterone levels and increasing oestrogen levels after luteolysis trigger the pre-ovulatory LH surge (Bartlewski et al., 2011). Thus an assay that is sensitive enough to detect these basal concentrations as well as the higher surge-related concentrations would be beneficial over one that fails to distinguish between the two states. This will be expanded upon in subsequent chapters.

The third hypothesis, that the bioassay would be specific to LH and show no cross-reactivity with FSH, was supported. Therefore, the increasing levels of FSH in samples taken from animals during the follicular phase of their oestrous cycle would not interfere with the detection of the LH surge if the samples were analysed using this bioassay. In the testis, the receptor for LH-CG is located on the Leydig cells (interstitial cells), while the receptor for FSH is located on the Sertoli cells within the seminiferous tubules (McLachlan et al., 1995). In a commercially available Leydig cell line it would be expected that the cell suspension is comprised almost exclusively of Leydig cells.
Nevertheless, four dilutions of hFSH were tested to check for cross-reactivity in the bioassay system. All dilutions of hFSH produced minimal to non-detectable levels of progesterone in the bioassay under the optimised conditions. At the highest FSH dose (neat) of 250 IU/ml there were detectable concentrations of progesterone produced in three out of four replicates, however, this is not necessarily due to a stimulatory effect of hFSH (implying it was binding to a receptor) as when examining pituitary LH preparations the blank wells (serum-free medium only) also produced detectable levels of progesterone. Panesar and colleagues (2003) detected progesterone after a time period of zero minutes when MLTC-1 cells had been exposed to hCG, though it was expressed as a function of the total RNA. Previous research involving bioassays using freshly sourced Leydig/interstitial cells demonstrated responses to ovine FSH (oFSH) in their bioassay (Yu and Wang, 1987). The authors claimed that the oFSH used in their assay contained traces of LH and cited this as the reason for FSH activity in the bioassay (Yu and Wang, 1987). This highlights one advantage of using a clonal Leydig cell line in an LH bioassay, and the use of pure recombinant FSH when assessing cross-reactivity.

Finally, a clonal Leydig cell line is advantageous over primary culture of cells as it firstly eliminates the use of live animals, secondly decreases interassay variability by sourcing cells from the same population and thirdly, potentially allows an unlimited number of cells to be grown that display similar responsiveness. It is important to note, however, that a clonal Leydig cell line is not an exact replicate of normal Leydig cell function in vivo (Rebois, 1982). The MLTC-1 cell line differs from freshly isolated mouse cells in being polyploid with 95±4 chromosomes per cell, though this does not have any adverse impact on activation of the hormone responsive adenylate cyclase system (Rebois, 1982). After prolonged culture, cells may undergo dedifferentiation which could impact on their ability to bind LH and secrete progesterone, and the ability of MLTC-1 cells to bind LH beyond 30 passages has not been elucidated (Rebois, 1982, Finaz et al., 1987). In the current
study, cells were not passaged beyond 25 cycles. In addition to this, normal Leydig cells in vivo, synthesise testosterone in response to LH, while it has been shown that MLTC-1 cells produce only very low amounts of both testosterone and androstenedione when compared with progesterone (ratio 1:20:60 respectively) (Panesar et al., 2003). Despite this, the commercial progesterone assay that was utilised in the present study has low cross-reactivity with other steroids including testosterone (<0.05%), thus the findings accurately reflect progesterone production in response to LH-CG stimulation.

In conclusion, using the optimised bioassay conditions with MLTC-1 cells, pituitary preparations of LH from eight species of mammals (human, monkey, porcine, rabbit, bovine, canine, equine and rat) produced physiological dose-response curves. Therefore it is likely that the assay can be used for the analysis of samples from diverse mammalian species, especially those lacking immunoassays. Therefore, it is envisaged that this bioassay will become a relevant tool in ex-situ breeding programs for threatened species that are hampered by the inability to accurately predict or identify ovulation due to the fact that commercial gonadotrophin immunoassays are only available for a limited number of species.
4. APPLICATION OF A BIOASSAY AS A TOOL FOR MEASURING LUTEINISING HORMONE (LH) IN TWO SPECIES OF MARSUPIAL

4.1 Introduction

The measurement of gonadotrophins (LH and FSH) is crucial when assessing the reproductive status of individual animals, diagnosing fertility problems and for using assisted reproductive technology when undertaking captive breeding programs to ensure the survival of threatened species (Graham et al., 2001, Matson et al., 2009). The chemical structure of the gonadotrophins shows considerable species heterogeneity and makes it hard to find a ‘one assay fits all’ way of measuring its secretion (Kalia et al., 2004). Earlier work investigating marsupial reproductive hormones demonstrated that marsupial gonadotrophins were of a lower potency relative to eutherian gonadotrophins in eutherian assays, with a eutherian assay being either a bioassay or radioreceptor assay with the particular hormone receptor being derived from eutherian gonadal tissue (Gallo et al., 1978, Farmer et al., 1981, Stewart et al., 1981). These results were improved upon when the tissue source of the hormone receptor was obtained from a marsupial (Gallo et al., 1978, Farmer et al., 1981, Stewart et al., 1981).

Even with the more recent development of techniques such as the RIA, problems were still encountered when attempting to measure reproductive hormones of marsupials. Matson and colleagues (2009) attempted to measure LH from western grey kangaroo plasma using an RIA based on polyclonal antibodies which were raised in rabbits against four different isoforms of ovine LH, and none of those antibodies were capable of detecting LH from the kangaroo. To overcome this, the authors adapted an EIA previously used for detecting LH in the elephant and which used a monoclonal antibody (518B7) anti-bovine LH-β subunit which had been described in
terms of its ability to bind LH from diverse mammalian species (Matteri et al., 1987, Brown et al., 2004, Matson et al., 2009).

In Chapter 3, the MLTC-1 bioassay was developed and optimised and it was found that the bioassay could measure LH from many different species. However, none of the peptides (species) tested were marsupial. In the current study, we investigated the potential of the cell-based bioassay developed in Chapter 3 to indirectly measure LH from two species of marsupial; the western grey kangaroo (*Macropus fuliginosus ocydromus*) and the black-flanked rock wallaby (*Petrogale lateralis lateralis*). While the previous studies that had limited success used bioassays with cells being sourced from either marsupial or eutherian testes, we investigated the potential use of a commercially available testicular clonal cell line derived from mice (Rebois, 1982). Previous studies using a different cell line (MA-10) demonstrated the ability of clonal cells to bind LH from different species of mammals; however, marsupials were not tested in this study (Dahl and Sarkissian, 1993). In order to determine if the clonal cells were able to detect changes in circulating levels of LH in marsupials, plasma samples were collected from animals before and after an administration of exogenous GnRH. It was hypothesised that firstly, the MLTC-1 cell-based bioassay would successfully detect LH in the plasma of marsupials, and secondly that the results would be comparable to those of an established EIA.

4.2 Materials and Methods

4.2.1 Kangaroo and Wallaby samples

Kangaroo blood plasma samples were a generous gift from Dr. Chris Mayberry (The University of Western Australia). During the months of February, May/June and October 2007, nine female western grey kangaroos (*M. fuliginosus ocydromus*) from the Harry Waring Marsupial Reserve (Perth, Western Australia; (Mayberry et al., 2010), were sedated before a blood sample was
collected from the jugular vein. The kangaroo was then given an intramuscular (i.m.) injection of GnRH (Gonadorelin; Fertagyl®) and 25 minutes after this a second blood sample was collected. Blood samples were separated by centrifugation (3,000rpm for 10 minutes) within 3 hours of collection and plasma was frozen at -20°C (Matson et al., 2009).

Wallaby blood plasma samples were a generous gift from Dr. Nicole Willers (The University of Western Australia). As part of a three year study from 2007 – 2009 during the months of August/September, December/January, and March/April six black-flanked rock wallabies (P. lateralis lateralis) from the Mt. Caroline Nature Reserve (170kms East of Perth, Western Australia; (Willers et al., 2011) were sedated as described for the western grey kangaroo above and a blood sample was collected. Following this, wallabies were given an i.m. injection of 2.5mg/kg GnRH (Gonadorelin; 0.5ml Fertagyl®) and 25 minutes after this a second blood sample was collected. In addition to this original group of wallabies, a second group of six wallabies were on a contraceptive, a GnRH super-agonist (Suprelorin; Peptech Animal Health, Macquarie Park, Australia) for approximately 4 months duration prior to sample collection. Two out of the six wallabies were on a single dose of Suprelorin (4.7 mg) while the other four were on a double-dose of Suprelorin (9.6 mg). This second group of wallabies also had blood samples collected before and after an i.m. injection of GnRH was administered. Blood samples were separated by centrifugation (3,000 rpm for 10 minutes) within 3 hours of collection and plasma was frozen at -20°C (Matson et al., 2009).

4.2.2 Bioassay procedure

Cells were transferred to a 96-well plate as detailed in Chapter 2, with a concentration of approximately 9,375 cells/well and allowed to adhere overnight. Following this, cells were washed twice with CMF-PBS and incubated with serum-free medium overnight. On the following day, a plasma sample (50 µl) either from the kangaroo or wallaby was added to each well in duplicate
along with 200 µl of serum free medium. The plate was incubated for two hours before culture supernatants were collected and stored at -20°C until required for progesterone analysis.

4.2.3 Statistical Analysis

Numerical data are presented as the mean ± standard error of the mean (s.e.m.). Statistical data was analysed using SPSS version 19 (IBM SPSS Statistics, 2010). Tests for normality were conducted using the Kolmogorov-Smirnov test with values of p> 0.05 not violating the assumptions of normality. Paired sample T-tests (two-tailed) were conducted to determine significant changes in circulating levels of LH before and after exogenous GnRH administration as measured by both bio- and immunoassay methods, as well as to determine if there were any significant changes in the overall magnitude of change (before to after) between the two methods (bioassay vs. immunoassay). Fold changes were calculated from individual samples and not the mean of all samples.

4.3 Results

4.3.1 Changes in circulating levels of LH in the western grey kangaroo before and after an exogenous dose of GnRH as determined by bio- and immunoassay methods

The bioassay method detected a 2.5-fold increase in circulating levels of LH in female western grey kangaroos after administration of exogenous GnRH (p= 0.016) (Table 4.1). The immunoassay method detected a 2-fold increase in circulating levels of LH in female western grey kangaroos after administration of exogenous GnRH (p= 0.01) (Table 4.1). There was no significant difference in the magnitude of increase in circulating levels of LH following a GnRH challenge in the western grey kangaroos shown by the bioassay and immunoassay methods (p= 0.742) (Table 4.1).
Table 4: 1Western grey kangaroos

Mean levels (± s.e.m.) of LH-stimulated progesterone production detected by the bioassay and endogenous LH detected by an enzyme immunoassay in nine female western grey kangaroos before and 25 minutes prior to an exogenous dose of GnRH (*before vs after: p<0.05). Fold change is calculated from the individual samples not the mean of these samples.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Time relative to GnRH injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Bioassay (pg/ml)</td>
<td>651.54 ± 236.4</td>
</tr>
<tr>
<td>Immunoassay (pg/ml)</td>
<td>5,733.33 ± 1,130.51</td>
</tr>
</tbody>
</table>

4.3.2 Comparing changes in circulating levels of LH in two groups of black-flanked rock wallabies before and after an exogenous dose of GnRH as determined by bio- and immunoassay methods

The bioassay method detected a 2-fold increase in circulating levels of LH in black-flanked rock wallabies (control animals) after administration of exogenous GnRH (p= 0.02) (Table 4.2). The immunoassay method also detected a 2-fold increase in circulating levels of LH in black-flanked rock wallabies after administration of exogenous GnRH, however this was not significant (p= 0.136) (Table 4.2). There was no significant difference in the magnitude of increase in circulating levels of LH following a GnRH challenge in the black-flanked rock wallabies (control animals) shown by the bioassay and immunoassay methods (p= 0.781) (Table 4.2).

