PITUITARY-OVARIAN RELATIONSHIPS IN THE TAMMAR WALLABY, MACROPUS EUGENII (DESMAREST)

by

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This thesis is my own work, except where specifically acknowledged.

Susan M. Evans
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ABSTRACT

Heterologous radioimmunoassays have been validated for the routine measurement of LH and FSH in tammar wallaby plasma. LH levels in plasma of normal females remain low, <0.2-2.0 ng/ml, except for large surges, usually >50.0 ng/ml, associated with ovulation: the LH surge lasts about 12 hours and ovulation occurs 20-40 hours after the LH peak. FSH levels are generally undetectable (<50 ng/ml) in plasma of intact females, but rise after ovariectomy to 100-400 ng/ml. LH levels also rise after ovariectomy.

In bilaterally ovariectomized wallabies with ovarian cortex grafts under the pouch skin, luteinization of the grafts was associated with a depression of plasma gonadotrophin levels to pre-castrate values, and the effects of steroid replacement in ovariectomized animals suggests that oestradiol-17β and progesterone may be major contributors to the ovarian control of gonadotrophin secretion, oestradiol-17β having both negative and positive feedback effects. I.v. injections of LH-RH also cause LH release, but no detectable changes in FSH levels in intact or ovariectomized females.

The tammar CL inhibits follicular growth during the first half of the oestrous cycle. Injection of oestradiol-17β, but not progesterone, for 7 days after lutectomy delayed the induced follicular growth and ovulation.

A model for the endocrine control of the oestrous cycle of the tammar wallaby is proposed. It is postulated that gonadotrophin secretion, and hence follicular growth, are regulated by changing levels of oestradiol in the circulation: during the first half of the cycle, luteal oestradiol probably inhibits gonadotrophin secretion and, hence,
follicular development, but it may be rising levels of oestradiol produced
by the maturing follicles that induces the preovulatory LH surge by positive
feedback.

Comparisons of the responses of wallabies to ovariectomy and
LH-RH stimuli during the breeding and non-breeding seasons has shown that
seasonal quiescence is associated with changes in the hypothalamo-
pituitary-ovarian axis which include a decreased capacity of the pituitary
to store LH and, probably, an increase in the sensitivity of the hypotha-
lamus to steroid feedback. Thus the original view that quiescence is
governed solely by a direct inhibition of the CL by prolactin has now
been extended.

An appraisal of the experimental data and that obtained from
normal wallabies in various physiological conditions has shown that
from studies on eutherian mammals, particularly sheep and primates,
parallels may be found for many of the endocrine mechanisms regulating
the oestrous cycle and the annual breeding cycle of the tammar wallaby.
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>UNITS OF HORMONE CONCENTRATION</td>
<td>xii</td>
</tr>
<tr>
<td><strong>CHAPTER 1: INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 The dual hormone concept</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Hypothalamic control of gonadotrophin secretion</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Ovarian control of gonadotrophin secretion</td>
<td>10</td>
</tr>
<tr>
<td>1.4 Endocrine control of the oestrous cycle: eutherian models</td>
<td>14</td>
</tr>
<tr>
<td>1.4.1 Follicular maturation</td>
<td>17</td>
</tr>
<tr>
<td>1.4.2 Hormonal control of ovulation</td>
<td>20</td>
</tr>
<tr>
<td>1.4.3 Influence of the corpus luteum upon oestrous cycle length</td>
<td>23</td>
</tr>
<tr>
<td>1.5 Patterns of reproduction in the Macropodidae</td>
<td>27</td>
</tr>
<tr>
<td>1.6 Reproductive biology of the tammar wallaby</td>
<td>30</td>
</tr>
<tr>
<td>1.7 Control of the ovarian cycle in macropod marsupials</td>
<td>31</td>
</tr>
<tr>
<td>1.8 The scope of the thesis</td>
<td>37</td>
</tr>
<tr>
<td><strong>CHAPTER 2: GENERAL PROCEDURES</strong></td>
<td></td>
</tr>
<tr>
<td>2.1 Animal maintenance</td>
<td>39</td>
</tr>
<tr>
<td>2.2 Blood collection</td>
<td>40</td>
</tr>
<tr>
<td>2.3 Surgical procedures</td>
<td>40</td>
</tr>
<tr>
<td>2.4 Autopsy procedure</td>
<td>41</td>
</tr>
<tr>
<td>2.5 Histology</td>
<td>43</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>3</td>
<td>A HETEROLOGOUS RADIOIMMUNOASSAY FOR TAMMAR WALLABY: LUTEINIZING HORMONE</td>
</tr>
<tr>
<td></td>
<td>3.1 Introduction</td>
</tr>
<tr>
<td></td>
<td>3.2 Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>3.3 Experimental procedures and results</td>
</tr>
<tr>
<td></td>
<td>3.4 Discussion</td>
</tr>
<tr>
<td></td>
<td>3.5 Summary</td>
</tr>
<tr>
<td>4</td>
<td>A HETEROLOGOUS RADIOIMMUNOASSAY FOR TAMMAR WALLABY: FOLLICLE-STIMULATING HORMONE</td>
</tr>
<tr>
<td></td>
<td>4.1 Introduction</td>
</tr>
<tr>
<td></td>
<td>4.2 Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>4.3 Experimental procedures and Results</td>
</tr>
<tr>
<td></td>
<td>4.4 Discussion</td>
</tr>
<tr>
<td>5</td>
<td>GONADOTROPHIN LEVELS IN NORMAL FEMALE WALLABIES</td>
</tr>
<tr>
<td></td>
<td>5.1 Introduction</td>
</tr>
<tr>
<td></td>
<td>5.2 Gonadotrophin levels in plasma during the oestrous cycle and pregnancy</td>
</tr>
<tr>
<td></td>
<td>5.3 The periovulatory period: temporal relationships of mating, the LH surge and ovulation</td>
</tr>
<tr>
<td></td>
<td>5.4 Seasonal variations in LH levels in plasma of female tammars</td>
</tr>
<tr>
<td></td>
<td>5.5 Discussion</td>
</tr>
<tr>
<td></td>
<td>5.6 Summary</td>
</tr>
<tr>
<td>6</td>
<td>EXPERIMENTAL MANIPULATIONS OF THE PITUITARY-OVARIAN SYSTEM</td>
</tr>
<tr>
<td></td>
<td>6.1 Introduction</td>
</tr>
<tr>
<td></td>
<td>6.2 The effects of ovariectomy during the non-breeding season upon gonadotrophin secretion</td>
</tr>
</tbody>
</table>
6.3 Diurnal variations in LH and FSH levels in plasma of ovariectomized and intact wallabies
6.4 Roles of the ovarian cortex and interstitial tissue in the feedback control of gonadotrophin secretion
6.5 Effects of steroids on gonadotrophin levels in ovariectomized and seasonally quiescent intact wallabies
6.6 Follicular growth after hypophysectomy
6.7 Effects of steroid replacement after luteectomy on follicular growth and the timing of ovulation
6.8 Induction of LH release by a single injection of oestradiol-17β
6.9 Hypothalamic control of LH secretion: responses to LH-RH
6.10 Summary

CHAPTER 7: GENERAL DISCUSSION

BIBLIOGRAPHY

APPENDIX 1: A SOLID PHASE RADIOIMMUNOASSAY FOR LUTEINIZING HORMONE

APPENDIX 2: LH LEVELS IN PLASMA OF 8 WALLABIES FROM d.25 RPY UNTIL AUTOPSY

APPENDIX 3. LH LEVELS IN PLASMA OF 2 WALLABIES SAMPLED THROUGHOUT 1975

APPENDIX 4: LH AND FSH CONCENTRATIONS IN PLASMA OF BILATERALLY OVARIECTOMIZED WALLABIES AND BILATERALLY OVARIECTOMIZED WALLABIES BEARING OVARIAN CORTEX OR INTERSTITIAL TISSUE GRAFTS UNDER THE POUCH SKIN FROM d.0 TO d.22-23 AFTER OPERATION (see Sections 6.2 and 6.4)

APPENDIX 5: LH AND FSH CONCENTRATIONS IN PLASMA OF:

1. ANIMALS BILATERALLY OVARIECTOMIZED DURING THE BREEDING SEASON,
2. Ovariectomized animals bearing ovarian cortex grafts under the pouch skin,

3. Ovariectomized animals bearing interstitial tissue grafts under the pouch skin, for 0-43 days after operation (see Section 6.4)

APPENDIX 6: PUBLICATIONS ARISING WHOLLY OR PARTLY FROM THE WORK PRESENTED IN THIS THESIS
# List of Figures

<table>
<thead>
<tr>
<th>Fig. No.</th>
<th>Title (abbreviated)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Separation of free iodine and iodinated hormone</td>
<td>54</td>
</tr>
<tr>
<td>3.2</td>
<td>Titration of Second Antibody</td>
<td>58</td>
</tr>
<tr>
<td>3.3</td>
<td>Cross-reactivities of eutherian LH's and FSH's with ovine LH antiserum.</td>
<td>59</td>
</tr>
<tr>
<td>3.4</td>
<td>Dose-response curves for 4 wallaby pituitary preparations</td>
<td>60</td>
</tr>
<tr>
<td>3.5</td>
<td>Dose-response curves for dilutions of wallaby plasmas</td>
<td>60</td>
</tr>
<tr>
<td>3.6</td>
<td>Effects of plasma on LH measurement</td>
<td>61</td>
</tr>
<tr>
<td>4.1</td>
<td>Cross-reactivities of eutherian FSH's and an LH with ovine FSH antiserum.</td>
<td>72</td>
</tr>
<tr>
<td>4.2</td>
<td>Dose-response curves for wallaby pituitary preparations</td>
<td>73</td>
</tr>
<tr>
<td>4.3</td>
<td>Dose-response curves for dilutions of wallaby plasmas</td>
<td>73</td>
</tr>
<tr>
<td>4.4</td>
<td>Effects of plasma on FSH measurement</td>
<td>74</td>
</tr>
<tr>
<td>5.1</td>
<td>a) LH levels in plasma of female wallabies during oestrous cycle and pregnancy.</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>b) Expanded time-scale for d.29 RPY for 4105.</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>Temporal relationships of mating, the LH peak and ovulation.</td>
<td>86</td>
</tr>
<tr>
<td>5.3</td>
<td>Changes in concentration of LH in plasma of oestrous female wallabies</td>
<td>88</td>
</tr>
<tr>
<td>5.4</td>
<td>LH levels in plasma of 2 females throughout 1975.</td>
<td>91</td>
</tr>
<tr>
<td>5.5</td>
<td>LH levels in plasma of female wallabies shot during December 20-29, 1974.</td>
<td>92</td>
</tr>
<tr>
<td>6.1</td>
<td>Changes in gonadotrophin levels in plasma after ovariectomy during the non-breeding season.</td>
<td>102</td>
</tr>
<tr>
<td>6.2</td>
<td>Diurnal variations in LH levels in ovariectomized and seasonally quiescent females.</td>
<td>106</td>
</tr>
<tr>
<td>6.3</td>
<td>Diurnal variations in FSH levels in ovariectomized and seasonally quiescent females.</td>
<td>106</td>
</tr>
</tbody>
</table>
6.4 Section of the ovary of a tammar wallaby.

6.5 Histological appearance of interstitial tissue and ovarian cortex grafts and uteri.

6.6 Histological appearance of ovarian cortex grafts and uteri.

6.7 (a)-(f) Changes in LH and FSH levels in bilaterally ovariectomized animals with or without ovarian cortex grafts, during the breeding season.

6.8 Changes in LH levels in ovariectomized and seasonally quiescent wallabies after steroid injections.

6.9 Changes in FSH levels in ovariectomized wallabies after steroid injection.

6.10 Changes in LH levels in wallabies after single injections of estradiol.

6.11 Changes in LH levels in an intact female after LH-RH.

6.12 Changes in LH levels in ovariectomized wallabies after LH-RH.

6.13 Changes in LH and FSH levels in female wallabies given 2 injections of LH-RH, in the breeding season.

6.14 Changes in LH levels in intact females after 1 or 2 injections of LH-RH, in the non-breeding season.

6.15 Changes in LH levels in ovariectomized wallabies after 1 or 2 injections of LH-RH, in the non-breeding season.

7.1 A model of the endocrine control of the oestrous cycle of the tammar wallaby.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title (some abbreviated)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Inter-assay variation in LH measurements.</td>
<td>63</td>
</tr>
<tr>
<td>3.2</td>
<td>LH levels in plasma of tammar wallabies.</td>
<td>64</td>
</tr>
<tr>
<td>4.1</td>
<td>FSH levels in tammar wallaby plasmas.</td>
<td>76</td>
</tr>
<tr>
<td>5.1</td>
<td>Time intervals between mating and LH peak and autopsy and autopsy results</td>
<td>89</td>
</tr>
<tr>
<td>6.1</td>
<td>Weights of urogenital systems and uterus in quiescent wallabies and bilaterally ovariectomized wallabies with or without cortex or interstitial tissue grafts, in October.</td>
<td>111</td>
</tr>
<tr>
<td>6.2</td>
<td>Autopsy results for bilaterally ovariectomized wallabies with ovarian cortex or interstitial tissue grafts.</td>
<td>115</td>
</tr>
<tr>
<td>6.3</td>
<td>Classification of ovarian follicles.</td>
<td>127</td>
</tr>
<tr>
<td>6.4</td>
<td>Distribution of follicles among the classes defined in Table 6.3 in ovaries of sham-operated or hypophysectomized wallabies.</td>
<td>129</td>
</tr>
<tr>
<td>6.5</td>
<td>Diameters and other characteristics of the three largest follicles in ovaries of hypophysectomized or sham-operated wallabies, 21 days after operation during the non-breeding season.</td>
<td>130-1</td>
</tr>
<tr>
<td>6.6</td>
<td>Mean maximum diameters of the largest follicle for hypophysectomized and sham-operated animals.</td>
<td>132</td>
</tr>
<tr>
<td>6.7</td>
<td>Results at autopsy of lutectomized wallabies given oil, progesterone, oestradiol-17β or both steroids for 7 days after operation.</td>
<td>139</td>
</tr>
<tr>
<td>6.8</td>
<td>Time intervals between LH-RH stimulus and associated peak, and peak LH levels in female wallabies.</td>
<td>148</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

CL  corpus luteum
CLX  luteectomy (removal of corpus luteum)
FSH  follicle-stimulating hormone
hCG  human chorionic gonadotrophin
HWP  plasma from a hypophysectomized wallaby
hypox  hypophysectomized
LH  luteinizing hormone
LH-RH  luteinizing hormone-releasing hormone
NWP  plasma from a normal wallaby
OVX  ovariectomized
PMSG  pregnant mare serum gonadotrophin
RIA  radioimmunoassay
RRA  radioreceptor assay
S.E.M.  standard error of the mean
ud  undetectable (i.e. below the sensitivity of the assay)
UGS  urogenital system

Conventional abbreviations are used for names of hormones and antibodies (eg. A-oLH = antiovine LH).

UNITS OF CONCENTRATION

Unless otherwise specified, LH and FSH levels are expressed as:

LH  ng NIH-LH-S19/ml plasma
FSH  ng NIH-FSH-S12/ml plasma

In the text, these are abbreviated to ng/ml.
CHAPTER 1

INTRODUCTION

1.1 THE DUAL HORMONE CONCEPT

1.2 HYPOTHALAMIC CONTROL OF GONADOTROPIN SECRETION

1.3 OVARIAN CONTROL OF GONADOTROPIN SECRETION

1.4 ENDOCRINE CONTROL OF THE OESTROUS CYCLE: EUTHERIAN MODELS

1.4.1 Follicular maturation

1.4.2 Hormonal control of ovulation

1.4.3 Influence of the corpus luteum upon oestrous cycle length

1.5 PATTERNS OF REPRODUCTION IN THE MACROPODIDAE

1.6 REPRODUCTIVE BIOLOGY OF THE TAMMAR WALLABY

1.7 CONTROL OF THE OVARIAN CYCLE IN MACROPOD MARSUPIALS

1.8 THE SCOPE OF THIS THESIS
1.1 THE DUAL HORMONE CONCEPT

In all vertebrates, reproduction is an essentially cyclic phenomenon. In middle and high latitudes, many animals display an annual breeding pattern, geared to produce offspring at the most favourable time of year. The annual cycle is thus governed by environmental influences. In mammals, this is superimposed upon a cycle of ovarian activity which has evolved to ensure that the prenatal requirements of the young are met. During the oestrous cycle, then, the sequence of hormonal events is not concerned only with follicular maturation and ovulation. Through the action of the corpus luteum (CL), the uterus is prepared to receive the embryo, and, if pregnancy ensues, synchrony between the embryo and the uterine environment is maintained for the duration of pregnancy.

The concept that the rhythmicity of ovarian activity in mammals is not solely an intrinsic property of the ovaries is of fairly recent origin. Only 50 years ago, Smith and Engle (1929) published an account of the pituitary in the development and regulation of the reproductive system and put forward the hypothesis that "the periodic liberation of gonadal-simulating hormone of the pituitary may explain the periodic ripening of groups of follicles", and Fee and Parkes (1929) demonstrated that some pituitary factor is also necessary for ovulation to occur. Thus there grew the dual hormone concept of the control of the mammalian oestrous cycle, and, concurrently, of male reproductive processes.

It has now been established that in eutherian mammals the pituitary produces two gonadotrophic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are chemically and functionally distinct. (Prolactin is also luteotrophic in some species, but will not be discussed here). The LH and FSH molecules have some important structural features in common both with each other and with thyroid-
stimulating hormone (TSH) and human chorionic gonadotrophin (hCG). All four are glycoproteins, consisting of a carbohydrate moiety and two dissimilar polypeptide chains, the α- and β-subunits. Alterations to the carbohydrate portion, which contains a large proportion of sialic acid, may result in changes in immunologic and biologic activity, notably causing considerable decreases in the half-life of the hormone in the circulation (Vaitukaitis et al., 1976). However, the hormonal activity of the molecule is a function of the polypeptide chains.

The chemistry, including amino acid sequencing, of the polypeptide portions of the glycoprotein hormones from various species has been reviewed recently by Papkoff et al. (1973), Sairam and Papkoff (1974) and Vaitukaitis et al. (1976), among others, and will not be discussed here in detail. The α- and β-subunits are noncovalently bonded, and may be dissociated under appropriate conditions. From structural and immunologic studies, it has emerged that the hormonal specificity of the complete molecule is a property of the β-subunit. While in each species the α-subunits of the glycoprotein hormones are essentially identical, their β-subunits differ from each other but show considerable homologies with the β-subunit of the corresponding hormone from another species. Thus the within-species cross-reactivity shown by the glycoprotein hormones resides in the species-specific α-subunit, while the hormone-specific β-chain confers between-species cross-reactivity (Vaitukaitis et al., 1976).

However, to show that LH and FSH not only are different molecules, but have separate functions, it was necessary to show that the hormones presented different secretion profiles during the oestrous cycle. Although bioassays were able to provide support for the two-hormone theory of oestrous cycle control by the demonstration of changing ratios of LH and FSH in plasma and pituitary tissue, the relative insensitivity
of such assays precluded any detailed analyses of the patterns of hormone secretion during the cycle. Another problem was the lack of assay specificity due to the overlap of properties of LH and FSH, attributable either to intrinsic activity of one hormone in the bioassay for the other, or to cross-contamination of the standard hormone preparations used. Moreover, as Creaser and Gorbman (1939) pointed out, "the effectiveness of a gonadotrophic hormone in a foreign species tends to vary directly with the phylogenetic proximity of the donor and recipient species". Such problems of hormonal specificity and species specificity continue to plague hormone assayists using the more refined techniques now available.

The concept of the radioimmunoassay (RIA), first described by Yalow and Berson (1959), developed from their work on the binding of $^{131}$I-insulin to antibodies present in the blood of insulin-treated diabetics, which showed that antibodies to a protein hormone could indeed be produced. With Hunter and Greenwood's 1962 publication of a simple method for preparing $^{131}$I-labelled proteins of high specific activities, RIA soon became the most widely used method of measuring polypeptide and protein hormones in biological fluids, while competitive protein-binding assays, based on binding globulins naturally present in plasma, were developed concurrently by Ekins, Murphy and others for estimation of thyroxine and steroid hormones. Both types of assay depend upon the same principle: radioactively labelled hormone (tracer) bound to antibody or binding globulin is displaced competitively by unlabelled hormone in standard or unknown, the amount of tracer displaced being a measure of the unlabelled hormone present. The sensitivity and specificity of such assays are vastly superior to those of any standard bioassay, although a valid criticism of the radioimmunoassay is that it measures immunological activity which is not necessarily correlated with biological activity.
Development of radioimmunoassays for the gonadotrophins of various species has been both helped and hindered by the strong structural similarities of the four glycoprotein hormones, LH, FSH, TSH and hCG. Problems of assay specificity arise when one hormone shares enough antigenic determinants with another to cross-react significantly with an antiserum to it. Thus in early RIA's for human FSH, when the best FSH preparations still contained relatively large amounts of LH, excess hCG was added to each assay tube to neutralize antibodies to LH and to inhibit competitively binding of iodinated LH to anti-FSH antibodies (Midgley, 1967; Faiman and Ryan, 1967). Conversely, the first RIAs for an LH depended upon the strong cross-reactivity of human LH and hCG (Midgley, 1966; Odell, Ross and Rayford, 1966). Antisera raised to hCG, which, unlike human LH, could be obtained in large quantities, were used with iodinated LH or hCG to measure LH (and hCG) in human serum.

In a homologous RIA, the hormone preparation against which the antiserum is raised and the hormone used as tracer are of the same species origin. An example was the first RIA for rat LH (Monroe, Parlow and Midgley, 1968), which used crude rat LH for immunization and purified rat LH for iodination. However, the between-species cross-reactivity of the glycoprotein hormones has been exploited frequently in the development of hormone-specific heterologous RIA's. Although Midgley (1971) gives a narrower definition of a heterologous assay (see below), the term is also applied to assays in which antiserum to hormone of one species, often with preparations of the same hormone as both tracer and standard, is used to measure hormone of a different species. Suitable antisera contain a high proportion of antibodies directed against the hormone-specific β-chain, or may be raised to β-chain preparations if available. Heterologous RIA can therefore be a useful method for reducing interference in the assay
by other glycoprotein hormones. It also provides a means of estimating changes in hormone levels in a species for which highly purified hormone preparations are not yet available in sufficient quantities to allow development of homologous assays.

Development of radioimmunoassays for FSH in particular has, until fairly recently, been hampered by the lack of suitable purified FSH preparations. Homologous RIA's based on several different antisera to ovine FSH were found to be subject to marked interference by ovine albumin (Salamonsen et al., 1973), while problems of cross-reactivity with LH, TSH and hCG were widely encountered (Midgley et al., 1971; L'Hermite et al., 1972). Midgley (1971) suggests that the FSH-specific region of the FSH molecule is poorly antigenic compared with other regions which may share antigenic determinants with corresponding portions of other glycoprotein hormones, and are therefore likely to cross-react in any homologous assay.

These problems of cross-reactivity may, however, be obviated by using a heterologous assay as defined by Midgley (1971), i.e. antiserum raised to hormone of one species is used in combination with labelled hormone from another species. Such an assay is dependent upon inter-species cross-reactivity, which is likely to reside in the hormone-specific portion of the molecule which would be conserved during molecular evolution. Although, overall, a heterologous tracer would probably bind less well to the antiserum than the homologous tracer, it would select for those antibodies directed against the hormone-specific part of the molecule. The corresponding hormone from a third species (i.e. in the samples to be assayed) is therefore likely to bind as well as, or better, than the tracer and be able to displace it from antibody. Such an assay could be used to quantitate FSH in species for which highly purified FSH preparations were unobtainable.
The methodology of the radioreceptor assay (RRA) is similar to that of the RIA, but the binding proteins used are the hormone-specific receptors on the plasma membranes of target cells. The immediate appeal of such a system is that the assay specificity is directed towards the biologically active portion of the hormone molecule rather than the immunogenic sites, as in RIA's. Although membrane receptors for gonadotrophins have been demonstrated in both testis and ovary, testis preparations are most commonly used in RRA's, Leydig cells for measurement of LH and hCG (reviewed by Catt and Dufau, 1975) and tubular fractions for FSH (Reichert and Bhalla, 1974). However there are problems, including a lack of sensitivity, with the estimation of hormone concentration in plasma or serum samples, and for this reason RIA continues to be the preferred method for most routine hormone assays.

RRA's, however, depend only upon the binding of hormone to its receptor, not upon activation of the cellular response to the hormone. A further type of assay has been developed for LH and hCG which may be described as an *in vitro* bioassay. The LH-like activity of a sample is estimated by its ability to induce steroidogenesis in target tissues, i.e. testosterone production by dispersed Leydig cells (Lichtenberg and Pahnke, 1976) or progesterone production by dissociated ovarian cells (Kunzig, Potthoff and Leidenberger, 1976). The amount of steroid produced after a given incubation period is measured by RIA, and a standard curve is constructed on the basis of the amount produced in the presence of a certain concentration of LH or hCG standard. As with RRA's, serum interferes with these bioassays, causing significant deviation of dose-response curves from parallelism with those of serum-free standards, and hence the assays cannot yet be applied to the measurement of LH bioactivity in serum or plasma.
However, comparisons of the potencies of hormone preparations in RIA, RRA and in vitro bioassay have proved most useful in studies of the biochemical and physiological relationships between the gonadotrophins of different vertebrate classes. Testing of hormones in both homologous and heterologous assays has led to a greater understanding of the evolution of gonadotrophin structure and function. Licht and co-workers have made extensive studies of the gonadotrophins of non-mammalian vertebrates and their results, reviewed, with those of other researchers, in Licht et al., (1977) are of considerable significance to the understanding of the eutherian situation.

Although it was originally thought that non-mammalian vertebrates might produce only a single pituitary gonadotrophin, two chemically distinct gonadotrophin molecules have been isolated from pituitaries of representatives of every class of tetrapods, with the situation in fishes being unresolved at present. A second gonadotrophin does appear to be lacking in certain groups (eg. squamate reptiles), but Licht et al. (1977) consider this to represent a secondary evolutionary loss and the possession of two gonadotrophins to be a primitive characteristic of tetrapod vertebrates.

There is considerable biochemical and immunologic evidence that the gonadotrophins of the non-mammalian tetrapods are structurally homologous to the LH and FSH of eutherian mammals, although numerous instances of phylogenetic specificity indicate evolutionary changes in the biologically active portions of the molecules (non-mammalian hormones are relatively inactive on mammalian gonads). Duality of hormone action, however, is not always apparent. Phylogenetically speaking, the induction of ovulation is a much more general and ancient role of LH than its role in the formation of corpora lutea (Witschi, 1955), and in both amphibia and eutherian mammals, LH is specifically required for both ovulation and gonadal steroidogenesis. There is, however, little evidence for a unique action of FSH
in amphibia: growth of the anuran ovary, recruitment of new follicles and vitellogenesis may be stimulated by either gonadotrophin.

The reptiles differ from these groups in that neither LH nor FSH appears to have specific actions upon the gonads. For example, both hormones, whether of reptilian or non-reptilian origin, have a direct effect upon the Leydig cells (Licht, 1977). This lack of specificity is apparently due to evolution of the testicular receptors, rather than the structure of the gonadotrophins: FSH receptors occur on tissues which are usually specific targets for LH, and it is possible that common receptors exist for the two hormones (Licht, 1977; Licht et al., 1977).

Although the birds might be expected to follow the reptilian pattern of gonadotrophin specificity, their responses to mammalian hormones indicate that gonadal steroidogenesis specifically requires LH. However, studies with avian gonadotrophins have shown conflicting results, and the situation must be regarded as unresolved (Licht et al., 1977).

It appears, therefore, that a duality of gonadotrophin function with a specificity of LH action is a primitive characteristic of tetrapod vertebrates, the reptiles having diverged from this original condition. On that evolutionary basis, then, one would expect the marsupials to conform to the eutherian pattern.

The first study of gonadotrophins in any marsupial was that of Purves and Sirett (1959). Seven cell types have been identified histochemically in the pars distalis (anterior pituitary) of the scrub wallaby, Macropus rufogriseus (Ortman and Griesbach, 1958), and Purves and Sirett (1959) attempted to correlate distribution of these cell types within the pars distalis with distribution of hormonal activities assessed by bioassays of rostral and caudal parts of the gland, and tentatively identified certain basophilic cells distributed throughout the gland as gonadotrophin-secretory cells.
Hearn (1972) isolated a gonadotrophin fraction from tammar wallaby (*Macropus eugenii*) pituitaries and developed a homologous radioimmunoassay for wallaby gonadotrophin using an antiserum raised to this gonadotrophin fraction. He was thus able to measure "total gonadotrophin" in wallaby plasma, although he believed it was mainly LH that was measured (Hearn, 1974). Later, two gonadotrophin fractions were prepared from red kangaroo pituitaries (Farmer and Papkoff, 1974). These were shown to have LH-like and FSH-like biological activities and to be chemically similar to eutherian LH's and FSH's.

When I began my study in 1975, no further information was available on marsupial pituitary gonadotrophins. However, during the course of my project, highly purified LH and FSH preparations have been isolated from pituitaries of tammar wallabies (Gallo *et al.*, in press) and the two species of grey kangaroo (Papkoff *et al.*, to be published). However, since studies on these preparations have formed part of, or been intimately connected with, my own work, they will not be discussed here, but later in the thesis. It is now clear, however, that marsupials possess two gonadotrophins which are immunologically and biologically similar to the corresponding hormones in eutherians.

This section has concentrated upon the structural aspects of gonadotrophin duality, although some reference has been made to the general functions of the two hormones. The particular roles of LH and FSH in the control of the mammalian oestrous cycle will now be brought into focus, a detailed discussion of eutherian studies prefacing a survey of the relatively little data available from marsupials.

1.2 HYPOTHALAMIC CONTROL OF GONADOTROPHIN SECRETION

In order to understand how changes in gonadotrophin levels may influence ovarian events, it is first necessary to discuss how the
secretion of gonadotrophins is itself controlled. The pituitary cannot be regarded as an isolated unit: it is dependent upon hypothalamic input and its output is modified by target cell hormones acting as feedback controls at either the hypothalamic or the pituitary level.

Investigations on the role of the central nervous system in the control of pituitary function began in the 1930's, and were given great impetus by the discovery of the hypophysial portal system providing the anatomical link between hypothalamus and pituitary. Green and Harris (1947) showed conclusively that the blood flowed from the hypothalamus to the pituitary gland and postulated a neurohumoural mechanism for control of pituitary hormone secretion: "neurohumours" synthesised by nerve cell bodies in the hypothalamus are transported down their axons to the pituitary stalk where they are liberated into capillaries of the portal system, and thus transferred into the pituitary vascular bed. Although most of the "neurohumours" so far discovered stimulate release of a particular pituitary hormone and are known as releasing hormones or releasing factors, a few, such as prolactin-inhibiting factor, have the opposite effect.

At first it was believed that LH and FSH release were each controlled by a different hypothalamic hormone (McCann and Ramirez, 1964). However, when luteinizing hormone-releasing hormone (LH-RH) was finally purified from porcine (Schally et al., 1971a) and ovine hypothalami (Burgus et al., 1972), it was found to be a decapeptide, the structure of which does not apparently vary between species. A decapeptide has been synthesised (Matuso et al., 1971) which stimulates release of both LH and FSH, indicating that FSH release is an intrinsic property of the molecule, and not attributable to contamination of hypothalamic extracts.

Nevertheless, the question of the existence of a separate FSH-releasing hormone has not yet been completely resolved. Currie et al. (1973) obtained certain fractions from porcine hypothalami which
stimulated the release in vitro of much more FSH than could be attributed to the action of decapptide LH-RH. They claimed that this data revealed an unknown FSH-RH by differentiation of its activity from that of LH-RH. This group has also reported evidence for a hypothalamic inhibitory hormone that decreases both the basal release of LH and the response to LH-RH (Johansson et al., 1975).

LH-RH stimulation may have a priming effect on the pituitary such that a second stimulus will induce greater LH release than the first. This priming effect appears to be a direct effect of LH-RH upon the gonadotroph cells, and to be dependent upon protein and RNA synthesis (Pickering and Fink, 1976). In the sheep, this sensitization lasts for about 3 hours after the first LH-RH injection and is followed by a period of desensitization for at least 24 hours (Crighton and Foster, 1977). The guinea-pig appears to differ from the rat in that the ovaries are required for the self-priming effect of LH-RH (ter Haar, 1978). Presumably the potential response to the second LH-RH injection is mediated by gonadal steroid secreted in response to LH released by the first LH-RH stimulus.

If it is conceded that a single releasing hormone controls the secretion of LH and FSH, then some other mechanism must be invoked to explain the differences in plasma profiles of the two hormones. It is generally found, as in the ram (Lincoln and Peet, 1977; Lincoln, 1978) and the castrate male rat (Gay and Sheth, 1972) that LH secretion appears to be pulsatile, while FSH levels remain fairly constant.

Schally et al., (1971b; 1972) reviewed the available evidence and concluded that while there was only one gonadotrophin-releasing hormone, preferential release of LH or FSH may be mediated by the action of steroid hormones at the pituitary or hypothalamic level. This view is supported by more recent work (e.g. Tang and Spies, 1975; Apfelbaum
Nevertheless, the differential release of LH and FSH may also be due in part to intrinsic properties of the gonadotrophin-secreting cells. While there is a considerable body of evidence that pituitary LH stores are divided into two pools, a readily-releasable pool, which is secreted immediately after acute LH-RH stimulation, and a second pool, the release of which requires continued stimulation (Yen, 1977), Bremner and Paulsen (1974) found no evidence for a similar readily-releasable pool of FSH in human male pituitaries, and it has been suggested that a prolonged exposure of the pituitary to LH-RH is necessary for maximal FSH release (Arimura, Debeljuk and Schally, 1972).

The relationship between plasma levels of LH and FSH in rams and the implications of this with respect to the control of gonadotrophin secretion have been studied by Lincoln and co-workers, who have found that the differences in the plasma profiles of the two gonadotrophins are attributable, in part, to differences in the mechanisms of their storage and secretion. After a single injection of LH-RH, there is an immediate, substantial, but short-lived rise in plasma LH levels, but the FSH response is relatively small and more sustained, suggesting that only LH is available in a readily-releasable pool. Similar responses to endogenous LH-RH stimuli, compounded by the much slower clearance of FSH from the circulation, would explain the observed plasma profiles (Lincoln and Peet, 1977; Lincoln, 1978).

There are marked fluctuations in LH and FSH levels in ovariectomized ferrets (Donovan and ter Haar, 1977) and rhythmic oscillations in plasma LH levels have been reported in ovariectomized rhesus monkeys (Dierschke et al., 1970), sheep (Butler et al., 1972) and rats (Gay and Sheth, 1972). Since it is highly unlikely that there are rhythmic changes in clearance
rates, this presumably reflects variations in the rate of secretion by the pituitary. The cyclic changes in LH secretion suggest a short-loop feedback control system whereby the hormone, acting at the pituitary or hypothalamic level, controls its own secretion, further releases being inhibited until the circulating levels drop sufficiently (Butler et al., 1972). However, Dierschke et al. (1970) reported that large infusions of LH did not appear to influence endogenous LH secretion in ovariectomized rhesus monkeys.

In ovariectomized rats, the rhythmic LH secretion is attenuated by treatment with phenobarbitone, which acts on the central nervous system. However, in treated animals, a series of acute LH-RH challenges will still induce pulsatile release, indicating that pulsatile secretion is not an intrinsic property of the pituitary (Schuiling and Gnodde, 1976). The authors concluded that in the long-term ovariectomized rat the hypothalamus secretes LH-RH in pulses at about 20 minute intervals.

1.3 OVARIAN CONTROL OF GONADOTROPHIN SECRETION

In all species studied, plasma LH and FSH levels rise after ovariectomy, indicating that the ovary exerts a negative feedback control on tonic gonadotrophin secretion. Although other steroid hormones may be involved, the main effectors of this regulation in females are usually oestradiol and progesterone.

The effect of oestradiol upon gonadotrophin secretion is critically dependent upon the dose and duration of the stimulus. For example, short-term infusion of oestradiol-17β into ovariectomized rhesus monkeys (Yamaji et al., 1972) or implants of oestradiol into ovariectomized ferrets (Donovan and ter Haar, 1977) decreased LH concentrations in plasma to pre-castrate levels. A similar negative feedback effect of sustained oestradiol stimulation has been observed in several other
species, and most of the evidence from \textit{in vivo} experiments suggests that the site of action is the hypothalamus (Schally, Kastin and Arimura, 1972). Nevertheless, it does appear that oestradiol may also act on the pituitary itself, depressing its sensitivity to LH-RH both \textit{in vivo} (Coppins and Malven, 1976) and \textit{in vitro} (Schally \textit{et al}., 1972; Tang and Spies, 1975).

Paradoxically, though, oestradiol may also have a positive feedback, or stimulatory, effect upon gonadotrophin secretion. Relatively small doses of oestradiol given to intact sheep cause a rise in plasma LH in vivo hours later (Goding \textit{et al}., 1969). Oestradiol has also been shown to elicit LH release in several other species including the rat (Callantine, Humphrey and Nessel, 1966), guinea-pig (Donovan and Lockhart, 1972) and marmoset monkey (Hodges and Hearn, 1978), the general pattern being an initial depression of LH levels followed by a massive discharge some 12-24 hours later. Some reports indicate that oestradiol also has a positive feedback effect upon FSH secretion (Knobil, 1974), but generally FSH secretion is more sensitive to the negative feedback effect of the steroid and may be depressed by dose regimes which stimulate or do not affect LH release (Tsai and Yen, 1971; Cargille \textit{et al}., 1973).

There is evidence that rising oestradiol levels have a direct effect upon the pituitary, increasing its sensitivity to LH-RH (Mahesh \textit{et al}., 1975). Yen (1977), discussing the regulation of the hypothalamic-pituitary-ovarian axis in women, considered that the primary effect of oestradiol is on the pituitary gonadotrophs, controlling the relative sizes of the "readily-releasable" pool of LH and the second pool which requires continued stimulus for release. Rising oestradiol levels prior to ovulation cause a shift of LH into the readily-releasable pool and activate a self-priming effect of LH-RH to potentiate the mid-cycle LH surge.
It appears, therefore, that oestradiol controls tonic gonadotrophin secretion by its negative feedback effect and also initiates the preovulatory surge by its positive feedback effect. The dual roles of oestradiol are, in Yen's model, seen as a continuum, rather than as separate functions, the pituitary response depending on the time-course of the oestrogenic stimulation. However, Schwartz (1969) and Karsch et al. (1973) considered that in the rat and the rhesus monkey respectively the positive and negative feedback control systems act conjointly but involve functionally independent mechanisms, so cyclic LH release is viewed as being superimposed upon tonic LH release.

Rothchild (1965) suggested that although progesterone could inhibit the ovulatory surge of LH, it had no effect upon tonic LH secretion. Progesterone has been found to enhance the negative feedback of oestradiol upon LH secretion but to inhibit its positive feedback effect in rhesus monkeys (Knobil, 1974) and sheep (Scaramuzzi et al., 1971). Also, progesterone alone is unable to suppress plasma gonadotrophin levels in ovariectomized rhesus monkeys (Yamaji et al., 1972), ferrets (Donovan and ter Haar, 1977) or sheep (Goding et al., 1970; Scaramuzzi et al., 1971).

However, Karsch, Legan, Hauger and Foster (1977) considered that the inverse relationship between circulating LH and progesterone levels during the oestrous cycle of the ewe was circumstantial evidence for a negative feedback inhibition of LH secretion by the steroid in this species. Their own work showed that progesterone can indeed be a potent inhibitor of tonic LH secretion in both intact and ovariectomized ewes, but that full expression of this effect requires the presence of another ovarian hormone, possibly oestradiol. These revelations led them to propose a new model for the control of the oestrous cycle of the ewe (Hauger, Karsch and Foster, 1977), which will be discussed in Section 1.4.3.
1.4 ENDOCRINE CONTROL OF THE OESTROUS CYCLE: EUTHERIAN MODELS

1.4.1 Follicular maturation

Follicular growth to the preantral stage with 4 layers of granulosa cells is independent of gonadotrophin support, but the effects of hypophysectomy show that further development of the follicles and the initiation of steroidogenesis do require gonadotrophic stimulation (Young, 1961; Peters, Byskov and Faher, 1973). The exact nature of this requirement is unknown and the picture is complicated by differences in oestrous cycle lengths in different species. In women, for example, it seems that follicular growth begins at the end of each menstrual cycle when concentrations of LH and FSH in plasma rise during luteolysis (Ross et al., 1970). In the mare, one of the few species in which there have been observed marked changes in gonadotrophin levels other than those at oestrus, a surge of FSH in mid-dioestrus has been implicated as necessary for the maturation of follicles destined to ovulate 10-13 days later (Evans and Irvine, 1976). However, in animals such as the mouse, in which complete follicular maturation requires 14 days and the oestrous cycle is only 4 days long, the FSH and LH secreted during any one cycle must be responsible for initiation of growth of follicles destined to ovulate in a subsequent cycle (Schwartz, 1974).

In sheep, it has been reported that there are at least 2 waves of follicular growth during the cycle (Smeaton and Robertson, 1971; Brand and de Jong, 1973), although Turnbull, Braden and Mattner (1977) believe there is continual, asynchronous growth and regression of follicles during the luteal phase. However, the majority of these follicles will atrophy without ovulating. Indeed, at any one time, only about 30% of the follicles of any size class are not atretic (Turnbull, Braden and Mattner, 1977). In this and other mammalian species, typically only a
small number of follicles, or just one, escapes atresia and eventually ovulates: this implies some process of selection.

Cautery of the largest follicle on the ovaries of rhesus monkeys on cycle days 8-12 abolished the incipient preovulatory rise in plasma oestradiol, prevented the mid-cycle gonadotrophin surge and delayed ovulation for about 12 days (Goodman, Nixon, Johnson and Hodgen, 1977). These results illustrate several points which have emerged from numerous studies of other species in a variety of experimental conditions. First, no other follicle of that cohort is competent to ovulate: a new crop of follicles must mature before ovulation can occur. Second, the major source of oestradiol just prior to ovulation is the preovulatory follicle and, third, this rise in oestradiol triggers the surge of gonadotrophin necessary to initiate ovulation. These points will be discussed briefly.