The bioassay method detected a 2-fold decrease in circulating levels of LH in black-flanked rock wallabies that had been on a contraceptive for four months following administration of exogenous GnRH (p= 0.002) (Table 4.2). The immunoassay detected a 1-fold decrease in circulating levels of LH in black-flanked rock wallabies that had been contraceptive treated four months prior to the GnRH challenge, however this was not significant (p= 0.146) (Table 4.2). There was a significant
difference in the magnitude of decrease in circulating levels of LH following a GnRH challenge in the black-flanked rock wallabies (contraceptive animals) shown by the bioassay and immunoassay methods (p< 0.01) (Table 4.2).

**Table 4: 2 Black-flanked rock wallabies**

Mean levels (± s.e.m.) of LH-stimulated progesterone production detected by the bioassay and endogenous LH detected by an enzyme immunoassay in two groups of six black-flanked rock wallabies before and after an exogenous dose of GnRH (*before vs. after: p<0.05). Fold change is calculated from the individual samples not the mean of these samples.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Time relative to GnRH injection</th>
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<th>Change (fold)</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioassay (pg/ml)</td>
<td>681.71 ± 62.22</td>
<td>1,353.54 ± 207.82 *</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Immunoassay (pg/ml)</td>
<td>2,649 ± 700.84</td>
<td>6,560.83 ± 2,785.44</td>
<td>2</td>
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<table>
<thead>
<tr>
<th>Assay</th>
<th>Time relative to GnRH injection</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioassay (pg/ml)</td>
<td>815.7 ± 157.46</td>
<td>472.59 ± 145.22 *</td>
<td>2</td>
<td></td>
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<tr>
<td>Immunoassay (pg/ml)</td>
<td>4,708.33 ± 1,981.43</td>
<td>3,985 ± 1,621.97</td>
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</table>

**4.4 Discussion**

The MLTC-1 clonal cell line was able to bind marsupial LH and generate production of the steroid progesterone, thus supporting the first hypothesis. The MLTC-1 clonal cell line in the developed bioassay successfully detected LH in two species of Macropodid marsupials, the western grey kangaroo (*M. fuliginosus ocydromus*) and the black-flanked rock wallaby (*P. lateralis lateralis*). Furthermore, in both the western grey kangaroo and the black-flanked rock wallaby control animals the *in vitro* bioassay detected a significant increase in plasma LH following administration
of GnRH. The black-flanked rock wallaby experimental animals that had been on a contraceptive for four months prior to GnRH administration showed a significant decrease in circulating levels of LH in the bioassay. The ability of the bioassay to detect changes in marsupial plasma both before and after a GnRH challenge provides evidence that it could be used to detect both basal and naturally occurring surge levels of LH in marsupials, implying that it may have potential as a tool for monitoring hormonal changes during the oestrous cycle. Although at lower hormone concentrations results were not as conclusive.

The use of a clonal cell line as an indirect method to measure LH from marsupials has not been previously conducted. As previously stated, difficulties can arise from attempting to measure reproductive hormones in marsupials even using conventional methods such as immunoassays (Matson et al., 2009). Dahl and Sarkissian (1993) investigated the use of a different clonal cell line (MA-10) as an in vitro bioassay for measuring LH. The authors achieved dose-response curves from seven different species of mammals, though no marsupials were tested in this assay (Dahl and Sarkissian, 1993). Both the MA-10 cell line and the cell line used herein were derived from the same transplantable tumour (M548OP) and developed in separate labs, and both have been described in terms of their ability to bind LH and hCG with downstream stimulation of the adenylate cyclase pathway (adenosine-3’,5’-monophosphate; cAMP) culminating in production of progesterone (Ascoli, 1981, Rebois, 1982).

The second hypothesis, that the results of the bioassay method would be comparable to those of an established EIA, was partially supported. To further validate the applicability of the bioassay, the present findings were compared to results obtained from the same blood samples, but with LH content measured by an EIA originally optimised to detect LH in the plasma of elephants, and which utilises the monoclonal antibody 518B7 (Matteri et al., 1987, Brown et al., 2004). To
compare the two methods we examined the magnitude of change in LH levels between the before and after GnRH blood samples. In both the western grey kangaroo and the black-flanked rock wallaby control animals we found no significant difference between the assays in the detection of the magnitude of change of LH before and after administration of GnRH. However, with the black-flanked rock wallabies receiving the contraceptive, there was a significant difference in the magnitude of decrease in circulating levels of LH following the GnRH challenge between the bioassay and immunoassay methods. The bioassay indicated a significant 2-fold decrease in LH levels following the GnRH challenge, whereas the immunoassay indicated a non-significant 1-fold decrease in LH levels following the challenge. The contraceptive treatment of the wallabies with Deslorelin should have resulted in a down regulation of the pituitary GnRH receptors on the gonadotroph cells, and hence lower circulating levels of LH and an attenuated response to a GnRH challenge (Herbert, 2004). Both assay systems indicated an attenuated response to GnRH, however, the bioassay actually indicated decreased levels of LH following the GnRH challenge. Furthermore, comparing the samples before the GnRH challenge in the control and contraceptive-treated wallabies, neither assay system indicated a decrease in circulating LH levels in the contraceptive-treated wallabies, as would be expected. These results may indicate that both assay systems had difficulty measuring low LH concentrations in the wallaby samples.

As previously mentioned, past studies using bioassays or radioreceptor assays (RRA) had limited success measuring LH in marsupials using the rat Leydig cell method. Marsupial pituitary extracts of LH are less potent in rat Leydig cell bioassays than oLH (1% activity in the case of tammar wallaby (Gallo et al., 1978); 3.2% activity in the case of eastern grey kangaroos, and 2.9% of oLH activity in the case of western grey kangaroos (Farmer et al., 1981)). When Gallo et al. (1978) used opossum testes as the cell source in a bioassay, tammar wallaby LH was equipotent to oLH. Farmer et al. (1981) also tested marsupial LH in a RRA using porcine ovaries and found both eastern and
western grey kangaroo LH to be 6.5% as potent as oLH. However, using a RRA with kangaroo testes as the cell source increased potency of both kangaroo species LH to 33% that of oLH (Farmer et al., 1981). The potency of purified wallaby LH (ME-14B) has been compared to ovine, equine, and rat LH in both a RRA and bioassay, both using rat tissue (McFarlane et al., 1997). Different potencies for the four species were found when the two assay methods were compared (McFarlane et al., 1997). Potency levels (expressed as a value relative to wallaby LH) indicated that wallaby was least potent when measured by RRA, yet second most potent in the Leydig cell bioassay (McFarlane et al., 1997). The authors hypothesised that these findings were possibly due to carbohydrate moiety on the α-subunit asparagine 52, which is critical for biological activity, and that while wallaby LH was less able to displace hCG in the RRA the biological response in terms of testosterone production by Leydig cells was much greater (McFarlane et al., 1997).

In conclusion, this study has demonstrated that a readily available clonal cell line, MLTC-1, can be used in a bioassay 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. It is suggested that this method may prove useful in advancing the understanding of the reproductive endocrinology of Australian marsupial species, particularly understanding hormonal fluctuations during the oestrous cycle and ovulation.
5. verification of a bioassay for measuring luteinising hormone (LH) during the anovulatory and ovulatory LH surges of the Asian elephant oestrous cycle

5.1 Introduction

The ability to monitor the oestrous cycle is of great importance when artificial reproductive technologies (ART) are to be employed (Dahl et al., 2004). This information is also useful for understanding the endocrinology of the general elephant population and for specific individuals, particularly females, in order to establish their reproductive health and establish a timeline for breeding (Dahl et al., 2004, Hildebrandt et al., 2011). There are two extant genera of elephants, the African (Loxodonta africana and L. cyclotis) and the Asian (Elephas maximus) and both are considered spontaneous, monovular, polyoestrous breeders (Rohland et al., 2010, Hildebrandt et al., 2011). Female elephants have the longest oestrous cycle of any mammal studied to date with a duration of 13 – 18 weeks, including a 6 – 12 week luteal phase followed by a follicular phase of approximately 4 – 6 weeks, thus female elephants may express only 3 – 4 fertile cycles per year (Hermes et al., 2007, Thitaram et al., 2009, Hildebrandt et al., 2011). In addition to this, females can show high variability between cycles, both within and between individuals, and this combined with high incidences of abnormal cycles, makes it difficult to predict and time ovulation and artificial insemination (AI) (Dahl et al., 2004, Hildebrandt et al., 2011).

The oestrous cycle of female elephants differs from all other mammals studied to date in that they exhibit a double LH surge during the follicular phase of the cycle (Dahl et al., 2004, Hildebrandt et al., 2006, Hermes et al., 2007, Lueders et al., 2011). In both species of elephant the two surges are qualitatively and quantitatively indistinguishable, however, only the second surge culminates in
Detection of the first anovulatory surge in LH (anLH) can provide an accurate estimation of the timing of the occurrence of the second ovulatory surge (ovLH), as the two surges occur approximately three weeks apart (Brown et al., 1999, Dahl et al., 2004). The anovulatory surge occurs between 12 and 21 days after progestogens decline to basal levels, while the ovulatory surge occurs approximately 19 - 22 days after this (Hermes et al., 2007). The most widely accepted theory explaining the double LH surge is that the first surge of LH functions to luteinise large follicles from the first wave of follicular development which become the accessory corpora lutea present in the ovary, while the second surge culminates in the ovulation of a single follicle developed during the second follicular wave (Hildebrandt et al., 2006, Hermes et al., 2007, Lueders et al., 2010, Hildebrandt et al., 2011, Lueders et al., 2011). In addition to the double LH surge, and contrary to what is recorded in most other species of mammals, the dominant progestogen produced by the corpus luteum are the 5α-reduced metabolites of progesterone (5α-pregnane-3,20-dione and, 3α-hydroxy-5α-pregnan-20-one) and not progesterone itself (Lueders et al., 2010, Hildebrandt et al., 2011).

Current ex-situ populations of elephants are not self sustaining and the demand for knowledge of elephant reproductive biology is high (Hildebrandt et al., 2006). Reproductive techniques developed for domestic and lab animals cannot always be directly applied to elephants, as their different physiology (including the presence of different hormone metabolites) makes commercially available hormone kits unsuitable (Hildebrandt et al., 2006, Hildebrandt et al., 2011). This prompted the investigation into the potential of a cell-based bioassay to indirectly measure LH from the Asian elephant around the time of both the anovulatory and ovulatory surges. A previous study using a different cell line (MA-10) demonstrated the ability of clonal cells to bind LH from an elephant and produce a dose-response curve (Dahl and Sarkissian, 1993). In Chapter 3 the
MLTC-1 bioassay was developed and optimised and it was found that pituitary LH preparations from eight species of mammals produced good dose-response curves demonstrating the practicality of this bioassay to measure LH from diverse species. However, none of the peptides (species) tested were representative of animals that are currently the focus of captive breeding programs. In Chapter 4 the viability of the MLTC-1 bioassay for use in other species was extended by showing that physiological LH concentrations could be detected in the blood plasma from two marsupial macropods, the western grey kangaroo and the black-flanked rock wallaby. Furthermore, the bioassay detected a significant increase in plasma LH following administration of exogenous GnRH, mimicking what would be expected for an endogenous LH surge associated with ovulation.