The mechanism whereby the follicle(s) destined to ovulate is initially selected is not understood. However it appears that oestrogens and androgens produced by the theca interna of this follicle (Seamark, Moor and McIntosh, 1974) may promote both its own growth and the atresia of other follicles. The high intrafollicular oestrogen concentration sensitizes the follicle cells to LH, possibly by increasing the number of receptors (Channing and Kammerman, 1974), and acts with FSH to enhance mitotic activity and steroid biosynthesis in granulosa cells (McNatty and Sawers, 1975). Concurrently, the action of oestrogen at the hypothalamic level inhibits development of other follicles by a negative feedback effect on gonadotrophin levels (Midgley and Jaffe, 1968). Androgens produced by the preovulatory follicle have a local, intraovarian effect, also promoting the atresia of other follicles (Louvet et al., 1975) and there is also some evidence for an inhibin-like substance, produced by granulosa cells and acting at the hypothalamic level to inhibit FSH, but not LH, secretion (de Jong and Sharpe, 1976)
Rising oestradiol levels during the preovulatory period have been recorded in many species including the rat (Butcher, Collins and Fugo, 1974), dog (Concannon, Hansel and Visek, 1975), pig (Henricks, Guthrie and Handlin, 1972) and cow (Dobson, 1978). It appears that in spontaneous ovulators a critical duration of exposure to a critical level of oestradiol switches the negative feedback control system regulating gonadotrophin secretion into the positive mode, and thus triggers the massive ovulatory release of gonadotrophins.

In polyestrous rodents (rats, mice and hamsters), a neural signal dependent upon the time of day is also necessary for the induction of ovulation and may override the effect of an appropriate steroid milieu: if ovulation is blocked by drugs during the critical period, it will be delayed for 24 hours (Hoffman, 1969). It is proposed that in the rat ovarian oestradiol determines the day of the LH surge but the central nervous system determines its hour of onset (Bogdanove, 1972). No such neural timing device is evident in primates. In this group, it appears that the pattern of gonadotrophin secretion during the menstrual cycle is governed largely by changes in ovarian oestradiol output, and thus the ovary itself is the "Zeitgeber" or "pelvic clock", determining the timing of the gonadotrophin surge and, hence, ovulation (Karsch et al., 1973; Knobil, 1974).

In reflex ovulators, such as the rabbit, cat, vole and ferret, the presence of mature follicles is ensured by a continuous process of maturation and atresia (Hill and White, 1934). The primary stimulus responsible for LH release and hence, ovulation, is a neural one, originating in the genital area, usually as a result of copulation, and reaching the hypothalamus by an as yet unknown pathway. It was the observation that the rabbit ovulates within 12 hours after mating, and only after mating,
that led early workers to the concept that an acute release of LH, rather than the gradual attainment of some critical level, is the initiator of ovulation (Schwartz and Hoffman, 1972).

1.4.2 Hormonal control of ovulation

It is generally accepted that LH is the primary ovulating hormone. Numerous studies have shown that a depletion of pituitary LH and a sharp rise in the concentration of LH in plasma occur at a fairly precisely defined time before ovulation, 24-28 hours in the sheep (Cumming et al., 1973), 24-28 hours in the dog (Phemister, Holst, Spano and Hopwood, 1973), and about 37 hours in the rhesus monkey (Weick et al., 1973). The maximum concentration of LH attained may be two orders of magnitude greater than basal levels and the duration of the surge varies between species, being, for example, about 12 hours in the sheep (Goding et al., 1969) but 2-3 days in primates (Karsch et al., 1973).

Although most species studied show an acute ovulatory release of LH, an interesting exception is the mare, in which LH rises before FSH, early in the long period of oestrus, implying that LH may be important in the prolonged maturation of the preovulatory follicle (Evans and Irvine, 1976). LH levels remain high for several days, and Noden, Oxender and Hafs (1975) have suggested that this prolonged stimulation is necessary for the migration of follicles to the ovulatory fossa, another peculiarity of this species. Again unlike other species, maximum LH levels are not attained until 1-2 days after ovulation, which suggests that LH is luteotrophic in this species (Evans and Irvine, 1976).

In those species in which LH and FSH have been measured, an FSH surge has been observed approximately coincident with the ovulatory LH surge. This raises the question as to whether FSH is also required for ovulation or whether the function of this peak is the initiation of
follicular growth during the forthcoming cycle. LH and FSH appear to
act synergistically to promote ovulation in the rat (Labhsetwar, 1970) and
rabbit (Nalbandov, Kao and Jones, 1972), but while there is no doubt that
LH alone is an excellent ovulating hormone in experimental situations,
there are conflicting reports of the ovulating ability of FSH. For example,
Cobbs, Schwartz and Ely (1972) decided that in the rat ovulation does not
occur in the absence of LH, but Ying and Greep (1971) found that FSH could
cause ovulation in this species. In hamsters, it has been reported that
ovulation can be induced by either LH or FSH, although the initiation of
progesterone synthesis does require LH (Greenwald, 1974).

Many of the discrepancies among such studies may be attributed
to the difficulties of obtaining highly purified LH and FSH preparations.
Another approach to the problem has been the use of antiserum to neutralize
either hormone. Again, results have not been uniform, but Schwartz (1974),
reviewing the subject, concluded that on present evidence either an LH or
an FSH surge can induce ovulation, although only LH induces progesterone
secretion. There may, of course, be variation between species in the
hormonal requirements for ovulation: LH seems to be an absolute necessity
but FSH and prolactin may have permissive effects. It may, therefore, be
more correct to speak of the "ovulating hormone complex", rather than
consider LH alone to be the "ovulating hormone".

In the sheep, there is evidence that the ovulatory releases of
LH and FSH are independently controlled. Increased oestrogen secretion is
the trigger for the dual gonadotrophin surge, but only LH release is
blocked by injection of pentobarbitone, although during anoestrus both the
LH and FSH responses to oestrogen treatment are blocked by the drug. It
has been suggested that androstendione may enhance the FSH-releasing action
of oestrogen at normal oestrus (Daane and Parlow, 1971).
While the FSH surge coincident with the preovulatory LH surge may be concerned with the induction of ovulation, a second FSH peak has been observed about 24 hours after the first in both sheep (Salamonsen et al., 1975) and cows (Dobson, 1978). This second peak, occurring at about the same time as ovulation, may also be concerned with the ovulatory process or, more likely, with the initiation of follicular growth associated with the rise in oestradiol secretion 3-4 days after oestrus (Pant, Hopkinson and Fitzpatrick, 1977). It is not blocked by pentobarbitone (Daane and Parlow, 1971) and the stimulus causing its release is unknown. However Schwartz and Hoffman (1972) have suggested that the fall in oestrogen secretion, which occurs before ovulation is complete, may have a permissive effect upon FSH secretion necessary for the initiation of new follicular growth.

Oestrogen secretion by the preovulatory follicle is terminated by peak levels of LH during the ovulatory surge (Moor, 1974) and this probably inhibits further LH release. The LH surge is not terminated simply by exhaustion of the pituitary supply, as the pituitary is still able to respond to an LH-RH stimulus (Weick et al., 1973). However, Foster and Crighton (1974) have reported a greatly decreased sensitivity to LH-RH during the 24 hours after the LH peak in sheep, and it is likely that increasing pituitary refractoriness to LH-RH is important in ending the LH surge.

While the LH surge terminates oestrogen secretion by the theca interna of the preovulatory follicle, it also initiates luteinization and secretion of progestins by the membrana granulosa (Moor, 1974), and the corpus luteum develops to exert a dominant influence over ovarian events during the luteal phase. Further discussions here will be confined to those species in which spontaneous ovulation is followed by an active luteal phase, as these can more appropriately be compared with the typical
Influence of the corpus luteum upon oestrous cycle length

It may be argued that the primary role of the mammalian corpus luteum is the preparation for and support of pregnancy and much attention has indeed been given to this subject. Nevertheless, it was recognized very early that ovulation and oestrus do not generally occur in the presence of the CL, and Prenant (1898) was one of the first to postulate that the CL inhibits follicle growth by some 'chemical pathway'.

The complete luteal phase encompasses the growth, secretory activity and regression of the CL. The growth phase involves an increase in the number and size of granulosa and, in certain species, thecal cells of the ovulated follicle, with a concomitant rise in concentration of progesterone in plasma. Maximum size is reached by about 7 days after ovulation and by day 9-15 morphological regression has begun and progesterone levels are declining (Rothchild, 1965). Luteolysis signals the beginning of the new follicular phase leading to ovulation.

Progesterone treatment will inhibit ovulation (unless given within 24 hours of an expected ovulation, in which case it is stimulatory). Rothchild (1965), in an extensive review of the subject, found that in the rat, rabbit, guinea-pig, cow, pig, sheep and goat ovulation-inhibiting doses of progesterone prevented only the terminal stages of follicular maturation. This finding was consistent with numerous reports that follicular growth continues during the luteal phase, and Rothchild concluded that the inhibition of ovulation by progesterone secreted by the CL is due primarily to the prevention of the preovulatory growth phase.

Inhibition of follicular maturation during the luteal phase can be demonstrated by removal of the active CL: this causes premature ovulation. The time interval to this ovulation, however, varies markedly between
species, being correlated with the length of the follicular phase of the oestrous cycle. Thus animals such as the sheep re-ovulate 2-3 days after luteectomy (Inskeep et al., 1963), but in primates such as the rhesus monkey the time interval is about 14 days (Goodman et al., 1977). This means either that follicular growth is extremely rapid in the sheep compared with the primate or, more likely, that the follicular phase of the sheep oestrous cycle represents only the final stages of follicular maturation: in the mouse, an elegant study using tritiated thymidine to label growing follicles has shown that 10-17 days are required for a small follicle with 4 layers of granulosa cells to reach preovulatory maturity (Pedersen, 1970).

Baird, Baker, McNatty and Neal (1975) have proposed an attractive hypothesis relating these variations in length of the follicular phase to the origin of the oestradiol secreted during the luteal phase. Their specific examples are sheep and women, although where possible confluent evidence from other higher primates is presented. The basis of their argument is that progesterone produced by the CL of both primates and sheep acts only to inhibit the positive feedback effect of oestradiol, preventing ovulatory discharges of LH during the luteal phase: it has little effect upon tonic LH or FSH secretion (Knobil, 1974; Scaramuzzi et al., 1971).

However, the human corpus luteum is unusual in that it secretes oestrogens and androgens as well as progestagens (Savard, Marsh and Rice, 1965). Thus the rise in concentration of oestradiol in peripheral plasma during the luteal phase, about 8 days after ovulation, is due to secretion of this steroid by the CL concurrently with progesterone. Follicular growth is markedly decreased during the mid- to late luteal phase (Block, 1951) and there is little oestradiol secreted by the follicles at this stage (Baird and Fraser, 1975). Baird et al., (1975) therefore postulate that
the oestradiol secreted by the CL has a negative feedback effect upon gonadotrophin secretion, accounting for the low levels of LH and FSH in plasma during the mid-luteal phase (Midgley and Jaffe, 1968). Hence follicular growth is suppressed until regression of the CL lifts the inhibition of gonadotrophin secretion and a full 14 days is then required for a new crop of follicles to reach preovulatory maturity.

In contrast, the sheep CL does not secrete oestradiol (Short, McDonald and Rowson, 1963) and the source of the oestradiol in peripheral plasma during the luteal phase is the largest non-atretic follicle (Moor et al., 1973). Thus the follicular compartment retains control of gonadotrophin secretion, and gonadotrophin levels can never fall so low as to inhibit follicular growth. Follicles may reach a diameter of 5 mm before becoming atretic (Brand and de Jong, 1973), and hence only a short time is needed to complete preovulatory maturation after luteolysis removes the progesterone block of the positive feedback effect of oestradiol (Baird et al., 1975).

In the sheep, however, the follicular phase may be lengthened artificially by exogenous oestradiol given as daily injections for 20 days from d.4 of the cycle. After luteolysis occurs with the cessation of treatment, the subsequent ovulation may be delayed by several days to over 3 weeks (Piper and Foote, 1968). This suggests that the oestradiol suppressed gonadotrophin levels such that follicular maturation did not continue, and so a new crop of follicles had to complete the full gonadotrophin-dependent phase of development before reaching preovulatory maturity.

The view that in primates oestradiol controls both tonic gonadotrophin secretion and the cyclic ovulatory surge, with progesterone having a permissive effect only upon the positive feedback of oestradiol, is supported by the work of Knobil's group on rhesus monkeys (Karsch et al.,
1973; Knobil, 1974). However, the notion that progesterone does not affect tonic LH secretion in sheep has been challenged by Karsch, Legan, Hauger and Foster (1977) who noted an inverse relationship between circulating levels of LH and progesterone during the oestrous cycle of the ewe and considered that this was circumstantial evidence for a negative feedback effect of progesterone upon LH secretion. Conversely, the parallel secretion patterns of LH and oestradiol during the large part of the cycle seemed to preclude a negative feedback relationship (Hauger, Karsch and Foster, 1977).

The experiments of this group with ovariectomized ewes did indeed show that progesterone has an inhibitory effect on LH levels (Karsch et al., 1977). They considered that earlier studies (e.g. Goding et al., 1970; Scaramuzzi et al., 1971) may have failed to achieve an effective level or pattern of progesterone secretion and/or failed to detect relatively small decreases in circulating LH. They also criticised the use of the long-term ovariectomized ewe as an experimental model, and pointed out that earlier studies on the feedback effects of oestradiol are difficult to interpret because the levels of oestradiol in plasma due to treatment were not measured or compared with physiological concentrations, and because the sensitivity of the ewe to the negative feedback action of oestradiol varies markedly during the year.

Hauger, Karsch and Foster (1977) therefore present a working model for the control of the oestrous cycle of the ewe, incorporating progesterone as a primary negative feedback agent, with LH as an important determinant of oestradiol secretion rather than this steroid having a powerful negative feedback effect on gonadotrophin secretion. In brief, tonic levels of LH, relatively high in the post-ovulatory period decrease to a minimum as progesterone levels increase and do not rise again until luteolysis occurs. Waves of follicular growth continue through the luteal
phase, but oestradiol output increases markedly only when tonic LH levels are relatively high, during the early and late luteal phases. Progesterone is viewed as the "organizer" of the sheep oestrous cycle, regulating both the tonic and surge mode of LH secretion. The presence of the CL therefore inhibits follicular development and ovulation, and its removal, by luteolysis or lutectomy, permits these events to occur.

This scheme is attractive in that it accounts for the observation that oestradiol levels rise during the late follicular phase. If gonadotrophin stimulation is necessary for steroidogenesis but oestradiol has an inhibitory effect on gonadotrophin secretion, one would expect curtailment of oestradiol secretion before it reaches a level sufficient to trigger the positive feedback system. Conversely, it does not explain why apparently only the preovulatory stages of follicular growth are inhibited during the luteal phase: separate control of FSH secretion should perhaps be postulated.

However, all such models are necessarily over-simplified. Although they may provide sound bases for directing further experimentation, it is clear that, at this stage, they cannot be treated as dogma but as working hypotheses to be altered or discarded as new information becomes available. For the many species which are not common laboratory species, much basic knowledge of their reproductive endocrinology must be gathered before such control systems may be proposed. Despite a long-standing general interest in the peculiarities of marsupial reproduction, for example, relatively little is known about the details of the regulatory systems involved, largely because more data is needed on the changes in hormone levels which effect this regulation.

1.5 PATTERNS OF REPRODUCTION IN THE MACROPODIDAE

The Macropodidae is among the most widespread families of Australian marsupials, including the familiar kangaroos and wallabies (Macropodinae)
and the smaller rat-kangaroos (Potoroinae). As a group, they have been highly successful, perhaps largely due to their ruminant-like digestion, which makes them the most efficient herbivores among the Australian fauna (Tyndale-Biscoe, 1973).

Patterns of reproduction in the Macropodidae vary according to the habitat of the particular species. For example the desert-dwelling red kangaroo (Megaleia rufa) and euro (Macropus robustus) are opportunistic breeders, able to produce young at any time of the year if conditions are favourable, but in the grey kangaroos (Macropus giganteus and M. fuliginosus), which occupy a less arid environment, young leave the pouch only in spring and early summer, when fresh green feed is available (Sharman, Calaby and Poole, 1966). Despite such differences, the reproductive physiology of all members of the group is basically similar.

All macropods studied so far are polyoestrous and spontaneous ovulators, with fairly long oestrous cycles, ranging in length from 22-46 days. If fertilization occurs, the ensuing pregnancy does not interrupt the oestrous cycle. This feature is common to all marsupials, but the relative duration of gestation and the oestrous cycle vary. In many species, the young are born at the end of the luteal phase, and prooestrus and ovulation are suppressed during lactation. In most macropods, however, gestation and the oestrous cycle are approximately the same length, and in most species ovulation occurs within a few days before or after parturition. In these cases, full development of the CL and, hence, the uterine luteal phase is inhibited by the suckling stimulus. Furthermore, if fertilization has occurred, embryonic development is arrested at the unilaminar blastocyst stage, and the blastocyst remains quiescent until the CL has resumed development, generally after lactation ceases. (Tyndale-Biscoe, Hearn and Renfree, 1974).
The absence of mitoses in both blastocyst and CL distinguish this dormancy from delayed implantation as exhibited by several groups of eutherian mammals, in which embryonic development does continue, albeit very slowly, and the CL is functional (Hearn, Short and Baird, 1977). The phenomenon observed in macropods is termed embryonic diapause, and appears to be a unique characteristic of this family of marsupials (Sharman and Berger, 1969).

Although the most obvious selective advantage of embryonic diapause is the prompt replacement of a lost pouch young and return of the mother to breeding condition, Tyndale-Biscoe (1973) has suggested that its primary function is to ensure embryo-uterine synchrony, by arresting embryonic development until the uterine environment is suitably prepared. He further suggests that diapause may, therefore, be a normal phase of development in all marsupials, and that macropods are unusual only in that the signal to end diapause is inhibited by the suckling stimulus, so that diapause is extended until the end of lactation. Nevertheless, the ecological advantages of embryonic diapause have been well exploited by the macropods, and must have contributed significantly to their radiation into a wide variety of habitats.

1.6 REPRODUCTIVE BIOLOGY OF THE TAMMAR WALLABY

The oestrous cycle of the tammar wallaby, *Macropus eugeni*, is about 29 days long, and the gestation length is such that the female comes into post-partum oestrus about 24 hours after parturition. If conception occurs, the CL and blastocyst enter quiescence at the stage reached about 8 days after oestrus (Tyndale-Biscoe, 1978).

The tammar and the Tasmanian subspecies of red-necked wallaby, *Macropus rufogriseus fructicus*, are the only macropods which are strictly seasonal breeders, this feature probably representing parallel adaptations to
habitats with distinct seasonal climatic changes. The breeding season is initiated at the summer solstice, presumably by decreasing photoperiod (Sadleir and Tyndale-Biscoe, 1977), and lasts until about June. 80% of females given birth (and mate post-partum) at the end of January, the pouch young normally being carried until September. If, however, the pouch young is lost during the breeding season, the quiescent CL and, hence, the blastocyst, resume development, the young is born 27 days later and the female again comes into post-partum oestrus. During the first half of the year, therefore, diapause is initiated and maintained by the suckling stimulus and the females are said to be in lactational quiescence.

If, however, the pouch young is lost or experimentally removed during the second half of the year, reactivation does not occur until the summer solstice. During this period, an environmental cue, most probably photoperiod, is the proximate factor controlling diapause and the female condition is termed seasonal quiescence. This is not a true anoestrus, as exhibited by the quokka (*Setonix brachyurus*), because even in non-lactating animals the uteri never regress to the extent seen in the hypophysectomized animal (Tyndale-Biscoe, Hearn and Renfree, 1974). Lactation is not necessary for the initiation of diapause since young female tammars reaching sexual maturity in October do come into oestrus, but if they conceive, the CL and blastocyst enter quiescence and do not reactivate until the solstice (Berger and Sharman, 1969).

Because the tammar wallaby is subject to seasonal quiescence as well as the lactational quiescence exhibited by other macropods, it is possible for a female giving birth at the end of January to conceive post-partum and carry the resulting quiescent blastocyst for up to eleven months, until the next summer solstice (Berger, 1966). This period of embryonic dormancy is rivalled among eutherian mammals only by the European badger, which also conceives post-partum in February, but implantation is delayed
until the following December (Enders, 1963).

1.7 CONTROL OF THE OVARIAN CYCLE IN MACROPOD MARSUPIALS

There is a paucity of information upon all aspects of this subject, although Hearn's (1972) study of the tammar wallaby did begin to bring into focus the central role of the pituitary in the control of reproduction in this species. Most experimental work has been done on tammar wallabies, quokkas and the red and grey kangaroos, which provide a fair spectrum of the modes of reproduction seen in the Macropodidae. Two other species of marsupials, the American opossum *Didelphis virginiana* (Didelphidae) and the brush possums *Trichosurus vulpecula* (Phalangeridae), have been favoured laboratory animals, and results from these species will also be discussed for comparative purposes.

The little data available suggests that gonadotrophins of eutherian origin when administered to marsupials will evoke responses comparable to those observed in eutherian mammals, i.e. follicular development with or without ovulation and luteinization. For example, in the opossum, *Didelphis virginiana*, pregnant mare serum gonadotrophin (PMSG) induced the formation of large cystic follicles, but not ovulation and human chorionic gonadotrophic (hCG) with or without PMSG causes luteinization of follicles without ovulation (Morgan, 1946). PMSG or acetone-dried horse or kangaroo pituitary extracts stimulated follicular growth in anoestrous quokkas (*Setonix brachyurus*) again without inducing ovulation (Tyndale-Biscoe, 1961), but PMSG or hCG will cause ovulation in *Sminthopsis crassicaudata* (Dasyuridae) (Smith and Godfrey, 1970). Such experiments may suggest that marsupial gonads possess receptors which recognise eutherian gonadotrophins and that these hormones elicit similar responses in marsupials and eutherians.
By hypophysectomy, Hearn (1972) has shown that the pituitary of the tammar is necessary for follicle growth and ovulation but that the CL can continue and complete its growth cycle and induce a normal luteal endometrium in the absence of the pituitary. Cook and Nalbandov (1968) found that ovine LH, but not FSH nor prolactin, could stimulate progesterone synthesis by luteinized opossum ovaries in vitro, and postulated that a single release of ovulating hormone, presumably LH, could trigger all the mechanisms necessary to complete the oestrous cycle, with the uterus having neither a luteotrophic nor a luteolytic function. Their suggestion that marsupials could have the simplest form of ovarian regulation found in mammals is given some support by Hearn's finding that the total gonadotrophin concentration, measured by homologous RIA, in the plasma of tammar wallabies showed significant changes only in the day of oestrus (Hearn, 1972). However, since his assay probably detected primarily LH (Hearn, 1974), independent measurement of LH and FSH would be of great benefit to further elucidation of the gonadotrophic regulation of ovarian events.

However, nothing is known of the control of gonadotrophin secretion in marsupials, although the presence of portal vessels in the pituitary stalk (Green, 1951) suggests that, as in eutherian mammals, the pituitary is under hypothalamic control. Information on possible steroid feedback systems is sparse. Injection of oestrogen into intact or castrate females will duplicate the changes that occur in the uterus and vaginal complex during proestrus as the Graafian follicle enlarges (Bolliger, 1946; Sharman, 1959), and changes in the concentration of progesterone in plasma have been measured in the tammar wallaby (Lemon, 1972; Hinds, unpublished results) and the brush possum (Shorey and Hughes 1973). The role of progesterone in the feedback regulation of gonadotrophin secretion may be inferred cautiously by consideration of the
effects of lutectomy in these two species.

In the brush possum, removal of the ovary bearing the CL or ablation of the CL itself on days 2-7 after oestrus resulted in premature oestrus and ovulation 8-9 days later, but if lutectomy was performed after day 7 ovulation occurred at the normal time (Shorey and Hughes, 1975). It is clear that the CL is inhibiting follicular growth during the first week of the cycle, but since circulating progesterone levels are low during this period, rising sharply on day 8 to reach a peak on day 12, it is unlikely that progesterone is the agent for this inhibition.

In the tammar wallaby, lutectomy on day 0, 6 or 12 of the cycle was followed by premature oestrus and ovulation about 12 days later, but if the CL were removed on day 18, ovulation occurred at the normal time, on day 26 to 28 (Tyndale-Biscoe and Hawkins, 1977). As with the brush possum, the periods of maximal inhibiting influence of the CL on follicular growth and of high progesterone secretion do not coincide, as progesterone levels are maximal during the second half of the cycle (Lemon, 1972, Hinds, unpublished) when follicular growth is occurring. Furthermore, in the quokka, removal of the ovary bearing the CL during the first half of the cycle resulted in premature ovulation 8-12 days later, but injections of progesterone for 5 days after the operation did not postpone this ovulation, even though a luteal endometrium was developed (Tyndale-Biscoe, 1963). Here again it seems that some hormone other than progesterone must be sought as the agent of the CL responsible for the inhibition of follicular growth during the early part of the cycle.

In macropods, oestrus and ovulation are also suppressed during lactation, but the inhibitory mechanism varies, although in each case the pituitary is probably involved. The grey kangaroos, *Macropus giganteus* and *M. fuliginosus*, are unusual in that the gestation length is somewhat
shorter than the oestrous cycle. The young is born at the end of the luteal phase and proestrus is suppressed by suckling, as in non-macropod marsupials. If the pouch young is lost during the breeding season, the female re-ovulates about 10 days later (Poole and Pilton, 1964; Poole and Catling, 1974). Lactation is not, therefore, associated with a quiescent corpus luteum, although in *M. giganteus* the female may come into oestrus and ovulate towards the end of the long lactation period, and if conception occurs this blastocyst and CL do enter quiescence until the young leaves the pouch (Clark and Poole, 1967). *M. fuliginosus* is the only macropod in which embryonic diapause has never been observed (Poole and Catling, 1974).

In the red kangaroo, *Megaleia rufa*, ablation of the quiescent CL during lactation causes premature ovulation about 18 days later, while the interval between lutectomy and ovulation in non-lactating animals is only about 12 days (Sharman and Clark, 1967). This suggests that while the quiescent CL is important in the inhibition of follicular growth during lactation, the suckling stimulus also has some inhibitory effect.

As previously mentioned, the tammar wallaby exhibits both lactational and seasonal quiescence. Lactating females lutectomized during the breeding season returned to oestrus at the same time as non-lactating, lutectomized animals, showing that in this species the CL alone is the agent of follicular inhibition. During the non-breeding season however, the response to lutectomy was more variable. Only half of the experimental animals ovulated (10-14 days after operation) and not all of these came into oestrus (Tyndale-Biscoe and Hawkins, 1977). This suggests that although the tammar is potentially capable of ovulating at any time of the year, it is less responsive to lutectomy during the non-breeding season, possibly due to an increased pituitary sensitivity during this time to the negative feedback of ovarian steroids (Hearn, Short and Baird, 1977).
The two pituitary hormones prolactin and oxytocin are concerned with lactation in eutherian mammals and it was therefore considered likely that one of these could be the inhibitory agent. An early experiment with red kangaroos showed that oxytocin injections delayed reactivation after removal of pouch young, but some of the water-injected control animals were also delayed (Sharman, 1965). Tyndale-Biscoe and Hawkins (1977) suggested that this effect may have been due to prolactin release caused by handling stress. Their own experiments showed that prolactin, but not oxytocin, injected for 7 days after hypophysectomy, could delay reactivation, or completely inhibit it, in hypophysectomized tammar wallabies.

Interestingly, a single dose of bromocryptine, which depresses prolactin secretion, given to lactating females caused their quiescent CL's to reactivate. They gave birth 26-27 days later, came into post-partum oestrus and ovulated while still suckling the original pouch young (Tyndale-Biscoe and Hinds, manuscript in preparation). This shows that only a transient fall in prolactin levels is necessary for the CL to escape the tonic inhibition imposed upon it by the pituitary, and that once it is reactivated, it becomes independent of the pituitary. The experiment also confirms that in the tammar wallaby follicular maturation is not inhibited by suckling, but by the quiescent CL (Tyndale-Biscoe and Evans, in press).

In macropod marsupials, therefore, it seems that prolactin has the unusual role of tonically suppressing the corpus luteum. Among eutherian mammals, it frequently has a stimulatory, or luteotrophic, influence, although, while low doses are stimulatory, high doses of prolactin will inhibit steroidogenesis by granulosa cells of human follicles (McNatty, Sawers, and McNeilly, 1974) or mouse ovaries in vitro (McNatty, Neal and Baker, 1976). If the prolactin influence is absent or removed,
the macropod CL appears to function quite autonomously, presumably regulating its own growth and regression: there is as yet no evidence for a uterine luteotrophic or luteolytic effect in any marsupial.

A picture therefore begins to emerge of the corpus luteum having central control of the ovarian cycle in macropod marsupials, being itself subject to inhibition by the pituitary during lactation and/or the non-breeding season. The other major role of the CL is the preparation for the maintenance of pregnancy, although in macropods, unlike other marsupials, the foeto-placental unit may assume part of this role during the latter part of gestation and hence determines the total gestation length (Tyndale-Biscoe, Hearn and Renfree, 1974). These authors expressed the view that the ovarian cycle, controlled by the CL-follicle interaction, and gestation, controlled by the CL and placenta, are semi-independent processes linked only by the dual regulatory role of the CL during the early part of the cycle. This corresponds to the long-held belief that in all marsupials the oestrous cycle is not affected by pregnancy. However, in the agile wallaby, *M. agilis* (Merchant, 1976) and the tammar wallaby (Merchant, personal communication), the interval from mating or removal of pouch young to next oestrus was 2-4 days shorter in pregnant than in non-pregnant females, which implies that the foeto-placental unit also has some influence upon ovarian events in these two macropods.

The endocrine mechanisms underlying reproductive processes in marsupials in general and macropod marsupials in particular are far from understood. Hearn's work on the tammar wallaby (Hearn, 1972; 1973; 1974) showed that one should be cautious in extending parallels from the eutherian to the marsupial situation. It also demonstrated the importance to further studies on marsupial reproduction of both the classical endocrinological technique of gland ablation and the ability to measure hormone levels in the circulation. Until we have some idea of the changing
profiles of the various hormones, steroids, gonadotrophins, prolactin and perhaps others, in plasma during the reproductive cycle, little progress will be made towards a more complete understanding of the endocrine system controlling the basically similar modes of reproduction in this group of mammals.

1.8 THE SCOPE OF THIS THESIS

The aim of my project was to examine the roles of LH and FSH in the regulation of the oestrous cycle and pregnancy and in the control of the annual cycle of reproduction in the tammar wallaby. The only previous study of the role of pituitary hormones in the control of reproduction in a marsupial species was that of Hearn, who developed the technique of hypophysectomy of the tammar wallaby (Hearn, 1974) and a homologous RIA based on a crude gonadotrophin fraction extracted from wallaby pituitaries (Hearn, 1972). With this assay, Hearn measured total gonadotrophin levels in plasma of female tammars at various stages of their reproductive cycles.

My study thus arose as a progression from Hearn's work. Although, the roles of LH and FSH in the control of mammalian reproduction are interrelated, the functions of each hormone, as this Introduction has shown, are also distinct. Thus to gain any further insight into the gonadotrophic control of ovarian activity in the tammar, it was important to measure circulating levels of each hormone separately.

My first objective was, therefore, to confirm that the tammar pituitary produces two gonadotrophic hormones, comparable to the LH's and FSH's of eutherian mammals, and to establish the secretory patterns of the hormones in the normal female tammar. To do this, RIA's were developed for both hormones. When I began my study, the highly purified wallaby and kangaroo LH and FSH preparations (Gallo et al., in press) were
not available, so instead of trying to develop homologous RIA's for the tammar gonadotrophins, I decided to adapt heterologous assays already in use for measurement of LH and FSH in other species. The advantages of heterologous RIA's have been discussed in this Introduction and the difficulties other workers have encountered in developing satisfactory RIA's for FSH have also been pointed out.

The validation of heterologous RIA's for the measurement of LH and FSH in wallaby plasma is described in Chapters 3 and 4 of this thesis, while Chapter 5 documents the changes in LH and FSH levels in plasma of normal female wallabies during the oestrous cycle and pregnancy and at different times of the annual cycle. Emphasis has been placed upon the relationship of LH levels to oestrus and ovulation.

With the RIA's as the major experimental tools and some basic information on gonadotrophin levels in normal females, the interactions between the pituitary, the ovary and the hypothalamus in this species could then be investigated. In the experiments described in Chapter 6, evidence was sought for a duality of function of the two gonadotrophins and for any differences in the operation of the hypothalamo-pituitary-ovarian axis in this marsupial compared to the eutherian species which have been studied. By performing similar experiments at different times of year, some insight was also gained into the endocrine changes associated with seasonality.

The discussion in Chapter 7 integrates the data presented in preceding chapters into an overall view of the control of reproduction in the tammar wallaby based upon present knowledge of this and eutherian species. A working model of the endocrine control of the oestrous cycle is proposed and I also present some new thoughts on the control of seasonality in this marsupial.
CHAPTER 2

GENERAL PROCEDURES

2.1 ANIMAL MAINTENANCE

2.2 BLOOD COLLECTION

2.3 SURGICAL PROCEDURES

2.4 AUTOPSY PROCEDURE

2.5 HISTOLOGY

2.6 VAGINAL SMEARS
2.1 ANIMAL MAINTENANCE

Wallabies were provided from the breeding colony maintained at C.S.I.R.O., Division of Wildlife Research, Gunghalin, A.C.T. The original stock had been brought from Kangaroo Island, South Australia in 1973-74. The animals were kept in open paddocks with free access to water, and their diet was supplemented with oats and lucerne chaff.

For certain experiments, animals were held in pens in the Animal houses at the Zoology Department, A.N.U. or at C.S.I.R.O., Division of Wildlife Research. These pens varied in size from 1.8 m x 1.4 m to 2.8 m x 2.5 m: up to five wallabies could be kept together in the larger pens for several days. While held indoors, they were fed on oats and lucerne chaff with water ad lib. Pen floors were covered with sawdust, and sacking shelters were erected to provide darkened retreats for the animals.

2.2 BLOOD COLLECTION

Routine blood samples were drawn from the tail vein into heparinized glass syringes, using No.19 needles. The blood was placed in centrifuge tubes on ice until centrifuged, as soon as possible, in a bench centrifuge for 10 minutes. Plasmas were decanted and stored frozen at -10°C.

Two other methods of blood sampling were used in certain experiments. For intensive sampling over a period of some days, jugular catheters were inserted. Catheters could be kept patent for over 4 weeks by daily flushing with sterile 0.9% saline and re-filling with dilute heparin (250 U/ml). However, when animals were kept together in pens, they tended to pull out each other's catheters. Hence, in some experiments
with LH-RH, when the animal was required once only, blood samples were taken using disposable winged infusion sets (Surflo, Terumo) inserted into the tail vein. The set consisted of a No.19 needle with a 12" extension tube (volume 0.5 ml) attached. The tube was filled with dilute heparin after each blood sample was taken.

2.3 SURGICAL PROCEDURES

All surgery was carried out under aseptic conditions. Instruments were sterilized. The operation site was shaved, washed and swabbed with Cetavlon antiseptic solution. Wounds were dusted with Sulphanilamide (BDH) powder and each animal was injected with 1 ml Vetspen Plus Injection penicillin (Claxovet) after operation.

Anaesthesia was induced by Surital (Parke-Davis), a barbiturate, injected through a No.19 needle into the tail vein. The initial dose was 4 - 6 ml of a 4% saline solution, depending upon the weight of the animal, and anaesthesia was maintained during surgery by further doses of 0.5 or 1 ml as required.

Ovariectomy: this procedure is described fully by Renfree and Tyndale-Biscoe (1978). A 5 cm, mid-ventral incision was made through the pouch skin, the underlying muscle layer and the peritoneum. Each ovary was exposed in turn and carefully removed from the bursa. A Mersilk No.3 suture was tied around the hilus, the ovary removed with scissors and the site cauterized. The muscle and peritoneum were sutured together with Mersilk No.3 thread and the skin incision closed with clips.

Luteectomy was performed similarly to ovariectomy. Each ovary was examined for the presence of the corpus luteum, identified by its pinkish colour compared to the white corpora albicantia. New CL show an
ovulation tip. The appropriate ovary was gently clamped at the hilus and the CL removed using iridectomy scissors. In early experiments, the socket was cauterized using a fine cautery needle, but later it was simply plugged with Gelfoam absorbable gelatin sponge (Upjohn) as it was felt that cautery might be adversely affecting the rest of the ovary.

_Jugular catheters_ were inserted usually into the left external jugular vein after the method of Than and McDonald (1976). The vein was exposed by a 4 cm incision over the vein about 5 cm posterior to the angle of the mandible. The vein was tied off with Mersilk at the anterior end, usually just below the branch of the posterior facial vein. A small nick was made in the vein wall with fine scissors and the catheter inserted to just above the heart, a distance of about 10 cm. Two Mersilk sutures were used to tie the catheter firmly into the vein. The catheter was polyethylene tubing (Intramedic) inside diameter 1.2 mm, outside diameter 1.7 mm, soaked in aqueous Cetavlon antiseptic, but flushed with sterile 0.9% saline before insertion. A polyethylene collar was attached about 10 cm from one end of the catheter, and nylon fishing line threaded through this collar was used to anchor the catheter to surrounding tissue.

Using a hollow trocar, closed at one end to act as a probe and open at the other end to carry the catheter, the catheter was threaded under the skin to emerge at the back of the neck through a small incision made with the cautery knife. The first incision was then closed with clips, the catheter trimmed to about 8 cm and plugged with a stainless steel plug. Throughout the operation, the catheter was kept full of sterile 0.9% saline and was finally filled with dilute heparin solution (250 U/ml in 0.9% saline).
2.4 AUTOPSY PROCEDURE

The animal was killed either by an overdose of Surital or by a blow to the back of the neck followed by cervical dislocation and cutting of the throat.

The entire urogenital system was removed and weighed. The two uteri were dissected free and weighed. Blastocysts and unfertilized eggs were recovered by flushing each uterus with 2 x 2.5 ml of 0.9% saline through a blunt 18 gauge needle inserted into the cervix. A small cut was made in the utero-tubal junction to allow the fluid to escape easily. If nothing was recovered by flushing, the uterus was slit open and everted under saline. Blastocysts and ova were examined in saline under a binocular dissecting microscope, and the diameters of the shell membrane and protoderm measured using a micrometer eyepiece. Ovaries were weighed, and examined under the binocular microscope. The diameters of large follicles or corpora lutea were measured with Vernier calipers. Tissues were fixed in Bouin's fluid if required for histology. Pituitary glands were removed and frozen at -40°C to add to a collection of wallaby pituitary tissue made by Dr. C.H. Tyndale-Biscoe for purposes of hormone extraction.

2.5 HISTOLOGY

Tissues were fixed in Bouin's fluid, blocked in paraffin wax, sectioned at 8 - 10 µ and stained with haematoxylin and eosin.

2.6 VAGINAL SMEARS

"Vaginal" smears were taken as described by Poole and Catling (1975). Briefly, the cloaca was everted and a smooth-ended glass tube inserted into the urogenital sinus. A dampened cottonwool swab carried
inside the tube was used to take the smear. Smears were immediately fixed in a 1:1 mixture of diethyl ether and 95% alcohol and stained by the method of Shorr (1941).

Mid-cycle smears were characterized by a majority of green-staining epithelial cells, while post-oestrous smears contained mainly red-stained cornified or partially cornified cells. After mating, large numbers of small, round, green-staining prostatic bodies could be found in the smear.
CHAPTER 3

A HETEROLOGOUS RADIOIMMUNOASSAY FOR TAMMAR WALLABY LUTENIZING HORMONE

3.1 INTRODUCTION
General principles of radioimmunoassay
Heterologous radioimmunoassay for wallaby LH

3.2 MATERIALS AND METHODS
Reagents and hormone preparations
Iodination procedure
Assay procedure
Calculation of results

3.3 EXPERIMENTAL PROCEDURES AND RESULTS
Titration of second antibody
Cross-reactivities of eutherian LH's and FSH's
Cross-reactivity of wallaby LH:
   a) Dose-response curves for wallaby pituitary preparations
   b) Dose-response curves for wallaby plasmas
Effect of plasma on the estimation of LH
Inter-assay variation
LH levels in plasmas of tammar wallabies

3.4 DISCUSSION

3.5 SUMMARY
3.1 INTRODUCTION

General principles of radioimmunoassay

The radioimmunoassay (RIA) is based upon a competition between radioactively labelled hormone (tracer) and unlabelled hormone (standard or sample) for a limited number of antibody binding sites. As the amount of unlabelled hormone increases, increasing amounts of tracer are displaced from the antibody, and on this basis a standard curve may be constructed. The hormone concentration in an unknown sample is deduced by measuring the amount of tracer displaced from the antibody in the presence of the sample and comparing this with the standard curve.

With any new RIA, two objectives must be achieved before the assay is judged to be suitable for routine use. Firstly, assay conditions must be adjusted so that the desired range of concentrations of unlabelled hormone is measurable. Secondly, it must be shown that the assay gives a valid measure of the hormone for the species in question and in the particular samples to be assayed, usually plasma and pituitary extracts. In the validation of a RIA, attention must be given to the following parameters. Definitions are adapted from Midgley, Niswender and Rebar (1969) unless otherwise stated.

Sensitivity is defined as the smallest amount of unlabelled hormone that can be distinguished from zero hormone. Sensitivity therefore depends upon the error in the determination of \( \frac{B}{B_0} \), which is the ratio of the amount of radioactivity bound (B) at a certain dose level to the amount of radioactivity bound in the absence of unlabelled hormone \( (B_0) \), and upon the slope of the standard curve. All other factors being equal, the most sensitive standard curve is obtained by using the minimum amount of tracer while retaining adequate counting accuracy.
**Precision** may be defined as the extent to which a given set of measurements of the same sample agree with their mean, i.e. precision is the variation in the estimation of unlabelled hormone. It is largely a function of methodological errors, including the radioactive counting error. It is also important that results should be reproducible from assay to assay, i.e. the *inter-assay variation* should be as small as possible.

**Specificity** may be defined as the extent of non-interference by other factors in the estimation of the hormone concentration. Specificity may be affected by non-specific factors or by immunological factors.

In establishing the specificity of an assay, it must be shown that any differences in incubation media between tubes do not affect the estimate of hormone concentration. Factors likely to cause any such interference are ionic strength, pH, heparin (Henderson, 1970), protein concentration or the presence of other substances in plasma. In routine assays, the most likely source of any such variation is the presence or absence of plasma, or the assay of different volumes of plasma. The non-specific effect of plasma on hormone measurement can be assessed by determining the *recovery* of hormone added to plasma, the amount added being compared with the amount measured by comparison with a standard curve prepared in assay buffer alone.