The aim of this study is to extend the species range of the bioassay to include elephants, which are currently the focus of a worldwide captive breeding program, and to detect endogenous LH surges in this species. It was hypothesised that firstly, the MLTC-1 cell-based bioassay would successfully detect LH in the blood plasma of the Asian elephant, secondly that anovulatory and ovulatory LH surges could be determined, and thirdly that the results would be comparable to those of an EIA that had previously been developed to measure elephant LH. In addition to this, to give greater applicability to the findings, an additional aim was to calculate the time taken from sample acquisition to progesterone/LH determination.

5.2 Methods

5.2.1 Asian Elephant plasma samples
Blood plasma samples from a female Asian elephant (*E. maximus indicus*) were a generous gift from the Perth Zoo in Western Australia. Siput, a 20 year old elephant (at the time of sample collection) was undergoing daily or twice daily blood sample collection (unananaesthetised) from an
ear vein, as part of the Zoo’s Asian elephant breeding program. All samples were centrifuged and plasma stored at -80°C. It should be noted that the samples had been through a single freeze/thaw cycle prior to the use in the bioassay in this study. Due to regular endocrine monitoring of the oestrous cycles of this elephant for several years, records indicated that she had three cycles per year usually occurring in April, August and December, thus samples were collected over three known oestrous cycles occurring in December 2009, April 2010 and, August 2010.

5.2.2 Experimental procedure

**Bioassay**
Cells (MLTC-1) were processed for use in the bioassay as detailed in Chapter 2. Briefly, cells were transferred to a 96-well plate with a concentration of approximately 9,375 cells/well and allowed to adhere overnight. Following this, cells were washed twice with CMF-PBS and incubated with serum-free medium overnight. On the third day of the experiment, a plasma sample (50µl) was added to each well in duplicate along with 200µl of serum free medium. The plate was incubated for two hours before culture supernatants were collected and stored at -20°C until required for progesterone analysis. NB; progesterone levels directly correlate with the amount of LH in the sample (see Chapter 3). The minimum detectable concentration of progesterone is 0.954 nmol/L.

**Enzyme Immunoassay**
The procedure for the enzyme immunoassay utilised by the Zoo for detection of elephant LH has been outlined previously (Brown et al., 2004). Briefly, a microplate is coated with a non-specific antibody (anti-mouse IgG) and incubated for 24 hours at room temperature. Following this, to reduce non-specific binding, the plate is coated with a protein blocking buffer and incubated at room temperature for a further 24 hours. On the third day, standards of bovine LH (NIH-LH-B10), elephant plasma samples and primary antibody (518-B7) are incubated at room temperature overnight or for three hours in an incubator at 37°C. Next the biotinylated ovine LH label is added
to the plate and incubated for either four hours at room temperature or 2 hours in an incubator at 37°C. Finally, streptavidin solution is applied and incubated on a plate shaker for 40 minutes at room temperature before TMB substrate solution is prepared and incubated on a plate shaker for an additional 40 minutes. The reaction is then terminated by addition of the stop solution. The assay sensitivity was 0.038 ng/ml (Brown et al., 2004). For further details please refer to Brown (2005).

*Automated Progesterone Analyser*
An investigation was done to determine the feasibility of using an automated analyser to reduce the time taken from sample acquisition to obtaining the progesterone result. Samples from one cycle (anLH and ovLH) were run through the bioassay (see day three of the experiment above). When culture supernates were collected they were transported on ice to Fertility North, Joondalup Specialist Medical Centre, in order to determine progesterone concentrations. This was done using a Siemens Centaur CP automated analyser. The assay uses 20 µl in singlicate, obtains a result in 18 minutes, has a standard curve ranging from 1.77 to 196.84 nmol/L, and shows coefficients of variation at three levels of 12.8% (2.5 nmol/L), 4.9% (20.2 nmol/L), and 3.6% (63 nmol/L). Cross-reaction of the assay was detected with corticosterone (0.05%), pregnenolone (0.46%), 17α-hydroxyprogesterone (0.31%), and 11-deoxycorticosterone (0.08%).

5.2.3 Statistical Analysis
Statistical data was analysed using SPSS version 19 (IBM SPSS Statistics, 2010). Tests for normality were conducted using the Kolmogorov-Smirnov test with values of p>0.05 not violating the assumptions of normality. Luteinising hormone concentration profiles were analysed over time based on an algorithm using a non-linear statistical model where a surge was identified if its peak value exceeded the previous nadir by three standard deviations (SD). Increases in LH of greater
than one SD but less than three SD, though deemed not significant in the algorithm, were also noted.

5.3 Results

5.3.1 Detection of the anovulatory and ovulatory surge of LH in the Asian elephant during two consecutive cycles using bioassay and immunoassay methods.

December

During the expected anovulatory surge of the cycle, the first significant increase (greater than three SD above baseline) occurred on the 3rd December am until the 3rd December pm for both the bioassay and EIA methods (Figure 5.1). In addition, the bioassay results indicated elevated LH concentrations after this surge (greater than one SD above baseline) until the 5th December am, and the EIA results indicated elevated LH concentrations (1SD) until the 4th December pm. During the expected ovulatory surge of the cycle, the first significant increase (3SD) occurred on 23rd December pm until the 24th December am for both the bioassay and EIA methods (Figure 5.1). In addition the bioassay and EIA results indicated elevated LH concentrations (1SD) in the sample prior to the surge (23rd December am). During this oestrous cycle, the bioassay detected an 8-fold increase from basal levels to the anLH surge and the EIA detected a 37-fold increase for this surge. It must be noted at this stage that the 37-fold increase detected by the EIA is probably erroneous as there was a large standard error recorded for one of the readings (i.e. 54.86 ± 63.01 ng/ml). The bioassay detected an 11.5-fold increase from basal concentrations to the ovLH surge and the EIA detected an 8-fold increase for this surge (Table 5.1).

April

During the expected anovulatory surge of the cycle, the first significant increase (3SD) occurred on the 5th April am until the 5th April pm for both the bioassay and EIA methods (Figure 5.2).
Figure 5: 1 December 2009 oestrous cycle

Anovulatory and ovulatory surges of a female Asian elephant during December 2009, comparing LH-stimulated progesterone responses from the bioassay (black line) to LH responses from the immunoassay (grey line).
Figure 5: 2 April 2010 oestrous cycle

Anovulatory and ovulatory surges of a female Asian elephant during April 2010, comparing LH-stimulated progesterone responses from the bioassay (black line) to LH responses from the immunoassay (grey line).
Table 5: Change (fold) from basal to surge concentration of LH for two oestrous cycles

Basal and surge concentrations, and change (fold) from basal-to-surge concentration, for the anLH and ovLH surges of a female Asian elephant during two consecutive oestrous cycles, in December 2009 and April 2010, as measured by both LH-stimulated progesterone production (bioassay) and endogenous LH (immunoassay) methods. Consistently low samples were used to calculate a mean basal concentration, while high samples were used to calculate a mean surge concentration in either the anLH or ovLH cycle. Surges were quantified as those values exceeding the previous nadir by 3 standard deviations.

<table>
<thead>
<tr>
<th>Assay</th>
<th>anLH (mean basal)</th>
<th>anLH (surge)</th>
<th>Change – basal to surge (fold)</th>
<th>ovLH (mean basal)</th>
<th>ovLH (surge)</th>
<th>Change – basal to surge (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay (nmol/L)</td>
<td>2.92 ± 1.97</td>
<td>23.64 ± 0.26</td>
<td>8</td>
<td>1.96 ± 1.62</td>
<td>23.15 ± 8.69</td>
<td>11.5</td>
</tr>
<tr>
<td>Immunoassay (ng/ml)</td>
<td>1.47 ± 0.42</td>
<td>54.86 ± 63.01</td>
<td>37</td>
<td>2.23 ± 0.7</td>
<td>17.8 ± 17.23</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 5: December 2009 Cycle

<table>
<thead>
<tr>
<th>Assay</th>
<th>anLH (mean basal)</th>
<th>anLH (surge)</th>
<th>Change – basal to surge (fold)</th>
<th>ovLH (mean basal)</th>
<th>ovLH (surge)</th>
<th>Change – basal to surge (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay (nmol/L)</td>
<td>3.37 ± 3.5</td>
<td>17.19 ± 0.76</td>
<td>5</td>
<td>3.32 ± 2.63</td>
<td>20.31 ± 0.48</td>
<td>6</td>
</tr>
<tr>
<td>Immunoassay (ng/ml)</td>
<td>0.87 ± 0.05</td>
<td>1.24 ± 0.1</td>
<td>1.4</td>
<td>2.92 ± 0.78</td>
<td>8.69 ± 2.05</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5: April 2010 Cycle
In addition, the bioassay results indicated elevated LH concentrations (1SD) for the sample prior to the surge (4\textsuperscript{th} April pm) and the EIA results indicated elevated LH concentrations (1SD) for the sample prior to the surge (4\textsuperscript{th} April pm) and the sample after the surge (6\textsuperscript{th} April am). For the expected ovulatory surge of the cycle, the first significant increase (3SD) occurred on the 24\textsuperscript{th} April pm and lasted until the 25\textsuperscript{th} April am for both the bioassay and EIA methods (Figure 5.2). In addition, the bioassay results indicated elevated LH concentrations (1SD) for the sample prior to the surge (24\textsuperscript{th} April am) and the EIA results indicated elevated concentrations (1SD) for the sample prior to the surge (24\textsuperscript{th} April pm) and the sample after the surge (25\textsuperscript{th} April pm). During this oestrous cycle, the bioassay detected a 5-fold increase from basal concentrations to the anLH surge and the EIA detected almost a 1.5-fold increase for this surge. The bioassay detected a 6-fold increase from basal levels to the ovLH surge and the EIA detected a 3-fold increase for this surge (Table 5.1).

5.3.2 Detection of the anovulatory and ovulatory surge of LH in the Asian elephant during a single cycle using bioassay, immunoassay and Siemens centaur CP automated analyser methods.

\textit{July/August}

During the anovulatory surge, the only significant increase (3SD) occurred on the 30\textsuperscript{th} July am for the bioassay, EIA and automated analyser methods (Figure 5.3). In addition, the bioassay results indicated elevated LH concentrations (1SD) after the surge until the 31\textsuperscript{st} July pm, the EIA results indicated elevated LH concentrations (1SD) from two days prior to the surge (28\textsuperscript{th} July am), and the automated analyser results indicated elevated LH concentrations (1SD) on the 29\textsuperscript{th} July am and the 31\textsuperscript{st} July pm. For the ovulatory surge, the first significant increase (3SD) occurred on the 18\textsuperscript{th} August am until the 18\textsuperscript{th} August pm for all three methods (Figure 5.3). In addition, the bioassay results indicated elevated LH concentrations (1SD) after the surge until the 19\textsuperscript{th} August am, the EIA
Figure 5: 3 August 2010 oestrous cycle

Anovulatory and ovulatory surges of a female Asian elephant during July and August 2010, comparing LH-stimulated progesterone responses from the bioassay (black line) and, automated analyser (dotted line) to LH responses from the immunoassay (grey line).
results indicated elevated LH concentrations (1SD) after the surge until the 19th August am, and the automated analyser results indicated elevated LH concentrations (1SD) for the sample before the surge (17th August pm) and also on the 19th August pm. During this cycle, the bioassay detected a 6-fold increase from basal concentrations to the anLH surge, the EIA detected an almost 2-fold increase, and the automated analyser detected an almost 7-fold increase for this surge. The bioassay detected a 5-fold increase from basal levels to the ovLH surge, the EIA detected an almost 4.5-fold increase, and the automated analyser detected a 3.5-fold increase for this surge (Table 5.2).