To validate a RIA, it is necessary also to show that the hormone in the samples to be assayed and the standard hormone behave identically in the assay system. A necessary but not sufficient condition for identical reactivity is that the amount of hormone measured falls linearly with the dilution, or volume of plasma, at which it is assayed, i.e. the dose-response curves for standard and unknown must be superimposable (Yalow and Berson, 1971). This is usually demonstrated by showing that
the slopes of suitably transformed, i.e. linearized, plots (eg. log dose versus \( B/B_0 \) or log dose versus logit \( B/B_0 \)) of the dose-response curves are parallel. Besides such similarity to the hormone to be assayed, the criteria by which a standard preparation should be chosen include stability during storage, availability in quantities sufficient for use over an extended period in several laboratories and freedom from contaminants which would interfere in the assay.

For the purposes of this thesis, the cross-reactivity, or potency, of a test preparation with respect to a standard is defined as the percentage weight of that preparation required to displace 50% of the total bound tracer (i.e. \( B/B_0 = 0.5 \)) relative to the weight of standard required to displace the same amount of tracer.

There is no requirement, however, that labelled and unlabelled hormone behave identically in the assay system. In fact, the sensitivity of a heterologous assay may be improved by using a tracer for which the antibody has lower affinity than for the standards and unknown. Nevertheless, a specific RIA ideally requires the presence of only a single molecular species of labelled hormone (Midgley, Niswender and Rebar, 1969). Since antisera are usually raised against hormone preparations which are at best of no greater purity than the one used for labelling, the antiserum is likely to contain antibodies to contaminating substances. Heterogeneity of the tracer therefore implies the presence of more than one immunoreactive system with consequent loss of specificity.

Finally, the validation of a RIA requires that the measured hormone levels be consistent with what is known of the physiology of the hormone (Yalow and Berson, 1971). Thus, if the gland which secretes it is removed, circulating levels of the hormone should fall to zero.
Administration of known inhibitors or stimulators of hormone release should decrease or increase, respectively, the concentration measured in samples, while removal of some component of, say, a negative feedback control system should result in elevated hormone levels (e.g. gonadotrophin levels should increase after ovariectomy).

During the development of the RIA's for tammar wallaby LH and FSH, described in Chapters 3 and 4 of this thesis, due consideration was given to all the points discussed in this introductory section.

**Heterologous radioimmunoassay for tammar wallaby LH**

Although purified wallaby LH and FSH have recently been prepared in small quantities (Gallo *et al.*, in press), these were not available when this study was begun. In view of the inherent difficulties in preparing such material from limited supplies of wallaby pituitary tissue, it was decided to take the alternative approach of developing heterologous, rather than homologous, RIA's for both wallaby LH and wallaby FSH.

Initial experiments with a solid-phase assay system, using ovine LH antiserum with ovine LH (o-LH) as tracer are described in Appendix 1. Although originally appearing promising, the solid-phase assay proved to be unsuitable for quantitation of LH in wallaby plasma and was abandoned in favour of another ovine-ovine RIA based upon Dr G.D. Niswender's o-LH antiserum, GDN-15.

This chapter describes the development and validation of an assay for wallaby LH using GDN-15 o-LH antiserum and an o-LH tracer. The double-antibody method was used to separate the bound and free fractions. This technique was first applied to RIA by Morgan and Lazarow (1962) and Utiger, Parker and Daughaday (1962) and involves the use of a second
antibody to precipitate the soluble antigen-antibody complexes formed by the first reaction. Thus if the hormone-specific antiserum was raised in a rabbit, the second antibody would be an anti-rabbit gamma globulin (A-RGG). The precipitated antibody-bound fraction is separated by centrifuging and decanting the unbound fraction in the supernatant.

3.2 MATERIALS AND METHODS

Buffer solutions

**PBS**

Throughout this thesis, the abbreviation PBS refers to 0.05 M sodium phosphate buffer containing 0.14 M sodium chloride (NaCl) pH 7.4.

**Diluent**

The diluent was PBS containing 0.2% (w/v) bovine serum albumin (BSA, Fraction V, Calbiochem) and 0.01% (w/v) sodium azide. Diluent was stored at 4°C and fresh solution prepared weekly.

**PBS-EDTA-NRS**

Normal non-immune rabbit serum (NRS) was diluted 1:400 with 0.05 M sodium phosphate buffer containing 0.14 M NaCl and 0.05 M ethylenediamine-tetracetic acid (EDTA) pH 7.0. This solution was stored frozen in 20 ml lots.

**Antiserum**

Rabbit anti-ovine LH (A-oLH) GDN-15 was prepared and supplied by Dr G.D. Niswender. Antibody was raised in an adult female rabbit by 3 subcutaneous injections of 125-175 µg of NIH-LH-S12 emulsified in complete Freund's adjuvant given at intervals of 3 weeks. Ninety days after the last injection of NIH-LH-S12, a booster injection of 350 µg of a more highly purified ovine LH (lot no. 2418-289-1, 1.9 x NIH-LH-S1, Abbott) was administered (Niswender *et al.*, 1968).
One vial of the lyophylized preparation was dissolved in 10 ml distilled water to give a 1:400 dilution of the antiserum in 0.05 M EDTA - 0.05 M phosphate-buffered saline pH 7.0. 200 µl aliquots of this dilution were stored at -20°C, and each was diluted to 20 ml with PBS-EDTA-NRS, i.e. to 1:40,000 A-oLH, for use in the assay.

**Second antibody**

Sheep anti-rabbit gamma globulin (A-RGG) was prepared and supplied by Dr M.R. Brandon, Australian National University, Canberra. The antigen, highly purified rabbit gamma globulin, was prepared from whole rabbit serum by gradient elution (0.20 M - 0.40 M phosphate buffer, pH 8.0) on a 50 × 2.5 cm DEAE A50 Sephadex column. The first protein peak to elute was IgG, and this material was collected, concentrated and stored at -20°C until used for immunization. One Merino ewe was immunized three times at 14 day intervals with 5 mg of antigen emulsified in Freund's complete adjuvant. The animal was killed 14 days after the last injection following confirmation of significant A-RGG activity by immunoelectrophoresis.

The antiserum was stored at -20°C in 1 ml aliquots, which were diluted 1:40 in diluent for use in the RIA.

**Standard LH preparation**

The standard was ovine LH (oLH), NIH-LH-S19. This preparation has a potency of 1.01 × NIH-LH-S1, contains < 0.050 NIH-FSH-S1 units/mg (i.e. < 5% FSH) and the TSH contamination is estimated at 0.11 USP units/mg (specifications supplied by the National Institute of Arthritis, Metabolic and Digestive Diseases, Maryland, USA).

The lyophylized preparation was dissolved in PBS and stored at -20°C in 100 µl aliquots, each containing 100 ng oLH. For each assay a
fresh vial was thawed and diluted to 5 ml with diluent. From this, a range of standards, 0.019 ng - 10.0 ng/500 µl, was prepared by serial dilution (1:1) in diluent.

**Tracer**

Initially, the highly purified oLH G3-222B, supplied by Dr H. Papkoff, was used as the tracer, but it was later replaced by another highly purified oLH preparation, LER 1374 A, supplied by Dr L.E. Reichert Jr. These preparations were iodinated to a specific activity of 30 - 50 µCi/µg by the method outlined below.

**Other hormone preparations**

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<th>SUPPLIER</th>
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<td>N.I.A.M.D.D.</td>
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<td>Papkoff Ex 241</td>
<td>Dr. H. Papkoff</td>
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<td>G4-I50C</td>
<td>Dr H. Papkoff</td>
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<tr>
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</tr>
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<td>wallaby FSH</td>
<td>Papkoff Ex 240D</td>
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</tr>
<tr>
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<tr>
<td>wallaby gonadotrophin fraction</td>
<td>JH 74.9 (1)</td>
<td>Dr J. Hawkins</td>
</tr>
</tbody>
</table>

1 Purified from the crude wallaby gonadotrophin fraction JH.74.9 (1). This material (J.H.74.9(1)) was prepared from 2.6 g fresh frozen pituitaries from male tammar wallabies. The pituitaries were ground with dry ice and extracted twice with 0.1 M ammonium sulphate pH 4.0. After centrifugation, the supernatant was dialyzed against distilled water for 24 h and then freeze-dried. This fraction was tested in the RIA. Besides the gonadotrophins, it probably contains adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH) (Neill and Reichert, 1971).
Purification of wallaby LH (wLH) and wallaby FSH (wFSH) from the JH.74.9 (1) gonadotrophin fraction is described by Gallo et al. (in press). Briefly, after alkaline extraction of the lyophilized preparation and precipitation with 0.2 M HPO₃ to remove residual growth hormone, LH and FSH were separated by ion-exchange chromatography on sulfoethyl-Sephadex (SE-CSO) and DEAE-cellulose, final purification of fractions being by gel filtration on Sephadex G-100.

Wallaby pituitary homogenate

Two fresh pituitaries from female wallabies were homogenised in 3 ml cold diluent in a hand-homogenizer. The homogenate was centrifuged at 3,000 rpm for 5 min and the supernatant was diluted 1:1000 with diluent and stored in small aliquots.

Iodination procedure

The iodination procedure was based upon that of Greenwood, Hunter and Glover (1963). During the iodination reaction, free iodine is liberated from sodium iodide (NaI) by the oxidizing agent chloramine-T and then substitutes into the protein. At pH 7.5, the substitution is primarily or wholly into tyrosine residues. Although the original method used NaI¹³¹, the I²⁵ isotope is now more frequently used, its main advantages being a higher efficiency of counting and a half-life of 60 days as opposed to 8 days for I¹³¹. The reaction is stopped by the addition of a reducing agent, sodium metabisulphite. Various chromatographic and electrophoretic methods may be used to separate the iodinated hormone from unreacted iodine, the method chosen here being gel chromatography on Sephadex G-50.

The iodination was carried out in a fume-hood, behind a thick perspex screen. The reaction vessel was a 1 ml snap-cap vial which already contained 2 µg hormone in 5 µl PBS, freshly thawed. To the vial
were added in sequence:

1. 20 µl PBS
2. 10 µl sodium phosphate buffer pH 7.6
3. 1 mCi NaI\textsuperscript{125}, carrier free, in 10 µl NaOH pH 8-11
4. 20 µg chloramine-T in 20 µl PBS.

The reactants were vortexed and reacted for 30 sec at room temperature.

5. The reaction was stopped by the addition of 30 µg sodium metabisulphite in 500 µl PBS. 100 ng potassium iodide (KI) in 100 µl PBS was added to act as carrier for the unreacted sodium iodide.

6. The mixture was vortexed and then layered onto a 15 × 1 cm column of Sephadex G-50 which had been pre-washed with 20 ml PBS containing 0.2% BSA (i.e. assay diluent) to reduce non-specific binding of protein to the column.

7. The column was eluted with PBS. 500 µl fractions were collected into tubes containing 100 µl 5% BSA in PBS to minimize adsorption of iodinated hormone to the walls of the tubes.

8. A 10 µl sample of each fraction was counted for 0.2 min in the gamma counter (< 100 - 350,000 counts/0.2 min/sample). Fig. 3.1 shows a typical elution profile. Higher molecular weight substances are eluted earlier, so the protein peak precedes the free iodine peak.

9. The fractions selected from the protein peak for use as tracer in the RIA were the fraction of highest activity and the one immediately following it, i.e. the trailing edge of the peak. For the separation shown in Fig. 3.1, fractions no. 8 and 9 were chosen. Earlier fractions, presumably containing high molecular weights complexes, and later fractions, containing
Fig. 3.1. Separation of free iodine and iodinated hormone on a 15x1 cm column of Sephadex G-50.
hormone fragments, were discarded. The selected fractions were stored frozen.

**RIA procedure**

The assay procedure followed that of Niswender et al. (1968). Unless otherwise specified, samples and standards were assayed in duplicate. For routine assays, 100 µl or 200 µl plasma samples were assayed.

**Day 1**

Standards (0.019 - 10.0 ng NIH-LH-S19/500 µl diluent) or unknown were added to disposable polystyrene tubes (5 ml serology tubes, Medical Plastics of South Australia). When necessary, diluent was added to bring the total volume to 500 µl. Control tubes for estimation of total binding of tracer (B₀) in the absence of unlabelled hormone contained 500 µl diluent alone.

A-oLH (GDN-15) was diluted to 1:40,000 with PBS-EDTA-NRS and 200 µl added to each assay tube. Control tubes for measurement of non-specific binding (NSB) of tracer received 200 µl PBS-EDTA-NRS. All tubes were vortexed and incubated for 24 h at 4°C.

**Day 2**

125I-oLH was diluted in diluent to give ~20,000 cpm/100 µl and 100 µl of this solution was added to each assay tube.

All tubes were vortexed and incubated for 24 h at 4°C.

**Day 3**

Sheep A-RGG (second antibody) was diluted 1:40 in diluent and 200 µl of this added to each tube. (This optimal concentration of A-RGG had been determined by titration: see p.57). All tubes were vortexed and incubated overnight at 4°C.
Day 4

2.0 ml cold PBS was added to each tube to dilute the unbound fraction. The antibody-bound and unbound fractions were separated by centrifugation at 3,000 g for 40 min in a Sorval automatic superspeed refrigerated centrifuge, type RC-2.

The supernatants were aspirated off and the precipitates in the tubes counted for 5 min each in a Searle automatic gamma system, Model 1197. The accumulated counts ranged from about 2,000 counts for NSB to 40,000 for $B_0$, giving a counting error of 2.3 - 0.5%.

Assay characteristics

The antiserum was used at a dilution of 1:40,000, i.e. a final dilution of 1:160,000 in the assay tube. At this dilution, 32 - 45% of the $^{125}\text{I}-\text{oLH}$ was bound to antibody, depending mainly upon the age and specific activity of the tracer which could be used for about 4 weeks after iodination without appreciable change in the sensitivity of the assay. Non-specific binding (NSB) was never more than 5% of the total bound radioactivity.

The standard curve was usually plotted as log dose versus $B/B_0$, which is the amount of bound radioactivity ($B$) at a certain dose level relative to the amount of radioactivity bound in the absence of unlabelled hormone ($B_0$). NSB was subtracted before the data were plotted. Such a plot was generally linear over the range 0.1 - 1.5 ng NIH-LH-S19/tube, which represented ~20 - 80% binding of tracer. However, non-linear portions could also be used for calculations of sample potencies.

By inspection of the standard curves included in every assay, the sensitivity of the assay was estimated to be 40 pg NIH-LH-S19/assay tube. As 200 µl plasma samples were usually assayed, the working sensitivity was, therefore, 200 pg/ml plasma.
Calculation of results

For earlier assays, a standard curve was plotted as log dose versus $B/B_0$, the curve itself being drawn freehand. LH concentrations in standards were then calculated empirically by comparison with this standard curve.

Later details of a computer programme for the calculation of assay data were obtained from Dr. J.E.A. McIntosh, University of Adelaide, South Australia. The programme was modified by Dr. G. Brown, CSIRO Division of Wildlife Research, Canberra, for use with a Hewlett Packard 9825A calculator with graph plotter. The standard curve is calculated by a general iterative curve-fitting programme which generates a log-logistic function without transformation. The fitting function is derived from Healey (1972). Data transformed to $T/B$ (ratio of total counts added to counts bound) is calculated after the fitting procedure so that a plot of $T/B$ versus dose can be drawn if desired. Both measured and fitted data are displayed in the print-out.

3.3 EXPERIMENTAL PROCEDURES AND RESULTS

Titration of second antibody

The dilution of second antibody (A-RGG) must be chosen carefully so as to give optimum conditions for precipitation of the antigen-first antibody complex. Obviously, too little A-RGG will not cause maximum precipitation, but too much second antibody can also decrease precipitation by a prozone effect (Court and Hurn, 1971). Normal non-immune rabbit serum (NRS) is included in the incubation as a "carrier protein". It increases the volume of the precipitate and thus improves handling accuracy. Too large a precipitate can trap unbound hormone, so optimum concentrations of A-RGG and NRS must be determined by titration.
In this assay, NRS at a dilution of 1:400 is included in the PBS-EDTA-NRS used to dilute the A-oLH. To assess the optimum concentration of second antibody, the following experiment was performed.

To a series of tubes were added:

**Day 1:**
- 500 µl diluent with 0, 0.5 or 10 ng NIH-LH-S19/tube
- 200 µl A-oLH, 1:40,000 in PBS-EDTA-NRS
- 100 µl $^{125}$I-oLH in diluent, ~20,000 cpm/tube.

(Tubes for estimation of non specific binding were also prepared).

**Day 2:**
- 200 µl sheep A-RGG, undiluted or diluted 1:1 - 1:100 in diluent.

**Day 3:**
- Tubes were centrifuged and the precipitates counted.

(See RIA procedure for other details).

Fig 3.2 shows the titration curves obtained by plotting the dilution of the second antibody against the percentage of added counts precipitated in the presence of 0, 0.5 or 10 ng LH/tube. The difference between the three curves is due to the inhibition of binding of tracer to A-oLH by increasing amounts of unlabelled hormone. A similar pattern is, however, evident in all 3 curves, although obscured by the large displacement of tracer in the presence of 10 ng LH/tube.

A marked prozone effect is apparent, i.e. there is decreased precipitation in the presence of excess A-RGG. With increasing dilutions of A-RGG, the amount of antigen-first antibody complex precipitated reaches a maximum and plateaus (1/20 - 1/60 A-RGG), and subsequently it decreases as the amount of A-RGG becomes insufficient to precipitate all of the product of the first reaction. A dilution of second antibody suitable for use in the RIA lies in the middle of the plateau region. A dilution of 1/40 was therefore chosen.
Fig. 3.2.

Titration of Second antibody (Sheep A-RGG) with 0.0 (●), 0.5(●), or 10.0(△) ng NIH-LH-S19/tube.

% Radioactivity precipitated

Reciprocal of dilution of Second antibody
Cross-reactivities of eutherian LH's and FSH's

A number of highly purified eutherian LH and FSH preparations were examined for their cross-reactivities with the ovine LH antiserum. Each hormone preparation was assayed over a range of concentrations and the dose-response curves are shown in Fig. 3.3. For all four LH's, ovine (NIH-LH-S19 and Papkoff G3-222B), human (LER-960) and rat (NIAMDD-rat LH-1-4) LH, about 10 ng of the preparation caused almost complete displacement of ovine LH tracer and the four dose-response curves all appeared to be parallel. This confirms the ability of this antiserum to cross-react with LH's from a number of different species.

The dose-response curves for the corresponding FSH preparations are also shown in Fig. 3.3. Up to 100 ng of either rat or human FSH displaced very little tracer LH from the antibody. Both preparations showed <1% cross-reactivity compared to NIH-LH-S19 (i.e. to displace 50% of the tracer, >100 times as much FSH was needed). The 1.0% cross-reactivity of the ovine FSH (Pap G4-150C) may be attributable to contamination with LH molecules. Up to 5 I.U. of hCG, another glycoprotein gonadotrophin, did not displace tracer from antibody (for clarity, this is not shown in Fig. 3.3).

The assay therefore appears to be highly specific for eutherian LH's, with highly purified FSH's showing little or no cross-reactivity.

Cross-reactivity of Wallaby LH

a) Dose-response curves for wallaby pituitary preparations

The cross-reactivity of wallaby pituitary LH with the ovine LH antiserum was assessed by examining the behaviour of four different pituitary preparations in the assay system. Details of these preparations were given on p. 52 They were:
Fig. 3.3 Cross-reactivities of eutherian LH's and FSH's with ovine-LH antiserum (GDN #15): dose-response curves (semi-log plots) obtained in the ovine-ovine LH radioimmunoassay.

KEY

**LH preparations** = solid lines
- NIH-LH-S19 (ovine) : ●
- Papkoff G3-222B (ovine) : O
- LER-960 (human) : ▲
- NIAMDD-rat LH-I-4 : ■

**FSH preparations** = dotted lines
- Papkoff G4-150C (ovine) : ●
- NIH-FSH-HS1 (human) : ▲
- NIAMDD-rat FSH-I-3 : ■
1) Wallaby pituitary homogenate. Ultrasound and proteolytic digestion of the pituitary gland were performed.

2) Wallaby gonadotrophin fraction. Freshly collected testis and ovary powder was dissolved in diluent and a series of dilutions was performed to give a range of concentrations for the assay.

3) Highly purified wallaby gonadotrophin. The purified hormone was used in the assay.

4) Assay for wallaby gonadotrophin. A standard curve was prepared for the assay.

5) Comparative study of wallaby and bovine gonadotrophin.

To show that the assay is valid for wallaby plasma, a number of plasma samples were analyzed.

B/B₀
1) Wallaby pituitary homogenate, diluted 1:1,000 in diluent. Volumes from 5 - 500 µl were assayed.

2) Wallaby gonadotrophin fraction J.H. 74.9 (1). The lyophylized powder was dissolved in diluent and a range of concentrations 0.5 - 80.0 ng/500 µl (i.e. 0.5 - 80.0 ng/tube) was assayed.

3) Highly purified wallaby LH, Papkoff Ex. 241.