5.3.3 Comparison of preparation and incubation times of three methods

Determination of LH levels in a plasma sample can be crucial when ART is being employed. As the current study examined three methods of LH determination, comparison of the working times of these three methods was also investigated. For the bioassay method, it was assumed that the cells had been plated and serum starved in advance. Therefore, the bioassay requires two hours incubation with cells, and two one-hour incubations in the progesterone EIA. For the enzyme immunoassay we assumed that plate coating and blocking had happened in advance. Thus, the enzyme immunoassay required a three hour incubation with samples, a two hour incubation with biotinylated label, and two 40-minute incubations firstly with streptavidin and secondly with the substrate. The automated analyser uses culture medium supernates the same as the bioassay which requires two hours incubation, then samples are analysed in the Siemens Centaur CP automated analyser for progesterone. This process takes approximately 18 minutes (Table 5.3).

5.4 Discussion

The bioassay method successfully detected both anLH and ovLH surges in three consecutive cycles of an Asian elephant. All surge concentrations were greater than three standard deviations above
Table 5: 2 Change (fold) from basal to surge concentration of LH for one oestrous cycle

Basal and surge concentrations, and change (fold) from basal-to-surge concentration, for the anLH and ovLH surges of a female Asian elephant during a single cycle, in August 2010, as measured by bioassay, immunoassay and automated analyser methods. Consistently low samples were used to calculate a mean basal concentration, while high samples were used to calculate a mean surge concentration in either the anLH or ovLH cycle. Surges were quantified as those values exceeding the previous nadir by 3 standard deviations.

<table>
<thead>
<tr>
<th>Assay</th>
<th>August 2010 Cycle</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>anLH (mean basal)</td>
<td>anLH (surge)</td>
<td>Change -basal to surge (fold)</td>
<td>ovLH (mean basal)</td>
<td>ovLH (surge)</td>
<td>Change -basal to surge (fold)</td>
</tr>
<tr>
<td>Bioassay (nmol/L)</td>
<td>1.09 ± 0.35</td>
<td>6.84</td>
<td>6</td>
<td>2.31 ± 2.1</td>
<td>11.7 ± 1.99</td>
<td>5</td>
</tr>
<tr>
<td>Immunoassay (ng/ml)</td>
<td>1.19 ± 0.2</td>
<td>2.18</td>
<td>1.8</td>
<td>1.73 ± 0.53</td>
<td>7.62 ± 3.66</td>
<td>4.4</td>
</tr>
<tr>
<td>P₄ Analyser (nmol/L)</td>
<td>16.26 ± 8.94</td>
<td>112</td>
<td>6.9</td>
<td>38.8 ± 33.26</td>
<td>141 ± 1.41</td>
<td>3.5</td>
</tr>
</tbody>
</table>
mean basal concentrations in all cycles, thus supporting the hypothesis that the MLTC-1 bioassay would successfully detect LH in the blood plasma of the Asian elephant. These findings were also comparable to those of an established EIA, which demonstrated surge concentrations greater than three standard deviations above mean baseline levels in all cycles, and both methods detected the surges on the same dates. Current ex-situ populations of Asian elephants are not self-sustaining, and the application of ART (for example AI) are being used in many facilities on a global scale to improve reproductive output (Hildebrandt et al., 2006, Thitaram et al., 2008). The ability to detect the first anLH peak in the elephant is crucial in terms of scheduling AI to coincide with the ovLH peak 19 – 21 days later (Hermes et al., 2007).

The use of a clonal cell line as an indirect method to measure LH from elephant plasma samples collected during the anLH and ovLH surges has not been previously conducted. Dahl and Sarkissian (1993) investigated the use of a different clonal cell line (MA-10) as an in vitro bioassay for measuring LH. The authors achieved dose-response curves from pituitary LH preparations derived from seven different species of mammals, including elephant, but they did not investigate whether their assay could detect basal and surge circulating LH levels (Dahl and Sarkissian, 1993). The present study demonstrated that the bioassay method successfully detected differences in basal

<table>
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<th></th>
<th>Working time</th>
<th>Incubation time</th>
<th>Total time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay</td>
<td>2 hours</td>
<td>4 hours</td>
<td>6 hours</td>
</tr>
<tr>
<td>Enzyme Immunoassay</td>
<td>1 hour 40 min</td>
<td>6 hours 20 min</td>
<td>8 hours</td>
</tr>
<tr>
<td>Automated Analyser</td>
<td>2 hours</td>
<td>2 hours</td>
<td>4 hours</td>
</tr>
</tbody>
</table>
and surge concentrations of LH in the Asian elephant, thus indicating its potential for applicability in ART programs. The results from the bioassay also correlated well with those from the immunoassay used by the Perth Zoo for measuring LH in elephants. This assay utilises the monoclonal antibody 518-B7 (anti-bovine LH-β subunit) as antiserum, as this antibody has been demonstrated to cross-react with a range of different species LH in plasma (Matteri et al., 1987, Brown et al., 2004). However, it must be kept in mind that in addition to the antibody being sourced from bovine LH-β subunit, the standards are also derived from bovine LH while the biotinylated label is ovine LH and thus results are an expression of the bovine LH standard.

All of the methods employed successfully detected basal and surge concentrations of LH in the Asian elephant, though the extent (magnitude) of these changes differed between methods. In December, the bioassay detected an eight fold increase at the onset of the anLH surge, while the immunoassay detected a 37-fold increase. At the onset of the ovLH surge, the bioassay detected an 11.5-fold increase while the immunoassay detected an eight fold increase. The discrepancy between these results is most apparent at the onset of the anLH surge when measured by EIA; however, as there was a high degree of variability in these samples this may be the reason for the differences in fold changes detected by the two methods. Interestingly, when comparing the bioassay results to those of the automated analyser, the automated analyser detected greater basal and surge concentrations of LH. Both of these methods are essentially measuring the same endpoint (progesterone in culture medium supernates), yet in August the mean basal concentration as determined by the bioassay were 1.09 nmol/L and for the automated analyser were 16.26 nmol/L prior to the anLH surge. The anLH surge was detected at a concentration of 6.84 nmol/L by the bioassay, and 112 nmol/L by the automated analyser. When looking at the fold changes between these two methods, however, they are much more comparable than the absolute concentrations. For example, at the onset of the anLH surge there was a 6-fold increase
in circulating LH levels when measured by the bioassay and a 6.9-fold increase when measured by the automated analyser. At the onset of the ovLH surge, there was a 5-fold increase in circulating LH levels as detected by the bioassay method and a 3.5-fold increase when detected by the automated analyser method. Potentially, the reasons for the discrepancies between these methods when comparing absolute concentrations may result from differing sensitivities of the two assay systems, yet this is not of great concern as the magnitude of change was similar.

There are several disadvantages to the standard EIA approach described above for measuring LH in elephant samples, which include; i) preparation of biotinylated LH label, ii) two – three day turn around for results and, iii) requirement of antiserum that cross-reacts with elephant LH (only a few of which are available) (Brown, 2000). In the current study, to overcome some of these challenges, the use of a clonal cell line (MLTC-1) as a generic bioassay for measuring LH in Asian elephants was investigated. In an endocrine laboratory that is running a high volume of samples, the first two steps of preparing the EIA plate (plate coating and plate blocking) would be done in advance as plates can be stored at room temperature for 2 – 3 weeks or for 4 weeks if frozen (Brown, 2005). In the same way when routinely culturing cells for the bioassay, the first two steps (plating at the correct density and serum-starving) would be carried out prior to the arrival of a batch of samples. Thus in terms of the speed of obtaining a result (which may be critical for husbandry decision making) the incubation steps of both of these methods are the rate limiting steps. For the EIA method the quickest way to obtain a result is to incubate samples at 37°C, but even using this method the total run time is just over six hours. Using the bioassay method there is a two hour incubation period with samples plus two one-hour incubations when analysing samples for progesterone in the commercial EIA kit and these values do not take into account other working time. The disadvantages to the bioassay method are that it requires cell culturing facilities (laminar flow hood and temperature controlled CO₂ incubator), and staff with cell culturing experience;
however, these disadvantages are offset by its improved turnaround time and it’s applicability to diverse mammalian species.

To improve the practical applicability of the bioassay method in an ART program for elephant, the time from the acquisition of plasma samples until progesterone determination was calculated. It was determined to be four hours (including all incubation steps), if the progesterone values were determined by an automated analyser. The automated analyser is used in pathology laboratories to rapidly determine progesterone concentrations in human serum samples. In order to test its applicability at determining progesterone concentrations from elephant LH in a culture medium supernate, we first analysed a known basal and surge sample and when these were detected successfully using the standard progesterone EIA, we then analysed the same samples in the automated progesterone analyser. Although the absolute progesterone concentrations were different, (see above), both the standard progesterone EIA and the automated progesterone analyser detected the anLH and ovLH surges, with similar magnitudes of change from basal to surge progesterone levels. In current practices, AI in elephants is preformed on the day of the ovLH surge and the day after ovulation when using fresh semen (Dahl et al., 2004, Hermes et al., 2007). If using frozen semen then AI usually occurs on the day after the ovLH peak, however, at this stage the only reported case of pregnancy following the use of frozen-thawed semen resulted in late gestation miscarriage (Thongtip et al., 2009, Imrat et al., 2012). If the bioassay determination of LH surges can be conducted in four hours, then it has the potential to be included in the protocol of an elephant AI program using either fresh or frozen semen.

In conclusion, it was demonstrated that a readily available clonal cell line (MLTC-1) can be used as a bioassay to successfully detect differences between basal and surge concentrations of LH in the Asian elephant. Firstly, this method may prove useful in advancing the understanding of the
reproductive endocrinology of wildlife species, particularly understanding hormonal fluctuations during the oestrous cycle. Secondly, with the added benefits of automated progesterone analysers, i.e. an LH concentration can be detected in four hours, means that the bioassay could be used as a method for predicting ovulation in order to coordinate with AI attempts in an endangered species like the Asian elephant.
6. VERIFICATION OF A LUTEINISING HORMONE (LH) BIOASSAY FOR DETECTING THE RESUMPTION OF OESTROUS CYCLICITY IN THE SEASONALLY BREEDING MARE

6.1 Introduction

The assignment of August 1st (southern hemisphere) or January 1st (northern hemisphere) as the birth date for all foals born in a particular year, has driven the demand for artificially establishing an early return to oestrous cyclicity in order for mares to conceive sooner and produce a foal as close to these dates as possible in the following year (Hart et al., 1984). Since there is a desire to breed mares as early as possible in the season, most non-pregnant mares are still in the transition phase from winter anoestrus to normal ovulatory oestrous cycles (Irvine et al., 2000, Aurich, 2011). The transition period lasts between 60 and 80 days and is characterized by erratic oestrus behaviour and the growth and regression of ovarian follicles which fail to ovulate (Ginther, 1992, Donadeu and Watson, 2007).

The domestic horse (*Equus ferus caballus*) has an oestrous cycle ranging from 21 – 22 days duration (Farquhar et al., 2001, Aurich, 2011). The oestrus period (sexual receptivity) in the mare generally lasts between five and seven days (Farquhar et al., 2001, Crowell-Davis, 2007, Aurich, 2011), and is associated with circulating concentrations of LH increasing approximately nine days prior to ovulation and remaining elevated 1-2 days post ovulation (Ginther et al., 2010). Therefore, weekly monitoring of circulating LH concentrations, along with rectal ultrasonography of the ovaries, are valuable research tools for monitoring the resumption of oestrous cyclicity in the seasonally breeding mare. Despite the reproductive demands placed upon mares, a recent search indicated two American companies distributing LH ELISA kits that specifically target equines, thus
this technology is not easily accessible to researchers around the globe. The use of RIA still appears to be most widespread amongst research on horses often employing reagents from other species (e.g. ovine).

Horses are long day breeders and the shortened day light hours experienced in winter are associated with a decrease in gonadotrophin secretion and consequently a decrease in ovarian activity (Nagy et al., 2000, Aurich, 2011). Therefore, control of seasonal reproduction in horses is primarily due to photoperiod (Hart et al., 1984, Nagy et al., 2000). However, other factors can also influence seasonal reproduction in mares such as; environmental temperature, age, nutrition, reproductive state and body condition (BC) (Nagy et al., 2000, Donadeu and Watson, 2007, Aurich, 2011).

Inadequate nutrition that results in decreased percentage body fat is associated with poor reproductive performance in many livestock species, including horses, resulting in a delayed onset of puberty, induction of anoestrus and prolonged postpartum anoestrus (Day et al., 1986, Schillo, 1992, Rhodes et al., 1996, Gentry et al., 2002b). In addition to this, mares that enter the breeding season or foal in low BC have prolonged postpartum intervals, decreased conception rates and require more cycles per conception than their higher BC counterparts (Henneke et al., 1983). A BC score is often used to describe an animal’s percentage body fat and is measured by visual appraisal and palpation of the animal over key areas of its body identified as indicative of changes in stored body fat (Henneke et al., 1983, Carroll and Huntington, 1988, Gentry et al., 2002b). Gentry and colleagues (2002) noted that mares with high BC could continue to have oestrous cycles during the winter anoestrus period, while mares of low BC did not.

Seasonal changes in pelage (coat loss) can also be associated with the start of the breeding season in mares. Mares grow a thick insulating coat in winter, which they shed when the day length starts
to increase. Studies by Kooistra and Ginther (1975) and Sharp et al (1979), both indicated an association between the shedding of the winter coat and the seasonal start of oestrus activity in mares. However, in a study by Wesson and Ginther (1982) on puberty attainment in ponies, hair coat changes were disassociated from changes in reproductive activity, but there were small sample sizes in this study. Whether the postulated association between pelage and reproduction has any mechanistic link is unknown, although a study by Thompson and colleagues (1997) indicated a possible role of seasonal changes in prolactin affecting pelage and reproduction.

In Chapter 5 it was shown that the MLTC-1 bioassay could be used to detect endogenous surges of LH during the Asian elephant’s oestrous cycle. In Chapter 3 it was shown that the bioassay demonstrated good sensitivity with equine LH pituitary preparation, therefore extending on from the findings of Chapter 5, the aim of this study was to investigate whether the MLTC-1 bioassay could be used as a tool for determining the resumption of oestrous activity in acyclic mares approaching their breeding season. Moreover, as the ovary of a mare can be visualised by rectal ultrasound to determine ovulation (presence of a corpus luteum on the ovary), this animal model will be used to verify the LH bioassay against a definite ovulation event. Thus, while ovulation can be successfully determined in the mare by rectal ultrasound of the ovaries, verification of the bioassay against this positive event adds further credibility to the bioassay as a method which can successfully be applied to the detection of ovulation in mammals, as we are verifying against a biophysical event and not a biochemical event. An additional aim was to investigate several factors which may be associated with the resumption of oestrous cyclicity in the spring transitional period of the mare. To detect a return to oestrous cyclicity, blood samples were analysed for LH and progesterone using the clonal Leydig cell line bioassay described in previous chapters. The hypotheses tested in this study were: 1) that the MLTC-1 bioassay could be used to detect endogenous LH surges in the mare, and hence determine ovulation and the resumption of
oestrous cyclicity; 2) that seasonal changes in the pelage (coat) of the mare will be associated with resumption of cyclicity; and 3) that a better BC (or increase in BC) will be associated with an earlier resumption of oestrous cyclicity.

6.2 Materials & Methods

6.2.1 Domestic Horse Sample Acquisition

Twenty-three horses resident in the teaching herd on the Murdoch University farm situated 12 km south of Perth, Western Australia (31°57’S, 115°52’E) were used for this study. Mares were kept in two distinct herds; a group of lean mares (n=9; lower BC score) and a group of large mares (n=14; higher BC score). Once weekly blood samples were collected between 0930 and 1200 hours, prior to a rectal ultrasound to visualise the ovaries, every Monday during the months of July – October 2011. Blood was collected from the jugular vein using a 21G 1.5” needle into a 6 ml heparinised vacutainer. Samples were stored on ice for no more than 4 hours before being centrifuged at 3000g for 15 minutes, with plasma collected and stored at -80°C until required for progesterone analysis. Resident mares are used in a surrogacy program for clients at the equine veterinary clinic, therefore, mares in the lean group were given a feed supplement commencing on the 22nd August which aimed to increase their BC and hasten their return to oestrous cyclicity. Mares in the study ranged in age from 7 to 23 years, the mean age of the lean mares was 10.8 years (range 7 – 14 years), and the mean age of the large mares was 18.9 years (range 15 – 23 years).

At the time of blood sampling, each mare was also assessed for BC, coat condition (winter or summer) and amount of hair loss with brushing. Body condition was measured using the method described by Carroll & Huntington (1988). Briefly, condition is assessed visually and by palpation of three key areas of the horse, along the withers, the ribs and behind the shoulders (Plate 1A) and assigned a score ranging from 1 - 5, with 1 being poor and 5 being extremely fat. A coat condition
scoring system was developed with 0 representing a thin, summer coat, 2 representing a thick, shaggy winter coat and 1 representing a state between the two. A hair loss scoring system was developed where the amount of hair loss each mare was demonstrating was determined after two sweeps with a horse brush in a circular motion on the barrel (Plate 1B). A score of 0 represents no hair loss, 1 was minimal hair loss, 2 was more hair loss and 3 was substantial hair loss. For summary statistics for all mares in the study refer to appendix 9.5.

Weekly rectal ultrasound was performed using an Aloka SSD-500 with a 5 MHz linear transrectal probe to assess the ovaries with data recorded on follicular number and size. The presence of a recently ruptured ovarian follicle (corpus hemorrhagicum or CL) was also recorded and indicated that a mare had undergone the first ovulation of her breeding season, and this was recorded as day of ovulation, starting with January 1st as day 1 (e.g. July 11th is day 192).

Environmental data was recorded each week using the bureau of meteorology website (www.bom.gov.au) for temperature at 9am which was 30 minutes prior to sample collection. Photoperiod was calculated by recording sunrise and sunset times from the Australian Government geoscience Australia website (http://www.ga.gov.au/geodesy/astro/sunrise.jsp). All experiments and animal care procedure adhered to Murdoch University Animal Ethics Committee protocols (R2439/11).

6.2.2 Bioassay Procedure

Cells (MLTC-1) were processed for use in the bioassay as detailed in Chapter 2. Briefly, cells were transferred to a 96-well plate with a concentration of approximately 9,375 cells/well and allowed to adhere overnight. Following this, cells were washed twice with CMF-PBS and incubated with serum-free medium overnight. On the third day of the experiment, a plasma sample (50 µl) was added to each well in duplicate along with 200 µl of serum free medium. The plate was incubated
for two hours before culture supernatants were collected and stored at -20°C until required for progesterone analysis. NB; progesterone levels directly correlate with the amount of LH in the sample (see Chapter 3).

6.2.3 Statistical Analysis

Statistical data was analysed using SPSS version 19 (IBM SPSS Statistics, 2010). Tests for normality were conducted using the Kolmogorov-Smirnov test with values of p>0.05 not violating the assumptions of normality. Least squares liner regression was performed in order to determine a relationship between day of ovulation and age, change in BC, coat condition, hair loss, temperature and photoperiod at ovulation. Independent two sample t-tests were used to determine if there was any change in BC between large mares and lean mares from the start of sampling and between large and lean mares at the end of sampling. Paired sample t-tests were used to determine if there was a change in the BC of large mares or lean mares from the start until the end of sampling.

6.2.4 Calculating the success rate of the bioassay

In order to determine the success rate of the bioassay at showing an imminent or recent ovulation, hormone profiles of individual mares were examined on the date of ovulation (as identified by rectal ultrasound). Data was compared for LH concentrations only, progesterone concentrations only and, LH and progesterone concentrations combined to determine if ovulation was predicted accurately or not. An accurate prediction was based on the longitudinal hormone data obtained immediately preceding an ovulation (the presence of a CL as determined by ultrasound). In some cases the CL was recorded the week following the LH peak, and this was still recorded as an accurate prediction as LH concentrations in the mare remain elevated for several days (Aurich, 2011).
6.3 Results

6.3.1 Success rate of the bioassay to show an imminent or recent ovulation

When analysing LH-stimulated progesterone production of individual mares, the bioassay accurately detected ovulation in 85% of cases (Table 6.1). When analysing endogenous progesterone production of individual mares the bioassay accurately detected ovulation in 55% of cases (Table 6.1). When comparing LH-stimulated progesterone production and endogenous progesterone determination, the bioassay accurately detected ovulation in 55% of cases (Table 6.1). The highest source of error in this assay was false negatives which occurred in 5% of cases using LH-stimulated progesterone production alone, and in 35% of cases using endogenous progesterone alone or in combination with LH-stimulated progesterone production (Table 6.1). False positives accounted for the smallest amount of error; 10% regardless of which combination of hormone concentrations was analysed (Table 6.1).

Table 6: 1 Accuracy of the bioassay

Accuracy of the bioassay at detecting a recent or impending ovulation in the mare according to LH data alone, progesterone data alone or both hormones together. Ovulation was determined by rectal ultrasound identification of a CL.

<table>
<thead>
<tr>
<th></th>
<th>Accurate</th>
<th>False Positive</th>
<th>False Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH data alone</td>
<td>85%</td>
<td>10%</td>
<td>5%</td>
</tr>
<tr>
<td>Progesterone data alone</td>
<td>55%</td>
<td>10%</td>
<td>35%</td>
</tr>
<tr>
<td>LH and Progesterone data</td>
<td>55%</td>
<td>10%</td>
<td>35%</td>
</tr>
</tbody>
</table>

6.3.2 Comparing body condition scores between large and lean mares over the duration of the study.

An independent two sample t-test was carried out to compare the average BC scores of the larger and leaner mares at the beginning of the study period, and again at the end of the study period. At the start of the sampling period (11th July) the large mares had significantly greater BC scores than
the lean mares (P= 0.01), and by the end of the sampling period (31st October) there was no significant difference between the BC of large and lean mares (P= 0.546) (Table 6.2). A paired samples t-test was undertaken to determine if there was any change in average BC scores in the large mares from the start compared to the end of sampling, and in the lean mares from the start compared to the end of sampling. Large mares did not significantly change their BC scores from the start of sampling until the end of sampling (P= 0.72) (Table 6.2). Lean mares significantly increased their BC scores from the start until the end of sampling (P< 0.01) (Table 6.2).