4) Highly purified wallaby FSH, Papkoff Ex. 240D. For both wallaby hormone preparations, the lyophylized material was dissolved in diluent and diluted to give a range of concentrations 0.31 - 80.0 ng/500 µl for assay.

Fig. 3.4 shows the dose-response curves for these wallaby pituitary preparations. All four preparations contained some factor capable of displacing tracer from the antibody. The dose-response curves for the pituitary homogenate, the gonadotrophin fraction and the highly purified wallaby LH all appeared to be parallel to the NIH-LH-S19 standard curve, but due to a lower cross-reactivity the wallaby FSH curve is displaced too far to the right of the standard curve for a comparison to be made.

The crude gonadotrophin fraction J.H. 74.9 (1) is about 9% as cross-reactive as NIH-LH-S19 in the assay system. This cross-reactivity is probably attributable mainly to its LH content, as the wallaby LH fraction prepared from it was considerably more effective at displacing tracer from the antibody than was the purified wallaby FSH: the cross-reactivity of the wallaby LH was about 16% of that of NIH-LH-S19, while the wallaby FSH was <1% as potent as the standard.

b) Dose-response curves for wallaby hormones

To show that the assay is valid for measurement of LH in wallaby plasma, a number of plasma samples were assayed at different
Fig. 3.4. Dose-response curves (semi-log plots) for 4 wallaby pituitary preparations, a crude pituitary homogenate (•) J.H. 74.9(1) gonadotrophin fraction (●), highly purified wallaby LH Papkoff Ex.241 (○) and wallaby FSH Ex.240D (---) compared with the NIH-LH-S19 standard curve (●).
Dose-response curves (semi-log plots) for dilutions of plasmas from a male (•), an oestrous female (○), an ovariectomized female (●) and a hypophysectomized wallaby (□) compared with the NIH-LH-S19 standard curve (●).
dilutions. Fig. 3.5 shows the dose-response curves obtained for dilutions of these plasmas. The highest concentrations of the plasmas from a normal male wallaby, an oestrous female and an ovariectomized female all caused significant displacement of tracer. The dose-response curves for each plasma over the full range of concentrations appeared to be parallel to the standard curve. Up to 500 µl of plasma from a hypophysectomized wallaby (HWP) did not displace tracer from the antibody, providing evidence that the cross-reactive substance in wallaby plasma is of pituitary origin.

The identity of Papkoff Ex 241 as a wallaby LH fraction was inferred by its behaviour, compared to Ex 240D and eutherian gonadotrophin preparations, in the Steelman-Pohley bioassay for FSH, in bioassays based on androgen production by Leydig cell preparations, and in competitive binding assays using $^{125}$I-labelled human FSH or hCG with gonads from several eutherian mammals or opossum testis (Gallo et al., in press). This, together with the results described in (a) and (b), imply that the factor in wallaby pituitaries and plasma that cross-reacts with the ovine LH antiserum is wallaby LH. The results of this section also show that the behaviour of wallaby LH in the assay system is similar to that of ovine LH.

**Effect of plasma on the estimation of LH**

Experiments were performed to see whether plasma interferes with the estimation of LH concentrations in this heterologous assay system.

Standard curves were set up with or without 200 µl HWP in each tube (i.e. the concentrations of standard were 0.039 - 10.0 ng/300 µl, and either 200 µl diluent or 200 µl HWP was added to bring the total volume in each tube to 500 µl before the addition of antiserum). Fig. 3.6(a)
Fig. 3.6. Effects of plasma on LH measurement.

(a). Comparison of a standard curve in diluent alone (•) and a standard curve with 200 µl HWP/tube (○).
Fig. 3.6 (b).

Recovery of NIH-LH-S19 (•) or Papkoff Ex.241 wallaby LH (○) added to wallaby plasma: log-log plots of LH measured by comparison with a plasma-free standard curve versus LH added to plasma (500μl plasma/tube). See text for regression equations.

![Graph showing log-log plots of LH measured against LH added.]
shows that the standard curves obtained in the presence or absence of 200 µl HWP/tube were superimposable.

Also, the recovery of ovine LH added to wallaby plasma was assessed. Different amounts of NIH-LH-S19 were added to HWP and 200 µl of each concentration was assayed in duplicate. Recovery of ovine LH was determined by comparing the amount added to plasma (x) to the amount measured (y), the latter being calculated from the standard curve run in diluent alone. These results are shown in Fig. 3.6(b). The equation of the regression line is \( y = 1.005x + 0.001 \), and the correlation coefficient is 0.998, which shows that standard LH added to wallaby plasma is recovered quantitatively. It was not, therefore, considered necessary to include hormone-free plasma with the standards in routine assays.

When different amounts of wallaby LH (x) were added to plasma and assayed with a constant 500 µl plasma/tube, the relative amount measured in terms of NIH-LH-S19 (y) varied linearly with concentration (see Fig. 3.6(b)). The regression equation was \( y = 0.09x - 0.01 \) and the correlation coefficient was 1.00. This is further evidence that the dose-response curve for wallaby LH in plasma is parallel to the standard curve and that plasma constituents do not interfere with the quantitation of wallaby LH in this assay system.

**Inter-assay variation**

Inter-assay variation, or repeatability, was assessed by assaying the same plasma samples in several different assays. A freshly thawed aliquot of plasma was used for each assay. Table 3.1 gives the mean LH concentration in each plasma with the standard deviations and coefficients of variation of the means. The coefficient of variation gives the inter-assay variation of the estimate of LH concentration in each sample.
TABLE 3.1 Inter-assay variation in LH measurements.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>No. of assays</th>
<th>Mean LH (ng/ml)</th>
<th>Standard Deviation</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3972 (female)</td>
<td>5</td>
<td>3.1</td>
<td>0.5</td>
<td>16%</td>
</tr>
<tr>
<td>4039 &quot;</td>
<td>11</td>
<td>4.6</td>
<td>0.8</td>
<td>18%</td>
</tr>
<tr>
<td>3669 &quot;</td>
<td>10</td>
<td>20.2</td>
<td>2.0</td>
<td>10%</td>
</tr>
<tr>
<td>female pool</td>
<td>4</td>
<td>9.5</td>
<td>1.3</td>
<td>14%</td>
</tr>
<tr>
<td>male pool</td>
<td>10</td>
<td>4.8</td>
<td>0.8</td>
<td>17%</td>
</tr>
</tbody>
</table>

1 All plasmas were from castrate animals.

Within one assay, the error in fixing the zero and 2 ng LH points on the standard curve was assessed by comparing the cpm/tube for 20 replicates of each, and calculating the coefficients of variation of their means. These were 3.8% and 7.7% respectively.

LH levels in plasma of tammar wallabies

In the tammar wallaby, LH levels in plasma as measured by this heterologous RIA, vary with physiological state in a manner predictable from work on other species. Table 3.2 gives some representative values.

Table 3.2 shows that basal LH levels in plasma of normal female wallabies are very low, with many animals having LH levels at or below the level of detection of the assay (i.e.< 200 pg/ml plasma), but there are dramatic elevations of plasma LH associated with oestrus (see Chapter 5). LH levels increase markedly after ovariectomy, as can be seen from Table 3.2, but may be depressed by steroid replacement therapy (see Chapter 6 for further details). Administration of LH-RH also results in a rise in the LH concentration in plasma (see Chapter 6).
TABLE 3.2 LH levels in plasma of tammar wallabies.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of animals</th>
<th>Range of LH concentrations (ng NIH-LH-S19/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-oestrous female</td>
<td>30</td>
<td>&lt;0.2 - 1.9</td>
</tr>
<tr>
<td>Oestrous female</td>
<td>12</td>
<td>10.0 - &gt;50.0</td>
</tr>
<tr>
<td>Castrate female</td>
<td>11</td>
<td>1.7 - 7.0</td>
</tr>
<tr>
<td>After LH-RH (female)</td>
<td>15</td>
<td>9.5 - &gt;25.0</td>
</tr>
<tr>
<td>Intact male</td>
<td>15</td>
<td>0.2 - 6.0</td>
</tr>
<tr>
<td>Castrate male</td>
<td>4</td>
<td>2.2 - 8.8</td>
</tr>
<tr>
<td>Hypox (male or female)</td>
<td>8</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

1 i.e. below the level of detection of this assay

LH levels in plasma of normal male wallabies are somewhat higher than basal levels in females and are similarly elevated after castration. Plasma from hypopysectomized (hypox) wallabies has no detectable LH activity.

3.4 DISCUSSION

Assays based on Niswender's ovine LH antiserum GDN-15 and using an ovine LH tracer have been validated for measurement of LH in a wide variety of mammalian species, including the rat (Niswender et al., 1968), sheep and cow (Niswender et al., 1969), hamster (Blake, Norman and Sawyer, 1973) and the rock hyrax (Millar and Aehnelt, 1977). In addition, dose-response curves parallel to an ovine LH standard curve have been obtained with pituitary extracts and/or plasma from dog, cat, guinea-pig, elk, baboon, opossum, turtle and chicken (Midgley et al., 1971), ground squirrel, spring-hare, cheetah and several African ungulates (Millar and Aehnelt, 1977). It is believed that the majority of the antibodies in the GDN-15 antiserum may be directed against the β-subunit
of ovine LH, i.e. the hormone-specific subunit, rather than the species-specific α-subunit (Midgley et al., 1971). This would account for the great inter-species cross-reactivity of this antiserum, which suggested that it might also be suitable for measuring tammar wallaby LH.

The work presented in this chapter has confirmed that a radio-immunoassay based on GDN-15 ovine LH antiserum and using an ovine LH tracer is specific for eutherian LH's, and has shown that tammar wallaby LH also cross-reacts with the antiserum. Dose-response curves for crude or highly purified wallaby LH preparations and for wallaby plasmas with high LH concentrations appear to be parallel to the NIH-LH-S19 standard curve. While the highly purified wallaby LH preparation was about 16% as cross-reactive as the standard, highly purified wallaby FSH was <1% as potent as ovine LH. No wallaby TSH fraction was available to be tested for cross-reactivity, but none of the papers referred to in the preceding paragraphs reported any interference by TSH (or FSH) in the estimation of LH concentrations by similar assay systems using the GDN-15 antiserum. Wallaby plasma does not interfere non-specifically with measurement of LH, and inter-assay variation is comparable to those of published assays (eg. Millar and Aehnelt, 1977; Blake, Norman and Sawyer, 1973).

The LH levels in plasmas from wallabies in various physiological conditions are numerically comparable to those reported in sheep by Niswender et al. (1969), also using a GDN-15 assay.

Although the most highly purified wallaby LH preparation (Papkoff Ex 241) was only 16% as potent as NIH-LH-S19 in the assay system, it should not necessarily be concluded that the antiserum therefore has a lower avidity for wallaby LH than for ovine LH. In the present study, the wallaby LH was found to be less than twice as potent as the crude gonadotrophin fraction (J.H. 74.9(1)) from which it was prepared, but
Gallo et al., in press), using an almost identical RIA, reported it to be 36 times as potent as the starting material. This suggests that there may have been some loss of immunological activity during transport to or storage in this laboratory.

Due to the difficulty of collecting enough wallaby pituitary material for purification of reasonable quantities of LH and FSH, the work on marsupial gonadotrophins described in Chapters 3 and 4 of this thesis has been extended by Drs. F. Stewart and R.L. Sutherland, who have studied the behaviour of highly purified kangaroo LH and FSH preparations in the RIA's described here and in the radioreceptor assay (RRA's) for both LH and FSH, using either rat or Western grey kangaroo (Macropus fuliginosus melanops) testes receptor preparations (Stewart and Sutherland, manuscript in preparation).

Highly purified kangaroo LH (Papkoff WA9B) gave a dose-response curve parallel to those of highly purified ovine (G3-222B), human (LER-960) and rat (NIAMDD-rat LH-I-4) LH preparations in the LH RIA and in both kangaroo and rat testes RRA's for LH, but all LH's showed <1% cross-reactivity compared to the ovine FSH standard in the heterologous RIA for FSH. In the RRA's for FSH, the LH preparations again showed <1% cross-reactivity except for kangaroo LH, which was almost half as potent as kangaroo FSH in both the kangaroo and the rat testis RRA's. This may indicate that kangaroo LH has some inherent FSH-like biological activity, but is more likely to be due to contamination of the LH preparation with FSH which was not detectable by the RIA which is relatively insensitive to macropod FSH. These results provide strong evidence that the LH molecule of another macropod marsupial is very similar to eutherian LH molecules, with respect to both immunological and biological (receptor-binding) activity. Also, Stewart and Sutherland (manuscript in preparation) have
found that highly purified kangaroo LH preparations are almost as potent as highly purified ovine LH in the RIA for LH. This supports the idea that the low cross-reactivity of highly purified wallaby LH compared to the standard is not due primarily to a lower avidity of the antiserum for wallaby LH, but to a lower LH activity/unit weight of the wallaby material.

The main advantages of this heterologous RIA for LH over Hearn's homologous RIA for "total gonadotrophin" (Hearn, 1972) are its sensitivity (40 pg NIH-LH-S19) and its demonstrable specificity for LH. Although Hearn (1974) stated that he believed that his assay measured primarily LH, a major difference between the results obtained with the two assays is that LH levels in female wallabies are generally very low or undetectable by the heterologous RIA, reaching 10–>50 ng/ml plasma at oestrus but Hearn (1974) reported basal gonadotrophin levels of 2-5 ng gonadotrophin fraction/ml throughout the year, with peaks of 10-18 ng/ml at oestrus, i.e. the relative increase was less striking. During the present study, it has been found that the oestrous LH surge lasts only about 12 hours (see Chapter 5), so Hearn's lower values for the oestrous peaks may be due to infrequent blood sampling. However, the elevated basal levels of gonadotrophin probably reflect the impurity of the gonadotrophin fraction against which the antiserum was raised, the same preparation being used for both standard and tracer.

It is concluded that the heterologous RIA can be used to measure relative changes in LH levels in wallaby plasmas, and will be a useful tool in the investigation of the role of LH in the control of reproduction in this species.

3.5 SUMMARY

A heterologous RIA using GDN-15 ovine LH antiserum and a highly purified ovine LH tracer has been described. Ovine, rat, human and
wallaby LH preparations cross-reacted with the antiserum, but the corresponding FSH preparations did not. Dose-response curves for dilutions of wallaby plasmas and pituitary extracts appeared to be parallel to the NIH-LH-S19 standard curve, and wallaby plasma did not interfere non-specifically with the measurement of LH. The sensitivity of the assay was 40 pg NIH-LH-S19 (i.e. 200 pg/ml plasma when 200 µl plasma samples were assayed) and the inter-assay variation was 10-18%.
CHAPTER 4

A HETEROLOGOUS RADIOIMMUNOASSAY FOR TAMMAR WALLABY
FOLLICLE-STIMULATING HORMONE

4.1 INTRODUCTION

4.2 MATERIALS AND METHODS

Antiserum
Standard
Tracer
Second antibody, other hormone preparations and buffers
RIA procedure
Assay characteristics

4.3 EXPERIMENTAL PROCEDURES AND RESULTS

Cross-reactivities of eutherian FSH's and LH's
Cross-reactivity of wallaby FSH:

(a) Dose-response curves for wallaby pituitary preparations
(b) Dose-response curves for wallaby plasmas

Effect of plasma on the estimation of FSH
Inter-assay variation
FSH levels in plasma of tammar wallabies

4.4 DISCUSSION

4.5 SUMMARY
4.1 INTRODUCTION

In the selection of an assay system for FSH potentially suitable for the measurement of FSH concentrations in tammar plasma, the advantages of heterologous RIA's discussed in Chapter 1, were a major consideration.

Hodgen et al. (1976) published details of a sensitive and specific heterologous RIA for macaque monkey FSH using an antiserum against purified ovine FSH with highly purified human FSH as tracer. In their discussion, the authors refer to the cross-reactivity of this antiserum with FSH's from a number of different species. An added attraction of this assay was that the authors made available their antiserum (H-31) through N.I.A.M.D.D. This chapter describes the development of a heterologous double-antibody RIA for wallaby FSH, based upon this ovine FSH antiserum and using human FSH (NIH-FSH-HS1) as tracer. Due to the lack of a suitable reference preparation of wallaby origin, NIH-FSH-S12, an ovine FSH preparation, was used as the standard.

4.2 MATERIALS AND METHODS

Wherever possible, reagents and procedures were the same as those used in the RIA for LH described in Chapter 3.

Antiserum

Rabbit anti-ovine FSH (A-oFSH) H-31 was obtained through N.I.A.M.D.D. In the preparation of this antiserum, total doses of 30 µg of oFSH (Papkoff, GH-147c) in complete Freund's adjuvant were given to 10 rabbits by multiple intradermal injections. Eleven weeks later, the rabbits were given 10 µg of the same oFSH in incomplete Freund's adjuvant. High quality antiserum was harvested over 214 days beginning at the fourth week after the booster injection. During this time, the antibody titre remained stable without
further booster injections. The antiserum H-31 was selected for its superior sensitivity and specificity for macaque monkey FSH (Hodgen et al., 1976). The antiserum was diluted 1:25 in PBS-EDTA-NRS and stored at -20°C in 200 µl aliquots, each of which was diluted with 40 ml of PBS-EDTA-NRS for use in the assay.

**Standard**

The reference preparation was ovine FSH, NIH-FSH-S12, which has a potency of \(1.25 \times \text{NIH-FSH-S1}\) and an LH contamination of \(< 0.01 \text{NIH-LH-S1 units/mg, i.e. } <1\% \text{ LH (specifications supplied by N.I.A.M.D. D.).}\)

The lyophilyzed preparation was dissolved in PBS and stored at -20°C in 50 µl aliquots, each containing 5 µg FSH. For each assay, a freshly thawed aliquot was diluted with 5 ml diluent and this solution diluted further to prepare a range of standards, 5 - 200 ng/500 µl.

**Tracer**

Human FSH, NIH-FSH-HS1, was iodinated to a specific activity of 30 - 50 µCi/µg, using the iodination procedure given in Chapter 3 (p.53).

**Second antibody, other hormone preparations and buffers**

These were all as described for the LH assay in Section 3.2, Chapter 3.

**RIA procedure**

For convenience, the same RIA procedure was followed as for the LH double-antibody assay. The antiserum H-31 was used at a dilution of 1:5000 in PBS-EDTA-NRS, i.e. a final dilution of 1:20,000 in the assay tubes. The range of standards employed was 5-200ng NIH-FSH-S12/tube and these were prepared in diluent. About 20,000 cpm of \(^{125}\text{I-FSH}\) were added to each assay tube and the second antibody sheep A-RCC, was used in a 1:40 dilution, this optimal concentration having been determined by titration as described for the LH assay on p.53.
For routine assays, 200 µl plasma samples were assayed in duplicate.

Assay characteristics

At the dilution of antiserum used, 20-40% of the tracer was specifically bound to antibody, while non-specific binding was always <10% of this total bound radioactivity. Accumulated counts over a 5 min counting period ranged from <4,000 counts/tube for NSB to 40,000 counts/tube for Bo, so the counting error was 1.6 - 0.5%.

When plotted as log dose versus B/Bo, the standard curve was generally linear over the range 10 - 100 ng NIH-FSH-S12/tube, but non-linear regions of the curve were also used for estimating sample potencies. The sensitivity of the assay was 10 ng NIH-FSH-S12, or 50 ng/ml plasma when 200 µl plasma samples were assayed.

4.3 EXPERIMENTAL PROCEDURES AND RESULTS

Cross-reactivities of eutherian FSH's and LH's

To investigate the specificity of the assay for eutherian FSH, a number of FSH and LH preparations were tested for their cross-reactivities with the ovine FSH antiserum. A range of concentrations of each hormone were prepared in diluent and assayed as described in the RIA procedure.

The dose-response curves for 4 FSH preparations and one LH preparation, NIH-LH-S19, are shown in Fig. 4.1. The human FSH, NIH-FSH-HS1, was most effective at displacing the tracer (which was also NIH-FSH-HS1) from antibody. The highly purified ovine FSH G4-150C (Papkoff) and NIAMDD-rat FSH-I-3 were approximately equipotent but were only 20 - 25% as cross-reactive as the human FSH preparation. The dose-response curves for
Fig. 4.1. Cross-reactivities of eutherian FSH's and an LH with ovine FSH antiserum (H-31). Dose-response curves (semi-log plots) for NIH-FSH-HS1 human FSH (●), NIAMDD-rat FSH-1-3 (●), two ovine FSH's, Papkoff G4-150C (●) and NIH-FSH-S12 (●) and an ovine LH, NIH-LH-S19 (●).
the rat FSH and both ovine preparations appeared to be parallel. The second ovine FSH preparation, NIH-FSH-S12, has a relatively low FSH biological activity per unit weight (from the N.I.A.M.D.D. specifications) and was only 1% as active as the human FSH in this RIA system. However, the dose-response curve for S12, plotted as log dose versus $B/B_o$, was steep over the range 20 - 250 ng/tube, and hence it was judged to be an acceptable standard for routine assays in place of the more active preparations, which were less easily obtainable.

Up to 1 µg of ovine LH (NIH-LH-S19) did not displace tracer from the ovine FSH antiserum, as can be seen from Fig. 4.1. The same results were obtained with the highly purified ovine LH G3-222B (Papkoff), but for clarity neither these results nor those for PMSG were included in the figure. PMSG was assayed at concentrations ranging from 50 ng - 50 µg/tube. Even the highest concentrations did not cause displacement of tracer. The assay therefore appears to be specific for eutherian FSH's.

Cross-reactivity of wallaby FSH

a) Dose-response curves for wallaby pituitary preparations

The cross-reactivity of FSH in wallaby pituitaries with the ovine FSH antiserum was investigated using two wallaby pituitary preparations. These were the wallaby pituitary homogenate and the crude gonadotrophin fraction J.H. 74.9(1), details of which were given on p.52. The homogenate was diluted 1:1000 with diluent and 5 - 500 µl volumes of this were assayed. The J.H. 74.9(1) fraction was dissolved in diluent and a range of concentrations 10 ng - 10 µg was prepared for assay.

In Fig. 4.2, the dose-response curves, plotted as log dose versus $B/B_o$, for these two preparations are compared with that for the NIH-FSH-S12 standard. Over the ranges of concentrations assayed, both wallaby preparations effectively displaced tracer from the antibody, and
Dose-response curves (semi-log plots) for wallaby pituitary homogenate (•) and J.H. 74.9 wallaby gonadotrophin fraction (•) compared with the NIH-FSH-S12 standard curve (•)
Dose-response curves (semi-log plots) for dilutions of plasmas from two ovariectomized wallabies (△, □) and a castrate male wallaby (●) compared with the NIH-FSH-S12 standard curve (○).
the dose-response curves appeared to be parallel to the standard curve. The J.H. 74.9(1) gonadotrophin fraction was about 33% as cross-reactive as NIH-FSH-S12 in this assay system.

b) Dose-response curves for wallaby plasmas

For this RIA to be valid for the measurement of FSH in wallaby plasma, it was necessary to show that dose-response curves obtained when plasma samples are assayed at different dilutions are parallel to the standard curve.

Some representative dose-response curves for dilutions of wallaby plasmas are shown in Fig. 4.3. The plasmas were from two ovariectomized wallabies and one castrate male wallaby, all of which had a high concentration of FSH in the undiluted plasma. Even 500 µl of plasma with a high FSH titre would displace only 50-60% of the tracer, but the dose-response curves appeared to be parallel to the standard curve.

Up to 500 µl of plasma from a hypophysectomized wallaby did not cause any displacement of tracer from antibody, suggesting that the cross-reactive factor in the other plasmas is of pituitary origin.

The results obtained in (a) and (b) imply that wallaby FSH cross-reacts with the ovine FSH antiserum and that this assay is suitable for the measurement of FSH concentrations in wallaby plasma samples.

Effect of plasma on the estimation of FSH

To show that plasma does not interfere non-specifically in this assay system, the effect of plasma on the estimation of FSH was investigated.

Dose-response curves were constructed for the highly purified ovine FSH Papkoff G4-150C, using concentrations of 39 pg - 10 ng FSH/tube, with or without the inclusion of 250 µl HWP per tube. Fig. 4.4(a) shows
Fig. 4.4. Effects of plasma on FSH measurement.

(a) Comparison of a standard curve in diluent alone (○) and a standard curve with 250μl HWP/tube (●).
Recovery of NIH-FSH-S12 added to wallaby plasma.

Plot of FSH added (x) versus FSH measured (y) by comparison with a plasma-free standard curve.

The amount added (x) was compared with the amount measured (y) by comparison with a standard curve run in diluent alone, a regression line was fitted to these results and an intercept of 11.1 was obtained. The calculation was made by the equation $y = 0.98x + 11.1$. The intercept represents the amount of endogenous FSH present in the wallaby plasma.

The results showed no interaction when compared with standards for the determination of FSH. It was not possible to show the presence of exogenous FSH, as the wallaby plasma was free of added controls.

In a plasma with high FSH concentration (50 ng/ml) and in one where the FSH concentration (100 ng/ml) were assessed by prepurification, the recovery was 122%.

The errors within one aliquot of determinations were calculated.
shows that the curves obtained in the presence and absence of plasma do not differ significantly.

The recovery of ovine FSH added to wallaby plasma was also investigated. NIH-FSH-S12 was added to wallaby plasma to give different concentrations and 200 µl of each concentration were assayed in duplicate. Fig. 4.4(b) shows that the ovine FSH was recovered quantitatively. When the amount added (x) was compared with the amount measured (y) by comparison with a standard curve run in diluent alone, a regression line with the equation y = 0.98x + 11.1 was obtained. The correlation coefficient was 1.00. The intercept represents the amount of endogenous FSH in 200 µl of the wallaby plasma.

Since these results showed no interference by wallaby plasma in the estimation of FSH, it was not considered necessary to include hormone-free plasma with the standards for routine assays.

Inter-assay variation

Inter-assay variation was assessed by comparing the FSH concentrations measured in two different plasma samples in several different assays, freshly thawed aliquots being used in each assay. The plasmas used were from an ovariectomized female, 4039, and a pool of plasma samples from castrate males.

For nine assays, the FSH concentrations in 4039 plasma and pooled male plasma were, respectively 165 ± 25 and 212 ± 30 ng NIH-FSH-S12/ml plasma (mean ± standard deviation). For both plasmas, the inter-assay variation was 15%.

The errors within one assay of determining the FSH concentration in a plasma with low FSH concentration (<50 ng/ml) and a plasma with high FSH concentration (165 ng/ml) were assessed by comparing the cpm obtained
for replicates of each plasma within that assay. The coefficient of variation of the mean was 2.7% (n = 16) and 4.2% (n = 12) for the plasma with the low and high FSH concentration respectively.

**FSH levels in plasma of tammar wallabies**

Assay of plasma samples from female wallabies showed that FSH levels in plasmas of intact females were generally below the limit of detection of the assay (Table 4.1). Even when 10 wallabies were sampled frequently through the oestrous period, FSH levels were detectable in only 3 females, in single samples, coincident with the LH peak (see Chapter 5 for further discussion). However, after ovariectomy FSH levels are elevated consistently, as shown in Table 4.1, and may be depressed by steroid replacement (see chapter 6).

In the intact male wallaby, unlike the female, plasma FSH levels are generally above the limit of detection of the assay, and are further elevated after castration. Plasmas from hypophysectomized wallabies show no detectable FSH activity.

**Table 4.1** FSH levels in tammar wallaby plasmas

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Range of FSH concentrations (ng NIH-FSH-S12/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-oestrous female</td>
<td>30</td>
<td>&lt;50*</td>
</tr>
<tr>
<td>oestrous female</td>
<td>10</td>
<td>&lt;50 - 105</td>
</tr>
<tr>
<td>castrate female</td>
<td>11</td>
<td>180 - 600</td>
</tr>
<tr>
<td>intact male</td>
<td>12</td>
<td>&lt;50 - 460</td>
</tr>
<tr>
<td>castrate male</td>
<td>4</td>
<td>260 - 366</td>
</tr>
<tr>
<td>hypox male or female</td>
<td>6</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

* i.e. below the limit of detection of the assay.
4.4 DISCUSSION

In Chapter 1, it was pointed out that the development of RIA's for FSH has, in general, proved to be more difficult than the development of LH assays. The main problems experienced by workers in this field have been associated with the lack of sufficiently pure FSH preparations. For example, Salamonsen et al. (1973) found that several homologous assay systems based upon different ovine FSH antisera could not be used for measurement of FSH in ovine plasma because of interference in the assays by ovine albumin; this could probably be explained by the presence of labelled albumin in the tracer and antibodies to albumin in the antisera.

Contamination of the available FSH preparations also increased the problems of cross-reactivity with the other glycoprotein hormones (Butt and Lynch, 1968; L'Hermite et al. 1972). In some cases, such problems have been alleviated by pre-absorbing the antiserum with the cross-reactive hormone (e.g. Midgley et al., 1971).

A homologous RIA for bovine FSH has been characterized recently by Cheng (1978). This sensitive and specific assay is based upon a highly purified bovine FSH preparation with a biological potency of $160 \times$ NIH-FSH-S1. However, most workers have turned to heterologous RIA as a usually effective means of overcoming problems of between-hormone cross-reactivity. The heterologous RIA described by Salamonsen et al., (1973), which used human FSH antiserum with ovine FSH tracer, was potentially applicable to the measurement of FSH in sheep, pig, cow, rat and man. Other examples of heterologous RIA's for FSH include assays for equine (Evans and Irvine, 1976), bovine (Schams and Schallenberger, 1976), hamster (Bast and Greenwald, 1974) and macaque monkey (Hodgen et al., 1976) FSH, this last assay being...
the one upon which the present assay for tammar wallaby FSH is based.

With the heterologous RIA described in this chapter, dose-response curves for wallaby pituitary preparations and for dilutions of plasmas with high FSH concentrations appear to be parallel to the ovine FSH standard curve. Wallaby plasma does not interfere non-specifically with the estimations of FSH concentration, and the inter-assay variation is comparable to that of published assays (e.g. Evans and Irvine, 1976). The assay is specific for FSH.

However, basal FSH levels in plasma of intact female wallabies are usually below the limit of sensitivity of this assay (i.e. <50 ng NIH-FSH-S12/ml plasma). FSH levels do rise after ovariectomy and may be depressed by steroid replacement so presumably the hormone is present in intact females, but at very low levels. The responses to ovariectomy are discussed in Chapter 6. Even in plasma samples taken every 4 hours during an extended period around the time of oestrus (see Chapter 5, section 5.3), FSH levels remained very low. During this period, however, LH levels in plasma rose sharply, which is further evidence firstly for the lack of interference by LH in this assay and, secondly, for the existence of separate LH and FSH molecules in this marsupial.

Using the heterologous RIA's described in this thesis, Stewart and Sutherland (manuscript in preparation) have found that highly purified kangaroo (Papkoff EA11B), human (NIH-FSH-HS1), rat (NIAMDD-rat FSH-I-3) and ovine (Papkoff G4-150C) FSH preparations all caused displacement of tracer from the antibody in the FSH assay, although not all displacement curves were parallel. All four FSH preparations showed <1% cross-reactivity in RIA for LH. (The results for the eutherian hormones confirm those presented in this thesis.)
In RRA's for FSH, using either rat or kangaroo testes as the receptor source, the same FSH preparations all give parallel dose-response curves, while in corresponding RRA's for LH they showed <1% cross-reactivity. These results suggest that kangaroo FSH is similar to eutherian FSH's and is a molecular entity distinct from kangaroo LH, and, with the data presented in this thesis, support the idea that macropod marsupials possess chemically separate LH and FSH molecules.

However, it appears that the ovine FSH antiserum used here does have a lower affinity for macropod FSH's than for eutherian FSH's. This is shown, for example, by the failure of 500 µl of wallaby plasmas with the highest FSH levels to displace more than about 50% of the human FSH tracer. (For comparison, in the LH assay 100 µl of plasma from some oestrous females would cause complete displacement of tracer).

Nevertheless, it must be pointed out that the failure of this RIA to detect FSH in plasma of female tammars is also due partly to the lower levels of this hormone in females as compared to males. As shown in Table 4.1, FSH levels in plasma of male tammars are much higher, and are usually within the range 100 - 200 ng/ml in intact males (P. Catling, personal communication). Using the RIA described here, Catling and Sutherland (manuscript in preparation) have shown that FSH levels in plasma of male tammars vary with the season and the presence or absence of oestrous females, rise after castration and may then be depressed by steroid replacement. Thus this assay has been extremely useful in advancing an understanding of the roles of the gonadotrophins in the control of reproduction in the male tammar.

It must be concluded, however, that a more sensitive assay, perhaps based upon a macropod FSH preparation, will be necessary to
investigate in detail the role of FSH in the control of reproduction in the normal female tammar, in which the concentrations of FSH in plasma appear to be very low. Nevertheless, the present assay is applicable in certain experimental situations such as those described in Chapter 6 of this thesis, in which the ovariectomized female was used as a model in an investigation of some aspects of pituitary-ovarian feedback relationships in the tammar wallaby.
CHAPTER 5

GONADOTROPHIN LEVELS IN NORMAL FEMALE WALLABIES

5.1 INTRODUCTION

5.2 GONADOTROPHIN LEVELS IN PLASMA DURING THE OESTROUS CYCLE AND PREGNANCY

5.3 THE PERIOVULATORY PERIOD: TEMPORAL RELATIONSHIPS OF MATING, THE LH SURGE AND OVULATION

5.4 SEASONAL VARIATIONS IN LH LEVELS IN PLASMA OF FEMALE TAMMARS

5.5 DISCUSSION

5.6 SUMMARY
5.1 INTRODUCTION

With the RIA methods established, a study was made of the LH and FSH concentrations in plasma of normal female wallabies in different physiological conditions, i.e. during the oestrous cycle with and without intervening pregnancy, during lactational quiescence and seasonal quiescence. The aims were to attempt to relate changes in gonadotrophin levels in plasma to ovarian events in the normal wallaby and thus to provide a basis for the experiments described in Chapter 6.

5.2 GONADOTROPHIN LEVELS IN PLASMA DURING THE OESTROUS CYCLE AND PREGNANCY

Synchronous oestrous cycles were induced in 3 lactating wallabies by removal of pouch young (RPY) on 3.3.77, day 0 after RPY (d.0. RPY). The animals were known to be non-pregnant. They were kept in outdoor, sheltered pens.

Jugular catheters were inserted into two of these animals, 4102 and 4105 on d.6 RPY and they were thereafter bled daily until d.26 RPY. On d.26, a catheter was put into the third animal, 4132. It was planned that during the period d.26-d.31 RPY, when oestrous was expected to occur, blood samples (1 ml) would be taken every 3 hours from all 3 animals.

A male wallaby was introduced into the pen with 4102 and 4105 on d.26 RPY, but 4132 was kept separate as it was not intended that she became pregnant.

Unfortunately, both 4102 and 4132 lost their catheters by d.28 RPY and from then on daily blood samples only were obtained by venipuncture from the tail vein. Neither 4102 nor 4105 had mated by the morning of d.29 RPY and that afternoon a different male was substituted for the original one, which was small and perhaps immature. 4102 mated between 07.00 and 12.00 on d.30 RPY but 4105 did not mate.
All three females were bled every 2-3 days through their next cycle, which was an oestrous cycle for 4105 and 4132 and a pregnancy for 4102. 4102 gave birth on d.58 RPY, 28 days after mating.

LH levels in plasma remained at, or below, the limit of detection of the assay (200 pg/ml) during most of the oestrous cycle, and were always below 1.0 ng/ml, except in a few samples taken during the time oestrus was expected in 3/5 cycles (Fig. 5.1(a)). At these times, LH levels reached 7.0, 10.0 and 11.0 ng/ml plasma, but these concentrations were maintained for less than 24 hours.

The same pattern was observed in the pregnant cycle of 4102, i.e. very low levels of LH in plasma throughout the cycle except for a brief elevation near the time oestrus was expected.

An LH Surge was observed in plasma samples from 4105 on d.29 RPY, during the period of 3-hourly samplings. LH was not detectable in the sample taken at 01.00 but its concentration had risen to 2.0 ng/ml plasma by 07.00, reached a maximum of 10.0 ng/ml at 13.00 and had declined to 0.6 ng/ml by 19.00 (Fig. 5.1 (b)).

For the purposes of this thesis, an LH surge is defined as that period during which LH levels in peripheral plasma remain above 2.0 ng/ml. (Experience has shown that LH levels in plasma of non-oestrous female tammars rarely rise above 2.0 ng/ml). The duration of the LH surge in 4105 was, therefore, less than 12 hours. A more detailed study of periovulatory hormonal changes, described in the next section, confirmed that the pattern of the LH surge described here is typical for this species.

FSH levels in plasma of 4105, 4102 and 4132 were undetectable by the RIA (i.e.<50 ng/ml plasma) in every sample, including those in which LH levels were high.
Fig. 5.1

(a) LH levels in plasma of female wallabies during oestrous cycles and pregnancy.

- LH levels during oestrous cycles and pregnancy
- Expanded time-scale for d.29 RPY for 4105

(b) Expanded time-scale for d.29 RPY for 4105.
5.3 THE PERIOVULATORY PERIOD: TEMPORAL RELATIONSHIPS OF MATING, THE LH SURGE AND OVULATION

The previous section showed that there is a dramatic rise in the concentration of LH in plasma of tammar wallabies at or near the time of oestrus, and provided preliminary evidence that the total duration of this LH surge is about 12 hours. As discussed in Chapter 1, a similar LH surge is necessary to trigger ovulation in eutherian mammals and the time interval from the onset of LH release to ovulation is characteristic for each species. Since rising levels of oestradiol during the pre-ovulatory phase are believed to be important for the initiation of both the LH surge and behavioural oestrus (see Chapter 1), the timing of ovulation with respect to the onset of oestrus is also of interest. The experiment described in this section was designed to investigate the temporal relationships of the onset of behavioural oestrus, the LH surge and ovulation in the tammar wallaby.

Experimental design

Synchronous cycles were induced in 6 wallabies (Group 1) by RPY. The animals were brought into individual pens in the Animal House (C.S.I.R.O.), each with a male wallaby, on d.20 RPY, to accustom them to these surroundings. These pens were under natural lighting. Jugular catheters were inserted into all 6 females on d.23 and 24 RPY.

The animals were believed to be pregnant. The earliest births were expected during the night of d.25-26 RPY and the first animals expected to come into post-partum estrus about 12 hours later, on the morning of d.26 (Merchant, personal communication). They were therefore bled (2ml blood) and checked for signs of births or copulatory plugs every 8 hours from 12.00 on d.25-26: each pair of wallabies was observed briefly about
every 15 minutes for behavioural signs of imminent parturition or mating. During night-time, this was done by torchlight so each pair could be checked with minimal disturbance to the other animals. In this way, it was hoped that the time of onset of oestrus could be determined by noting the time of the animal's first mating.

However, no births or matings had occurred by d.29. It was decided that either the experimental conditions or surgery late in pregnancy may have been stressful, so the 6 females were returned to their outside pen with 2 males. They continued to be bled and checked at 08.00 and 16.00 daily and were watched unobtrusively during daylight and dusk.

When mating had been observed (2 females) or a copulatory plug found (1 female), the animal was transferred back to an indoor pen and bled every 4 hours so that the LH surge might be described more precisely. From Hearn (1972, 1974)'s data, and from the experiment described in Chapter 6 which showed that LH rises in peripheral plasma of tammar wallabies about 12 hours after oestrogen injection, it was predicted that the LH surge would begin about 12 hours after the onset of oestrus.

Ovulation was expected 12-24 hours later, i.e. 24-36 hours after the onset of oestrus. Animals were therefore autopsied at 24, 30 and 36 hours post-coitum to determine whether ovulation had occurred. The 3 animals which had not mated by d.32 were autopsied on that day.

Additional data were obtained from 3 other animals (Group 2) which were kept in outside pens with a male. To minimize disturbance, they were not catheterized and were not watched continuously. They were bled from the tail and checked at 08.00 and 16.00 daily from d.25 RPY until mated. They were then bled every 4 hours until autopsy.
In summary, animals were bled and checked for births or copulatory plugs at least twice a day from d.25 RPY. After mating, they were bled every 4 hours until autopsied at 24-60 hours post-coitum to see if ovulation had occurred.

All blood samples were assayed both for LH and for FSH.

Results

LH levels in plasma

Part of an LH surge was observed in 8/9 animals, peak LH levels measured in plasma being >23 ng/ml. In only 3/9 animals did a complete LH surge occur within the 4-hourly sampling period, and the results for these 3 animals, 3797, 4096 and 4215, showed a pattern similar to that described for 4105 in the previous section.

Fig. 5.2 shows the results for eight of the nine animals. The ninth is excluded because she did not mate, showed no evidence of any LH surge and had not ovulated by d.32 RPY. To allow more direct comparison between animals, the data for each individual have been centered upon the time of the LH peak (i.e. the highest LH concentration measured in plasma).* The results for 3797, 4096 and 4215 will be described in detail before general observations are made upon the group as a whole.

4215 was observed mating at 10.05 on d.30 RPY. Two hours before mating, LH was undetectable in her plasma. Two hours post-coitum(p.c.), LH levels were still low, but had risen to a peak of >50 ng/ml by 6 hours p.c. and returned to basal levels between 14 and 18 hours p.c. The LH surge, as defined in Section 5.2, therefore lasted about 12 hours. The animal was autopsied 24 hours p.c., i.e. 18 hours after the LH peak and a preovulatory follicle of 3.5 mm diameter was found.

* LH levels in all plasma samples taken during this experiment are given in Appendix 2.
Fig. 5.2 Temporal relationships of Mating, the LH peak and Ovulation in the tammar: results for 8 individuals.

(continued on next page)
Fig. 5.2 (continued).

-32 -24 -16 -8 0 8 16 24 32 40 48

Hours since LH peak

1 division = 10 ng NIH-LH-SG/ml plasma

4348

4488

4324

These three animals exhibited ovulatory activity during the preovulatory period. Mating occurred around

Hours since LH peak
4096 was observed mating at 08.20 on d.30 RPY. LH was undetectable in plasma samples taken 20 mins before mating and 4 hours after mating. At 8 hours p.c., the LH level was 1.0 ng/ml, but had risen to a peak of 35.0 ng/ml by 12 hours p.c. It may have increased further after this sample was taken, as it was still relatively high, 24.5 ng/ml, at 16 hours p.c.. By 20 hours p.c., however, it had fallen to 1.8 ng/ml, and had returned to basal (undetectable) levels by 24 hours p.c.. Again, the complete LH surge lasted about 12 hours. The animal was killed at 30 hours p.c. or 18 hours after the LH peak, and a 4.2 mm preovulatory follicle was found.