Table 6: 2 Comparisons of body condition scores

Comparisons of average BC scores within and between the two groups of mares; large (n=14) and lean (n=9) over the duration of the study. Body condition was assessed by palpation and scored on a scale of 1 (skinny) to 5 (obese). Data is presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Mare group</th>
<th>BC at start</th>
<th>BC at end</th>
<th>Comparing between the start and end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large mares</td>
<td>3.6 (± 0.5)</td>
<td>3.7 (± 0.43)</td>
<td>P=0.7</td>
</tr>
<tr>
<td>Lean mares</td>
<td>3.1 (± 0.33)</td>
<td>3.6 (± 0.33)</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Comparing between large and lean</td>
<td>P=0.01</td>
<td>P=0.5</td>
<td></td>
</tr>
</tbody>
</table>

6.3.3 Representative hormone profiles of two mares demonstrating a return to oestrous cyclicity

Two mare’s hormone profiles were selected as representative of demonstrating a period of acyclicity before the first ovulation of the new breeding season (Figure 6.1). The first mare, (395), demonstrated basal concentrations of endogenous progesterone up to and including the sample collected on the 29th August. On this date she also exhibited an increased concentration of LH-stimulated progesterone production of MLTC-1 cells, plus each of her ovaries had a follicle of 50mm diameter as determined by rectal ultrasound. By the following sampling period (5th
**Figure 6:** Representative hormone profiles – acyclicity

Representative hormone profiles from two mares that demonstrated acyclicity at the beginning of the study and resumed oestrous cyclicity by the end of the study.
September), mare 395 showed decreased LH concentrations, increased progesterone concentrations and a CL on her left ovary, all indicating that she ovulated during that time period. The second mare, (400), demonstrated basal progesterone production up to and including samples collected on the 24th October (Figure 6.1). On this date she also indicated increased LH concentrations and a 52mm follicle on her left ovary. On the following sample period (31st October) mare 400 had decreased concentrations of LH, increased concentrations of progesterone and a CL on her left ovary all indicating an ovulation during that time period.

6.3.4 Representative hormone profiles of two mares demonstrating fluctuations in their hormone profiles before the first ovulation was recorded

A number of mares during the course of the study demonstrated fluctuations in their hormone profiles before the first ovulation of the season were detected and two mares have been selected as representative of this state. The first mare, 347, demonstrated elevated concentrations of LH on the 1st August (greater than 2 standard deviations above mean basal levels) although at this stage she only had a 25- and 15 mm follicle present on her ovaries (Figure 6.2). The progesterone concentrations recorded for mare 347 did not increase in the week following (1st August), thus it is unlikely that there was an ovulation at this time. On the 29th August, both LH and progesterone concentrations for mare 347 were relatively low, and she had a 35- and 30mm follicle present on her ovaries. By the following sampling period (5th September) both LH and progesterone had increased and there was a CL on the right ovary. The second mare, (310) had increased concentrations of LH on the 18th and 25th July (both greater than 3 standard deviations above mean basal levels) and a number of 20mm follicles on her ovaries (Figure 6.2). She then experienced another increase in LH on the 15th August (greater than 3 standard deviations above mean basal levels) while having a single follicle of 20mm diameter. None of these LH increases were followed by increases in progesterone in the following week, thus it is unlikely that ovulation
Figure 6: 2 Representative hormone profiles – fluctuations

Representative hormone profiles for two mares demonstrating fluctuations in hormone concentrations before the first ovulation was detected.
occurred during this time. On the 17\textsuperscript{th} October, LH concentrations were slightly increased while progesterone was not. A 35- and 20mm follicle was recorded on the ovaries on this date. On the following sample period (24\textsuperscript{th} October) both LH and progesterone had increased and there was a CL on the right ovary.

6.3.5 Representative hormone profiles of two mares who failed to ovulate during the study period

Only two mares failed to ovulate (i.e. no positive identification of a CL) during the course of the study despite occasional increases in LH-stimulated progesterone production. The first mare, (200), experienced increased LH on the 26\textsuperscript{th} September, which was greater than 4 standard deviations above mean basal levels (Figure 6.3). Her progesterone concentrations on this date were relatively low yet she only had multiple small follicles (~10 – 15 mm) present on her ovaries. She also experienced an increase in LH (greater than 3 standard deviations above mean basal levels) on the 10\textsuperscript{th} October, though again only had multiple small follicles present on her ovaries. On the 24\textsuperscript{th} October, mare 200 had high concentrations of LH (greater than 4 standard deviations above mean basal levels), but still hadn’t developed follicles of an ovulatory size. Even by the 31\textsuperscript{st} of October, she only had multiple medium follicles (~20 – 25mm) present on her ovaries, and no detectable CL. The second horse who failed to ovulate during the course of this study was mare 311 (Figure 6.3). She demonstrated a slight increase in LH on the 1\textsuperscript{st} August (though it was less than 2 standard deviations above mean basal levels) and despite having a 45mm follicle present on this date, her progesterone levels appeared to decrease by the following week thus it is assumed that she did not ovulate at this time. Mare 311 also experienced increased (though insignificantly so) LH on both the 3\textsuperscript{rd} and 10\textsuperscript{th} of October, but her progesterone concentrations did not increase after either of these dates. Interestingly, on the 3\textsuperscript{rd} October she had multiple large follicles (≥35mm) present on her ovaries and on the 10\textsuperscript{th} October she had a follicle of 45mm
Figure 6: 3 Representative hormone profiles – no ovulation

Hormone profiles of the two mares who failed to ovulate during the course of the study.
diameter. On the 24\textsuperscript{th} October mare 311 had a follicle of 50mm diameter, but only low levels of LH, and following this on the last day of the study she had a 45mm follicle as well as both increased LH and decreased progesterone. It is possible that mare 311 ovulated the 50mm follicle from the 24\textsuperscript{th} October, based on her hormone profile; however, a CL was not positively identified on the last day of sample collection.

6.3.6 The effect of environmental data and measurements of the mare on resumption of oestrous cyclicity.

Two environmental conditions (temperature and photoperiod) and four observations of the mare (change in BC, coat condition, hair loss and age) were investigated for their influence on resumption of cyclicity in the seasonally breeding mare. After initial analysis coat condition was excluded from the regression model due to its colinearity with photoperiod, as photoperiod would be the major driving factor in seasonal changes in pelage. Only 19 of the 23 mares were used in the analysis due to two mares receiving deslorelin which would influence their return to oestrous cyclicity, and two mares failing to ovulate during the study period. A least squares linear regression was conducted to determine the effect that five variables (change in BC, hair loss, age, temperature and photoperiod) have on the resumption of oestrous cyclicity in the seasonally breeding mare. This model explains 99.7\% of variance in the day of ovulation ($R^2 = 0.997$). Day of ovulation was most strongly influenced by photoperiod ($t = 37.71, P < 0.001$). Change in body condition ($t = -2.88, P = 0.013$), and hair loss at ovulation ($t = 3.05, P < 0.01$) also contributed significantly. Temperature and age, however, did not significantly contribute to day of ovulation ($t = 1.53 P = 0.15$) ($t = -2.05 P = 0.06$) respectively.

Out of 21 mares who ovulated during the study, 43\% ovulated with a BC score of 3.5 and 38\% ovulated with a BC score of 4, while 57\% of mares were at the transitional phase between a thick,
shaggy winter coat and a light, thin summer coat, and 33% were undergoing more than average amounts of hair loss (2) at the time of ovulation. Daily temperature ranged from 10.4 to 21.7 °C over the duration of the study while photoperiod increased from 10 hours and 10 minutes to 13 hours and 20 minutes. The first mare to ovulate was 17 years old and the last two mares were 20 and 10 years old respectively.

6.4 Discussion

The bioassay method accurately detected ovulation in 85% of cases when using LH-stimulated progesterone production data alone. This supports our first hypothesis that the bioassay would be able to successfully detect equine LH surges indicative of a return to oestrous cyclicity in the seasonally breeding mare. Moreover, as the ovary of a mare was able to be visualised by rectal ultrasound to determine ovulation (presence of a corpus luteum on the ovary), this animal model further validates the bioassay by combining the LH data with a definite ovulation event.

The greatest source of error in the bioassay method was false negatives which accounted for 35% of error when using progesterone data alone or in combination with LH data. When using LH data alone false negatives accounted for only 5% of error in the method. This highlights the fact that the assumption that progesterone levels would be suppressed preceding an ovulation at the start of the breeding season is invalid. Progesterone levels would only be seen to be suppressed if there was a preceding CL that undergoes luteolysis prior to the start of the follicular phase of the oestrous cycle. At the start of the breeding season this would not occur as there haven’t been any previous ovulations to result in the formation of a CL. Therefore, progesterone levels prior to the start of the breeding season would be low anyway. The progesterone data was only really included in this study to indicate when an ovulation may not have been detected using the rectal ultrasound.
False positives, on the other hand, only account for 10% of error in the method regardless of which combination of hormones was examined. This small incidence of error was recorded from the mares who failed to ovulate (no visible CL on the ovary) during the course of the study. To identify ovulation in the mare, rectal ultrasounds were conducted to establish the presence of a CL (or a corpus hemorrhagicum) on the ovary. Of the 23 mares in the study, there were only two instances where the positive identification of a CL could not be made by the last sampling date of October 31st. Based on the follicular data and hormone profiles for one of these mares (311) it is likely that she did ovulate a 50mm follicle before the last day of sample collection, in fact there was a structure present on her ovary that resembled a CL but identification was not completely positive. If this was definitely a CL (which seems likely based on her hormone profile), it increases the accuracy of the bioassay method using LH data alone to 90%.

Some of the mares in this study presented a pattern of fluctuating hormone levels before the first ovulation of the season was detected. Despite their general seasonal reproductive nature, it would seem that a small proportion of mares do not enter into seasonal anoestrous every year, and that this apparent lack of seasonal reproduction does not follow any clear pattern from year to year (Fitzgerald and Davison, 1997, Davison et al., 1998, Fitzgerald and McManus, 2000). In one of the mares (347), it would appear that she was cycling from the commencement of the study (July 11th). Though her progesterone levels were low on the first day of sample collection, they were much higher by the following week which could be due to a CL as she could have ovulated between the 11th and 18th of July even though it was not detected by ultrasound. By the 8th August, mare 347 had decreased progesterone and increased LH levels which possibly indicates another ovulation as it happened 22 days after the first suspected ovulation (22 days is the duration of the mare’s oestrous cycle) (Farquhar et al., 2001). In addition to this, when ovulation was successfully detected in this mare it was another 22 days after the second suspected
ovulation. Whether mare 347 did not enter seasonal anoestrus, or whether she simply resumed cycling earlier than other mares we cannot completely discern due to the range of our sampling dates.

The other pattern in hormone concentrations that was noted in transitional mares during this study is that of very little hormonal activity prior to ovulation. Two mares demonstrating this state were presented here (mares 395 and 400). The mares both presented a state of seasonal anoestrus as indicated by low levels of circulating hormones (LH and progesterone) before the first ovulation of the season was detected. It has been noted that transition out of the anovulatory period in mares may be characterised by follicles which commence development and then become atretic (Guerin and Wang, 1994). Ginther (1990) found that some mares develop anovulatory follicular waves prior to the first ovulation, while others have no significant follicular growth before their ovulatory follicular wave. From our hormonal observations it is likely that mare 395 had one anovulatory follicular wave four weeks prior to her ovulatory wave, while mare 400 may have had several. However, to best quantify this, it would be desirable to have FSH concentrations as well as the LH and progesterone concentrations, as FSH is the key stimulus of early follicular development (Ginther and Bergfelt, 1993, Donadeu and Ginther, 2002).

The mares in this study were kept in two separate herds, one of larger mares and one of leaner mares. At the beginning of the study, the larger mares had a significantly heavier average BC than the lean mares. The equine department of the Murdoch Veterinary Hospital offer a surrogacy program to clients, thus once mares return to oestrous cyclicity they may be selected for embryo transfer. Several studies report the effect that nutrition has on reproduction in mares (Henneke et al., 1983, Gentry et al., 2002b). The BC of mares is often reported to reflect the amount of stored fat in the animal (Henneke et al., 1983, Carroll and Huntington, 1988), and those mares of a higher
BC often continue cycling through winter compared with their lower BC counterparts who experience a longer, deeper anoestrus period (Gentry et al., 2002b). Due to these reasons, the mares of the leaner herd were supplement fed commencing on the 22nd of August to improve their BC and hasten their return to oestrous cyclicity. The large mares did not significantly differ in their average BC from the start to the end of the study. The lean mares, however, significantly increased their average BC from the start to the end of the study. By the end of the study, there was no significant difference between the average BC of the large and lean mares. It has also been noted that mean body weight and estimated percentage body fat in the mare fluctuates over the year being higher in summer then decreasing to a nadir throughout autumn and into winter before increasing again in spring (Fitzgerald and McManus, 2000).

The results indicated that the strongest influence on resumption of oestrous cyclicity in the mare was photoperiod. As the mare is a seasonal (long day) breeder, it has been reported that the photoperiodic stimuli is primarily responsible for seasonal changes in reproduction (Fitzgerald and McManus, 2000, Nagy et al., 2000). The hypothesis that seasonal changes in pelage would be associated with resumption of oestrous cyclicity in the mare was supported. Despite changes in coat condition (thick winter coat or thin summer coat) being removed from the model due to its colinearity with photoperiod, changes in hair loss was significantly related to resumption of oestrous cyclicity. It is likely that photoperiod is the main factor driving seasonal changes in pelage (Kooistra and Ginther, 1975), though this change may be mediated by seasonal variations in prolactin concentration as suggested by Thompson and colleagues (1997).

The hypothesis that a better (higher) BC score or an increase in BC would be associated with day of ovulation was also supported. It has been well documented that inadequate nutrition has a negative impact on reproductive function which can manifest as delayed onset of puberty,
induction of anoestrous and prolonged post-partum anoestrous (Day et al., 1986, Schillo, 1992, Rhodes et al., 1996, Gentry et al., 2002b). Previous studies have noted that low BC in mares results in a longer and deeper period of anoestrous than mares of higher BC (Gentry et al., 2002a, Gentry et al., 2002b). The mares in our study, however, were all of a moderate to fat BC and we did not sample mares across the range of conditions (i.e. there were no skinny or obese mares), and this may have influenced our results.

The model demonstrated that temperature did not have a significant effect on resumption of oestrous cyclicity in the mare. Guerin and Wang (1994) examined temperature changes over a ten year period and the relationship between resumption of oestrous cyclicity in the mare. They concluded that the first ovulation varied from year to year and correlated with the annual variations in minimum and maximum temperatures (Guerin and Wang, 1994). The temperature samples in the present study were recorded at 9 am every Monday, thirty minutes prior to sample collection. A larger scale of temperature fluctuations over a longer time period may be required to determine more conclusively if it does have an influence on resumption of oestrous cyclicity in the mare.

Age did not have an effect on resumption of oestrous cyclicity in the mare. This is interesting as it has been noted that younger mares (2 to 5 years) of a lower BC score enter seasonal anoestrous earlier than mature mares (>10 years) with higher BC scores (Fitzgerald and McManus, 2000). Older mares tend to experience lower pregnancy rates and increased pregnancy loss than younger mares as well as longer inter-ovulatory intervals (Carnevale and Ginther, 1992, Carnevale et al., 1993). Comparisons between studies on the effect that age has on follicular activity in the mare need to be approached with caution, as there are inconsistencies in the definitions of age groups; for example one study had three groups based on the following ages: 5 – 6 years, 10 – 14 years
and >18 years (Ginther et al., 2008), and another had the following: 5 – 7 years, 15 – 19 years and 20 or more years (Carnevale et al., 1993). Mares in the current study ranged in age from 7 to 23 years, and most fell in an intermediate age range of 8 to 20 years. It has been noted that mares can become senescent from twenty years onwards, and coupled with a lack of young mares may have influenced our results (Ginther et al., 2008).

In conclusion, we have verified the ability of the bioassay to accurately detect a recent or imminent ovulation in the seasonally breeding mare. When using LH-stimulated progesterone production of MLTC-1 cells, the bioassay was accurate 85% of the time, which suggests that this method is highly reliable and highlights its practicality to be adopted by equine reproductive professionals. Photoperiodic stimuli, (increase in day length), is the primary driving factor of resumption of oestrous cyclicity in the mare, while changes in pelage and BC also have an effect.
Plate 6: Representations of sample collections in the mare

Representations of (A) areas of the mare that were palpated to determine body condition, and (B) areas of the mare that were brushed to determine hair loss and coat condition.
7. GENERAL DISCUSSION

In the general introduction it was stated that the overall aim was to develop a simple, rapid *in vitro* LH bioassay which would be able to detect both basal and surge concentrations of LH from a diverse range of mammalian species. The general hypothesis being that by utilising a clonal cell line in a bioassay, LH concentrations will be able to be measured, indirectly, in different mammalian species as the cells will be stimulated to produce progesterone regardless of the origin of the source of LH. As described in this thesis, the bioassay was applied to three taxonomically distinct species of mammals (four species in total) and it was able to detect changes in LH in all species. Furthermore, for the two species of marsupials and the Asian elephant, a comparison of the bioassay results to those obtained by the more routinely used EIA showed similar findings. Therefore, from the results of the development of the bioassay and the experiments validating it, the general hypothesis was supported.

*Development and validation of the bioassay*

In Chapter 3 the specific hypotheses tested were that an optimised bioassay would: (1) successfully detect LH pituitary preparations from a range of mammalian species; and (2) successfully detect LH within the normal physiological range. The findings from Chapter 3 demonstrated that under the optimised assay conditions of 9,375 cells per well incubated for 120 minutes (obtained during the optimisation phase described in this chapter), the MLTC-1 bioassay could detect dose-response curves from pituitary preparations of LH sourced from eight species of mammals. Comparing the findings to previous research described in the literature, the MLTC-1 bioassay displayed improved sensitivity over a similar bioassay, the MA-10 bioassay (Dahl and Sarkissian, 1993) for most species tested (bovine being the exception). The sensitivity of the MLTC-
1 cell line at detecting LH was compared to previous reports of endogenous LH concentrations in these species and it was noted that it was able to detect both basal and surge concentrations in all species except bovine, though the 4 ng/ml minimum detectability is still within the lower end of the physiological range for bovine samples. Therefore, both of the hypotheses from this experimental chapter were supported, and it was concluded that it was likely that the assay could be used for the analysis of samples from diverse mammalian species, especially those lacking immunoassays.

In Chapter 4 the specific hypotheses tested were that the bioassay would: (1) successfully detect LH in the plasma of marsupials; (2) detect artificially induced low and high circulating endogenous LH concentrations; and (3) produce results which would be comparable to those of an established enzyme immunoassay (EIA) for measuring LH in marsupial samples. The marsupial model was chosen to test the bioassay because earlier work demonstrated that marsupial gonadotrophins were of a lower potency relative to eutherian gonadotrophins in eutherian assay systems (Gallo et al., 1978, Farmer et al., 1981, Stewart et al., 1981). Additionally, the marsupial plasma samples used in this study were collected from animals that had undergone a GnRH challenge. In this animal model, a single dose of a GnRH agonist administered as an injection results in an increase in production of both gonadotrophins (LH and FSH). This increase can be reliably detected in the circulation 20 to 30 minutes after the GnRH injection. The MLTC-1 bioassay was able to detect the expected changes in circulating LH concentrations (significant increase) in western grey kangaroos and black-flanked rock wallabies that had received a single injection of GnRH.

Exogenous GnRH can also be used as a contraceptive; for example GnRH super-agonist implants flood the system with GnRH, thus masking the endogenous pulsatile secretory pattern necessary for stimulating the gonadotrophins (Kuhl et al., 1984). As GnRH levels remain elevated for a
prolonged period of time with the implant system, there is also a down regulation of pituitary GnRH receptors which influences post-receptor pathways that prevent the pituitary from responding to a GnRH challenge (Herbert, 2004). In a second animal model in Chapter 4, a contraceptive treated group of black-flanked rock wallabies had been given a GnRH super-agonist (deslorelin) implant four months prior to a GnRH challenge. The MLTC-1 bioassay detected the expected change (significant decrease) in circulating concentrations of LH in these animals. Interestingly, when comparing the before challenge samples from contraceptive-treated and a non-contraceptive (control) groups of wallabies, the contraceptive animals actually had a greater concentration of LH-stimulated progesterone production than the control animals in both the bioassay and EIA results. It would be expected that contraceptive-treated wallabies would have had a reduced concentration of circulating LH due to down-regulation of pituitary GnRH receptors, and the observed results imply that both the bioassay and EIA methods failed to accurately detect low levels of circulating LH in wallaby blood plasma. This may be a reflection of the species specificity of both the mouse clonal cell line (MLTC-1) and 518-B7 antibody (EIA) for marsupial LH. Therefore, the hypotheses from this experimental chapter were mostly supported given that the MLTC-1 clonal cell line was able to bind marsupial LH and generate progesterone (to indirectly measure LH), and that the bioassay and EIA were able to detect the expected increases in circulating LH concentrations, but not able to accurately detect low circulating LH concentrations. It was concluded that the bioassay could be used for both marsupial and eutherian mammal species, though it appeared 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 in marsupial samples.

In Chapter 5 the specific hypotheses tested were that the bioassay would: (1) successfully detect LH in the plasma of the Asian elephant; (2) detect anovulatory and ovulatory endogenous LH
surges; and (3) produce results which would be comparable to those of an established EIA for measuring LH in elephant samples. The elephant model was chosen firstly because current ex-situ populations of elephant are not self sustaining and the demand for knowledge of elephant reproductive biology is high. Consequently, because of the efforts to understand elephant reproductive biology, programs such as those at Perth Zoo mean that the elephant’s oestrous cycles are closely monitored. In addition, to be able to collect samples during known oestrous cycles, the unusual double LH surge during the follicular phase of the elephant’s oestrous cycle offers a unique opportunity to examine the accuracy of the bioassay. The elephant plasma samples were collected from a single female housed at the Perth Zoo over three consecutive cycles from 2009 until 2010. As the Zoo have been monitoring the oestrous cycles of this elephant for several years, records indicated that she had three cycles per year usually occurring in April, August and December. Analysis of the plasma samples by EIA occurred within a day of the last sample of each month (i.e. the last sample from December 2009 was collected on the 24th and the assay was run on that day). Analysis of samples by the bioassay occurred retrospectively, allowing selection of the samples so as to include the anLH and ovLH surge and a few samples before and after these events. As expected, the bioassay successfully detected elephant LH and all surges of LH from all cycles. The findings from the bioassay also correlated well to those of the established EIA.

An additional aim in Chapter 5 was to address some of the challenges that the current EIA method presents to researchers when routinely monitoring the elephant oestrous cycle. The rate-limiting step in the EIA method is the long incubation times required for LH determination (just over 6 hours). While the standard bioassay procedure described in this thesis has a shorter total incubation time (i.e. the incubation times in both the bioassay and the progesterone EIA) of four hours, for maximal speed an automated progesterone analyser was used to determine LH-
stimulated progesterone production in comparison to the standard progesterone EIA method. The absolute values of progesterone obtained by the standard EIA method and the automated analyser were quite different from each other, which was unexpected as both methods are essentially measuring the same end-product (progesterone in cell culture supernates). The magnitude of change was however similar implying that the differences may arise from differing sensitivities of the two methods. Overall, the hypotheses from this experimental chapter were supported given that the MLTC-1 clonal cell line was able to detect elephant LH, and that the bioassay and EIA were similarly able to detect endogenous LH surges during the elephant’s oestrous cycle. It was concluded that the bioassay could be used to successfully detect differences between basal and surge concentrations of LH (both anovulatory and ovulatory) in the Asian elephant. With the addition of an automated progesterone analyser into the system, an LH concentration can be detected in four hours, meaning that the bioassay could be used as a viable method for predicting ovulation in order to coordinate with AI attempts in Asian elephants.

In Chapter 6 the specific hypotheses tested were: (1) that the MLTC-1 bioassay could be used to detect endogenous LH surges in the mare, and hence determine ovulation and the resumption of oestrous cyclicity; (2) that seasonal changes in the pelage (coat) of the mare will be associated with resumption of cyclicity; and (3) that a better BC (or increase in BC) will be associated with an earlier resumption of oestrous cyclicity. The horse model was chosen due to the fact that rectal ultrasound is widely in practice in equine management, and can be used to successfully show an ovulation (identification of a CL), and that the horse has a prolonged LH surge compared to other species of mammals (Ginther et al., 2010). We were fortuitous in being able to time our sample collection to that of the routine monitoring of horses on the university farm. We successfully verified that the bioassay could detect endogenous changes in LH in horse plasma samples, and that the bioassay was accurate in 85% of cases, thus supporting our first hypothesis. We also
examined the effect that several factors had on resumption of oestrous cyclicity in the mare. Photoperiod was the strongest influence on resumption of oestrous cyclicity, which is in agreement with previous research (REF). Changes in pelage as determined by the change from a thick, shaggy winter coat into a light, thin summer coat and the amount of hair loss was also found to significantly relate to resumption of oestrous cyclicity in the mare. This supports our second hypothesis. Mares with a high or increasing BC were found to ovulate earlier than mares with a low or decreasing BC, thus supporting our third hypothesis.

**Limitations and applicability of the bioassay**

Out of all of the different species we tested under all the physiological situations (induced LH response, natural LH response) the only area where the bioassay method was relatively unsuccessful was at detecting low physiological levels of marsupial LH. In a captive breeding situation where the LH surge is used to determine ovulation and schedule AI or natural mating attempts, basal (low) concentrations are not as critical as surge (high) concentrations. Therefore, while the bioassay may not accurately portray low level (luteal phase) concentrations of LH, it should still accurately determine a LH surge indicative of impending ovulation.

The only unforeseen event during the procedure of optimising the bioassay was the degradation of the progesterone standard supplied in the commercial kit. Despite following all instructions for storage of the kit (-20°C) and preparation of the standard (diluted just prior to use in either EIA buffer or serum-free culture medium according to manufacturer’s instructions), a kit used within a 5 day period (each kit can run 5x 96-well plates) still showed degraded progesterone over this time period as indicated by a gradient shift in the standard curve. Early analyses of samples were deemed inaccurate due to the degradation of the progesterone standard which prompted us to develop our own progesterone standard for further use in all assays (either determining LH-
stimulated progesterone production or endogenous progesterone from blood plasma samples). Our standards were deemed acceptable for use in the assay when the relationship of percent binding and the standard concentration is inversely proportional (i.e. the relationship between the percentage of bound antigen to the concentration of the standard added) (Brown, 2005).

The collection of blood plasma samples is not always feasible for animals that have not been conditioned to routine sample collection, or for free-living species. Several studies have used urinary LH to determine oestrous cycle characteristics, and sometimes also detect ovulation for AI attempts (French et al., 1996, Robeck et al., 2004, Robeck et al., 2005). Urinary LH concentrations were determined using the monoclonal 518-B7 antibody in an EIA (bottlenose dolphin) and in a RIA (killer whale) (Robeck et al., 2004, Robeck et al., 2005). These methods were then applied to Asian and African elephant urine samples, however, urinary LH was unable to be detected by the EIA despite correlating serum samples demonstrating the relevant changes in LH associated with both the anLH and ovLH surges (Brown et al., 2010). While it may be possible that elephants do not excrete detectable amounts of LH in the urine, it would be interesting to see whether the bioassay (using MLTC-1 cells) is able to detect LH concentrations in urine from elephants or other species. This raises its own challenges however, as urine may be toxic to the cells due to its acidity, though one potential way of overcoming this may be to dilute the urine samples in culture medium prior to incubating with the cells.

There are some drawbacks to the bioassay method that may restrict the ease at which other researchers can adopt this method. Cell culturing requires a sterile working environment, and specific equipment such as a laminar flow hood and a CO₂ incubator. Zoological facilities will all differ in the equipment that they have access to on-site, however, it is increasingly common for
many zoos to have close ties to tertiary institutes which themselves may have access to cell
culturing equipment. Thus the bioassay method may contribute to collaborative research efforts in
mammalian reproductive endocrinology. In addition to this, as we noted with the Asian elephant
samples, the most time-effective way to analyse samples is to use an automated progesterone
analyser which can determine progesterone concentrations from culture medium supernates in
approximately 18 minutes. In the current study, we used an automated analyser which was
located at the specialist fertility centre at Joondalup Health Campus, and hence such a machine
may not be readily available to researchers wishing to adopt this method, which in turn increases
working time of the bioassay method.

The bioassay method described herein, has several practical advantages over traditional primary
culture bioassays, RIA and EIA methods. A clonal cell line eliminates the continual sacrifice of
animals as a source of cell populations, and the relatively difficult process of isolation of the
correct cell type at high purity. By sourcing cells from the same population interassay variability is
kept to a minimum (Dahl and Sarkissian, 1993). As cells can be frozen and resuscitated at differing
passes, there is the potential for an unlimited number of cells to be grown, all of which display
similar responsiveness (Dahl and Sarkissian, 1993). However, cells potentially can lose their
responsiveness to hormonal stimulation at greater passes though this hasn’t been
comprehensively studied for the MLTC-1 cell line past 30 passes (Rebois, 1982).

The RIA presents several drawbacks most of which relate to health hazards associated with radio-
labelled tracers, the high costs associated with safe disposal of these materials and the equipment
required (gamma counter) to analyse the results. It is also noted that the products have a short
shelf life and that RIAs are often inaccurate at detecting low concentrations of LH (Kalia et al.,
2004). While the EIA method utilising the 518-B7 antibody (which was used to validate the
bioassay) was able to detect LH in both marsupials and the Asian elephant, it is not readily available as a commercial kit. There is a finite source of 518-B7 antibody, and all other reagents must be purchased individually. Additionally, without a comprehensive endocrine manual (such as Brown (2005)), several parameters must be optimised (e.g. Antibody concentration, antigen dilution, standard curve preparation, time and temperature of incubation procedures) which restricts the applicability of this method.

Conclusions

It was successfully demonstrated that a clonal mouse Leydig tumour cell line is able to be used in a bioassay to indirectly measure concentrations of LH in taxonomically diverse mammalian species. The benefits of this assay are that it could be suitable for i) zoo and wildlife facilities that breed endangered wildlife, ii) native species (marsupial) research including work on fertility control measures and, iii) domestic and production animal research. The bioassay method could be adopted by researchers of mammalian reproductive endocrinology, especially those working on wildlife species for which commercial gonadotrophin assays are unavailable. In an ideal situation, researchers with strong collaborative ties with different institutions can benefit from a multi-species bioassay that can be carried out in four hours time, thus demonstrating the practicality of using this method in AI programs for threatened species. Finally, the overall aim of this thesis to develop and validate a simple, rapid in vitro LH bioassay which would be able to detect both basal and surge concentrations of LH from a diverse range of mammalian species was achieved.


# 9. APPENDICES

## Appendix 9: 1 Cell culture medium product specificities

RPMI-1640 culture medium product specificities obtained from Mediatech Inc, makers of Cellgro®

<table>
<thead>
<tr>
<th>Product</th>
<th>RPMI-1640 Powder – Phenol Red Free</th>
</tr>
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<tr>
<td><strong>Inorganic salts</strong></td>
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</tr>
<tr>
<td>Ca(NO$_3$)$_2$.4H$_2$O</td>
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</tr>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>MgSO$_4$ (anhydrous)</td>
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<tr>
<td>NaCl</td>
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</tr>
<tr>
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<tr>
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<td>L-Valine</td>
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</table>
Appendix 9.2 Plate map for bioassay optimisation

96-well plate map indicating cell concentrations of each column and hCG dilution. Five 96-well plates were prepared with the above specifications, each plate being incubated for a different time period; 15, 30, 60, 120 or 240 minutes.

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</table>
Appendix 9.3 Plate map for LH preparations: part 1

96-well plate map indicating species of LH preparation and concentration per sample. Each well contained approximately 9,375 cells and the plate was incubated for two hours.

<table>
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<td>Human 0.125ng</td>
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<td>Human 2ng</td>
<td>Human 4ng</td>
<td>Human 8ng</td>
<td>Human 16ng</td>
<td>Human 32ng</td>
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</tr>
<tr>
<td>B</td>
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<td>Human 0.125ng</td>
<td>Human 0.25ng</td>
<td>Human 0.5ng</td>
<td>Human 1ng</td>
<td>Human 2ng</td>
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<td>Human 16ng</td>
<td>Human 32ng</td>
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<tr>
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<td>Rabbit 0.5ng</td>
<td>Rabbit 1ng</td>
<td>Rabbit 2ng</td>
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Appendix 9.4 Plate map for LH preparations: part 2

96-well plate map indicating species of LH preparation and concentration per sample. Each well contained approximately 9,375 cells and the plate was incubated for two hours.

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<th>4</th>
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### Appendix 9.5 Summary statistics of mares

Summary statistics for all mares in the study indicating all variables measured on a weekly basis. Data presented is that recorded at ovulation.

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<th>CC Ovulation</th>
<th>HL Ovulation</th>
<th>Temp Ovulation</th>
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