3797 was a Group 2 animal, and gave birth on 28.3.78, having lost its original young 20-27 days before. The animal had not mated before the new young was found, but had a copulatory plug 6 hours later - the actual mating time is not known. LH was undetectable in plasma samples taken at both these checking times, and there was only 0.7 ng/ml plasma 4 hours after the plug was found. The LH peak of 37 ng/ml was reached 8 hours after the plug was found, the LH concentration declining to basal levels over the following 12 hours. The LH surge lasted about 12 hours. The animal was autopsied 28 hours after the copulatory plug was found (i.e. 28-34 hours p.c., and 20 hours after the LH peak), and a 4.3 mm preovulatory follicle was observed.

These three animals exhibited a common pattern of events during the preovulatory period. Mating occurs sometime after the beginning of oestrus, and an LH surge follows, the peak LH levels in plasma being reached 8-12 hours after mating. The results also show that the form of the LH surge described for 4105 in Section 5.2 is typical for this species.
As previously mentioned, complete LH surges were not observed in the other animals in this experiment. However, in both 4348 and 4488, LH levels were high at the time of mating and then found to decline with time in a manner indistinguishable from that observed in the first 3 animals considered. Data from 4348 and 4488, therefore, have been combined with the data from 3797, 4096 and 4215 to give a composite diagram of the LH surge, shown in Fig. 5.3. For times before the LH peak, therefore, n = 3, but for times 0-12 hours after the LH peak n = 5.

Fig. 5.3 shows that the total duration of the LH surge, defined as in Section 5.2, in the tammar wallaby is about 12 hours. The peak level of LH in plasma, which may be >50 ng/ml, is reached within about 4 hours, with LH concentrations returning to very low, or undetectable basal levels over the following 8 hours, i.e. the rise to the peak is rapid, with a slower decay back to basal levels.

FSH levels in plasma

FSH remained undetectable in plasma of all animals at all sample times except for three isolated cases: at the time of the measured LH peak, the FSH concentration in plasma of 4363 and 4234 was 65 ng/ml and 105 ng/ml respectively, while for 4096 the plasma sample taken 4 hours after the LH peak contained 85 ng FSH/ml. (These levels are about half those measured in plasma of ovariectomized wallabies.)

Time intervals between mating and the LH peak

Table 5.1 gives the times of mating for the 8 animals in which an LH surge was detected. Where mating was not observed but diagnosed from the presence of a copulatory plug, the latest time at which the animal was checked is given, as well as the time that the plug was found.
Fig. 5.3

Changes in concentration of LH in plasma of oestrous female wallabies, centered on the time of maximum measured LH concentration.

(Mean±S.E.M., data from 5 animals)
TABLE 5.1 Time of mating, time intervals between mating and LH peak (i.e. maximum LH measured in plasma) and between LH peak and autopsy, and autopsy results for 8 wallabies killed at different times after mating (Males were present continuously from d.25 RPY). All time intervals are in hours and follicle diameters given.

<table>
<thead>
<tr>
<th>ANIMAL NO.</th>
<th>TIME OF MATING</th>
<th>MATING → AUTOPSY</th>
<th>MATING → LH PEAK</th>
<th>LH PEAK → AUTOPSY</th>
<th>AUTOPSY RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4224</td>
<td>Not mated by d.32 RPY</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>Follicle 3.5 mm</td>
</tr>
<tr>
<td>4215</td>
<td>10.00 d.30 RPY</td>
<td>24</td>
<td>6</td>
<td>18</td>
<td>Follicle 3.7 mm</td>
</tr>
<tr>
<td>4096</td>
<td>08.20 d.30 RPY</td>
<td>30</td>
<td>12</td>
<td>18</td>
<td>Follicle 4.2 mm</td>
</tr>
<tr>
<td>3797</td>
<td>Young found 10.00 d.20-27 RPY. Copulatory plug at 16.00</td>
<td>28 - 34</td>
<td>8 - 14</td>
<td>20</td>
<td>Follicle 4.3 mm</td>
</tr>
<tr>
<td>4348</td>
<td>Young found 0.800 d.28 RPY. Mated 08.15</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>CL + egg in fimbria</td>
</tr>
<tr>
<td>4363</td>
<td>Between 20.00 d.30 RPY and 08.00 d.31 RPY (plug found)</td>
<td>26 - 38</td>
<td>(-)2 - (-)14</td>
<td>40</td>
<td>CL + oviducal egg</td>
</tr>
<tr>
<td>4488</td>
<td>Not mated 08.00, d.26 RPY. Young and plug found 08.00 d.27 RPY</td>
<td>49 - 61</td>
<td>0 - 24</td>
<td>48</td>
<td>CL (no egg found)</td>
</tr>
<tr>
<td>4234</td>
<td>Not mated by d.32 RPY (silent oestrus)</td>
<td>-</td>
<td>-</td>
<td>48</td>
<td>CL + uterine egg</td>
</tr>
</tbody>
</table>
As mentioned already, 4224 did not mate, but is included here because the plasma LH level was high at the time of autopsy. 4488 also did not mate, but autopsy showed that ovulation had occurred, presumably with silent oestrus. The only animals to give birth, and thus enter a post-partum oestrus, were 3797, 4348 and 4488, which were the Group 2 animals. The insertion of jugular catheters into Group 1 animals on d.23-24 RPY probably caused failure of pregnancies: Mr. R. Young (personal communication to Dr. C.H. Tyndale-Biscoe) has found that anaesthesia with Surital after d.20 often affects the course of pregnancy in tammars.

Time intervals between mating and the measured LH peak are given in the fourth column of Table 5.1. The LH peak occurred at or after the time of mating except in 4363. However, in this animal mating was not observed but diagnosed from the finding of a copulatory plug, so that the LH surge occurred before the 4-hourly sampling began and was not, therefore, closely defined. Because mating times were not known accurately for some animals, the time intervals from mating to the LH peak ranged from -14 to 24 hours. The mean time interval was $8 \pm 3$ (S.E.M.) hours, calculated using mid-points of those intervals where the actual mating time was unknown.

**Time intervals between the LH peak and ovulation**

The results of autopsies performed at various times after the LH peak are given in the last column of Table 5.1. Up to 20 hours after the LH peak, preovulatory follicles of increasing diameter were found, but by 40 hours after the peak corpora lutea were present: ovulation, therefore, must occur between 20 and 40 hours after the LH peak. Unfortunately, there were no results for intervals between 20 and 40 hours because animals had to be killed at set times after mating, this being
immediately observable, rather than after the LH peak. However, the follicle of 4.3 mm diameter found 20 hours after the LH peak in 3797 was the largest follicle ever observed in this species (Tyndale-Biscoe, personal communication). This suggests that ovulation was likely to occur very soon and, therefore, closer to 20 hours after the LH peak.

Two animals were autopsied 40 hours after the LH peak and newly ovulated eggs were recovered from the fimbria and oviduct in 4348 and 4363 respectively. No egg was recovered from either oviduct or uterus of 4488, killed 48 hours after the LH peak. It is likely that the egg was still in the fimbria and either lodged there or was lost into the body cavity during dissection. 4234 was also autopsied 48 hours after the LH peak, and in this case the egg had reached the uterus by the time of autopsy. If ovulation occurred 20-40 hours after the LH peak, then transport of the ovum to the uterus was accomplished in 8-28 hours.

5.4 SEASONAL VARIATIONS IN LH LEVELS IN PLASMA OF FEMALE TAMMARS

As discussed in Chapter 1, the tammar wallaby is a seasonal breeder. During the non-breeding season, the females enter seasonal quiescence (which may be imposed upon lactational quiescence), but they never enter a true anoestrum — this implies that basal levels of gonadotrophin are secreted throughout the year. Hearn (1972, 1974) concluded that there was no significant difference in basal levels of total gonadotrophin measured during the breeding and non-breeding seasons in plasma of the same six female tammars. However, Hearn's results for the non-breeding season are based only upon single plasma samples taken from each wallaby in August and in October of the same year. It seemed worthwhile, therefore, to carry out further investigations into the seasonal aspects of gonadotrophin secretion in the tammar wallaby.
Fig. 5.4 LH levels in plasma of 2 female wallabies sampled throughout 1975.
A series of plasma samples taken every 2-3 days during 1975 by Dr. J. Hawkins were assayed for LH to see if there was any variation in the levels of this hormone at different seasons. FSH was not measured since it had already been found that FSH remains undetectable by the present RIA in plasma of normal female wallabies. Plasma LH levels in the two females 3872 and 3894 during 1975 are shown diagrammatically in Fig. 5.4, the numerical data being given in Appendix 3. In both animals, LH levels generally remained within the same limited range at all times of the year. This basal range was higher in 3894 than in 3872, probably reflecting between-animal variation. The peak of 5.5 ng/ml on March 19th in 3872 was probably due to an ovulatory LH surge, occurring as it did on d.27 RPY. Other smaller peaks of LH, up to 2 ng/ml, were seen in both animals at various times throughout the year, and were not obviously correlated with any physiological events.

It was interesting to find that in both animals plasma LH levels were relatively high on December 22nd. This was especially marked in 3872, in which LH reached 17.6 ng/ml plasma on this day and was also elevated (7.1 ng/ml) on December 26th. In 3894, the LH concentration in plasma was 1.6 and 0.5 ng/ml on December 22nd and 26th respectively.

**LH levels in plasma at the time of the summer solstice**

The breeding season of the tammar wallaby appears to be initiated by changing daylength at the summer solstice (Sadleir and Tyndale-Biscoe, 1977). Since the previous experiment implied that LH levels in plasma may be elevated around December 22nd, the possibility that reactivation may be associated with a rise in LH levels was investigated. LH was assayed in a series of plasma samples taken by Drs C.H. Tyndale-Biscoe and Hawkins from female tammars shot on Kangaroo Island during the nights of December 20th/21st to 28th/29th, 1974. Five
Fig. 5.5 LH levels in plasma of female wallabies shot on Kangaroo Island during the nights of December 20-29, 1974.

![Graph showing LH levels in plasma of female wallabies shot on Kangaroo Island during December 20-29, 1974. The graph includes data points for different dates and LH levels. There is a peak at 25 ng/ml.](image-url)
animals were shot each night except for the first two nights, when only four were shot.

Unfortunately, no animals were shot before the night of December 20th/21st, but LH levels were low in plasma of all four animals shot that night (Fig. 5.5). LH levels tended to be higher in animals shot on December 21st/22nd and the days immediately following, and reached 25 ng/ml in one animal killed on December 22nd/23rd. By December 28th/29th, however, LH was undetectable in 4/5 samples. There is an indication, therefore, that LH levels in plasma of female tammars are elevated at the time of the summer solstice.

5.5 DISCUSSION

It has been shown that LH, as measured by the heterologous RIA, remains very low at all times during the oestrous cycle of the tammar wallaby except for the LH surge which occurs shortly after the onset of behavioural oestrus. This pattern is the same as that reported by Hearn (1972) for the changes in "total gonadotrophin" concentration in plasma of female wallabies. This, together with the observation that FSH levels are very low at all times in normal female wallabies, supports Hearn's contention that his homologous RIA measured primarily LH (Hearn, 1974).

The LH surge was found to reach a peak about 8 hours after the onset of oestrus, here taken as being the time of the first mating in females run continuously with males. For comparison, the LH peak occurs between the 5th and 12th hours of oestrus in the ewe and during the first 8 hours of oestrus in the cow (Hansel, Concannon and Lukaszewska 1973).
However, in the six animals which mated, there was considerable variation in the time interval between mating and the LH peak. Some of this variation can be attributed to inaccuracies in the timing of the LH peaks, because of infrequent sampling, and/or mating times, when mating was not actually observed. Furthermore, in the tammar, oestrus lasts about 12 hours (Berger, 1970) and mating may have occurred near the beginning or the end of this period, although the continual presence of males suggests that mating would have occurred fairly soon after the female came into oestrus.

The time of an LH peak in the tammar cannot therefore be predicted accurately from routine daily checks for copulatory plugs or post-oestrous vaginal smears. For example, in a group of 13 animals induced to ovulate by removal of the CL (see Section 6.7), an LH peak appeared 1-3 days after mating or a post-oestrous smear in 4 animals, on the same day as such signs of oestrus in 2 animals and 1 day after a post-oestrous smear in 2 animals and 1 day after a post-oestrous smear in another animal. The daily blood sampling failed to detect an LH surge in the remaining 6 animals although all 13 had indeed ovulated. This can now be explained by the results given in Fig. 5.3, which showed that the total duration of the LH surge is about 12 hours. Thus with daily blood sampling, the probability of detecting an LH surge is 0.33-0.5 and therefore tammars must be bled at least every 8-12 hours during the period when oestrus is expected to be sure of detecting the ovulatory surge.

Sampling only every 24 hours probably explains the variability reported by Hearn (1972) in the peak concentrations of total gonadotrophin in plasma of oestrous wallabies. However, Hearn (1972) also found that for the 10 oestruses in his sample, the gonadotrophin peak was higher in mated than in unmated females, and suggested that mating stimulates the
release of more gonadotrophin. During the present study, no evidence was obtained either for or against this idea. On subjective appraisal, the LH peaks measured during the experiment described in Section 5.3, in which most of the animals mated, were consistently higher than those measured during other experiments (e.g. the LH surge described for 4105, Fig. 5.1(b)). Also, in this experiment, the lowest LH peak was recorded in 4324, which went through a silent oestrus. However, 4324 was being bled only every 8 hours (see Appendix 2), so the measured LH peak probably does not represent the highest LH level attained in plasma. Furthermore, the highest LH peak (>50 ng/ml) was recorded in the unmated 4224 on the day of autopsy.

Hearn (1972) and Tyndale-Biscoe and Rodger (1976) have observed that in the tammar ovulation has not occurred by 24 hours after mating, which would, according to the results of the present study, be about 12 hours after the LH peak. This study has shown that there is a well-defined temporal relationship between the LH surge and ovulation in the tammar, ovulation occurring 20-40 hours after the LH peak. The large size of the follicle observed 18-20 hours after the peak is circumstantial evidence that the time interval is closer to 20 hours than to 40 hours, but further experiments will be required to confirm this. With hourly blood sampling, Cumming et al. (1973) were able to show that a high proportion of ewes ovulated between 23-24 hours after the start of the LH surge, and that all ewes ovulated within 21-26 hours after this. However, in the tammar, a much smaller animal, such frequent sampling could not be continued for long periods.

The newly ovulated egg had reached the fimbria or the oviduct by 40 hours after the LH peak, and an uncleaved egg was found in the uterus of one animal autopsied 48 hours after the peak, which was 8-28
hours after ovulation. This is another reason for suggesting that
ovulation occurs about 24 hours, rather than 40 hours, after the LH
peak in the tammar wallaby. Renfree and Tyndale-Biscoe (1978) state
that in the marsupials Didelphis virginiana (American opossum),
Trichosurus vulpecula (brush possum) and Setonix brachyurus (quokka), as
well as the tammar, passage of the egg to the uterus takes only about one
day, and cleavage usually takes place in the uterus. In this respect,
marsupials differ from eutherian mammals, in most of which the egg takes
3-4 days to reach the uterus, while in the dog and cat, for example,
the period is 7 days (Boyd and Hamilton, 1961).

The FSH levels in plasma of female wallabies remained undetectable
by the RIA at all times of the cycle, although there were hints of
elevated levels at the time of the LH surge. These could not be
ascribed to cross-reactivity of LH in the FSH assay as, firstly,
detectable FSH levels were not associated with the highest LH levels
and, secondly, the cross-reactivities of eutherian LH's in the RIA for
FSH were negligible, so it is unlikely that wallaby LH would cross-react
significantly. However, until a more sensitive assay is available,
suitable for measuring FSH in plasma of female as well as male wallabies,
the role of FSH in the control of the oestrous cycle in the tammar must
remain equivocal.

No difference was seen in the patterns of LH secretion during
the breeding and non-breeding seasons, except that preovulatory LH surges
occurred only during the breeding season. Basal levels of LH fluctuated
from undetectable levels to ~2 ng/ml throughout the year. It is possible
however, that there were subtle variations undetectable by the present
assay.

It seems, therefore, that Hearn's results with his homologous
assay for "total gonadotrophin" may have given a misleading impression
of the level of "basal" gonadotrophin secretion in the tammar. Hearn states that basal levels of 2-5 ng gonadotrophin/ml plasma were secreted throughout the year, but while the sensitivity of his assay was given as 1 ng gonadotrophin/ml, he also stated that the assay could not differentiate between levels of 2 to 5 ng/ml (Hearn, 1972). Therefore the impression of a fairly high level of basal gonadotrophin secretion is probably false, especially when one considers that FSH, as well as LH, levels in plasma, as measured by the heterologous RIA's are very low indeed compared to, for example, the levels measured in ovariectomized wallabies.

The association of the time of reactivation after the non-breeding season with somewhat elevated LH levels was unexpected, as Hearn (1972, 1974) found no rise in gonadotrophin levels in plasma during reactivation after RPY. This point will not, however, be discussed further at this stage, but will be considered later in a general discussion of some aspects of the seasonality of reproduction in the tammar wallaby (see Chapter 7).

5.6 SUMMARY

LH levels in plasma of female wallabies remain very low or undetectable during the oestrous cycle and pregnancy, except for the LH surge which occurs about 8 hours after the onset of oestrus. The total duration of the surge is 12 hours and ovulation occurs within 20-40 hours of the LH peak.

FSH levels remain undetectable at all times during the oestrous cycle and pregnancy, although there were hints of slightly elevated levels coincident with the LH peak associated with ovulation.
There was no evidence for a change in the pattern of LH secretion during the non-breeding season, but there appears to be an elevation in LH levels in plasma associated with reactivation at the time of the summer solstice.
CHAPTER 6
EXPERIMENTAL MANIPULATIONS OF THE
PITUITARY-OVARIAN SYSTEM

6.1 INTRODUCTION

6.2 THE EFFECTS OF OVARIECTOMY DURING THE NON-BREEDING SEASON UPON GONADOTROPHIN SECRETION

6.3 DIURNAL VARIATIONS IN LH AND FSH LEVELS IN PLASMA OF OVARIECTOMIZED AND INTACT WALLABIES

6.4 ROLES OF THE OVARIAN CORTEX AND INTERSTITIAL TISSUE IN THE FEEDBACK CONTROL OF GONADOTROPHIN SECRETION.

6.5 EFFECTS OF STEROIDS ON GONADOTROPHIN LEVELS IN OVARIECTOMIZED AND SEASONALLY QUIESCENT INTACT WALLABIES

6.6 FOLLICULAR GROWTH AFTER HYPOPHYSECTOMY

6.7 EFFECTS OF STEROID REPLACEMENT AFTER LUTECECTOMY ON FOLLICULAR GROWTH AND THE TIMING OF OVULATION

6.8 INDUCTION OF LH RELEASE BY A SINGLE INJECTION OF OESTRADIOL -17B

6.9 HYPOTHALMIC CONTROL OF LH SECRETION: RESPONSES TO LH-RH

6.10 SUMMARY
6.1 INTRODUCTION

Although it was originally believed that the cyclicity of the hypothalamo-pituitary-ovarian system originated solely in the hypothalamus, there is now increasing evidence that in many mammalian species the ovary itself is the protagonist, gonadotrophin secretion being controlled by variations in ovarian oestral output (Karsch et al., 1973; Knobil, 1974). Such a regulatory system requires at least three characteristics: first, an appropriate sequence of negative and positive feedback actions, second, a differential feedback on LH and FSH secretion (whether or not a separate FSH-RH exists) and, third, a local, intraovarian, regulatory effect on follicle growth and maturation, separable from but interrelated to, the gonadotrophin action (Yen, 1977).

Normal circulating levels of gonadotrophins are, therefore, the result of a complex series of endocrine interactions originating in the ovary, but different ovarian compartments may contribute to this in varying degrees at different times of the cycle. To try to understand the entire system, it is therefore necessary to consider each component separately.

In this chapter, some aspects of the control of reproduction in the female tammar are explored in a series of experiments, based mainly upon the classical endocrinological approach of, first, removal of the endocrine tissue in question and, second, replacement of its putative active agent to try to reverse the effects of removal. The basic conclusions drawn from each experiment are presented at the end of each section to establish a continuity of argument, but all major discussion has been reserved for the General Discussion in Chapter 7.
6.2 THE EFFECTS OF OVARIECTOMY DURING THE NON-BREEDING SEASON UPON GONADOTROPHIN SECRETION.

The levels of LH and FSH in plasma of ovariectomized wallabies are considerably higher than in non-oestrous intact females (Tables 3.2 and 4.1), which implies that the ovary has a negative feedback effect on gonadotrophin secretion. However, the rises in gonadotrophin levels do not occur immediately after ovariectomy, and in this experiment the time-course of the hormonal changes during the three weeks after surgery was investigated.

The tammar ovary is, however, composed of three major tissues, the interstitial tissue, the corpus luteum and the cortex containing the follicles, each of which may contribute to the inhibition of gonadotrophin secretion. In another study, concurrent with the experiment described here, ovarian cortex or interstitial tissue was grafted under the pouch skin of ovariectomized animals to see if either type of graft was capable of producing steroids with an inhibitory effect on gonadotrophin secretion. The bilaterally ovariectomized animals served as controls in this experiment, which will be described in Section 6.4.

Experimental procedure

The experiment was performed in October, during the non-breeding season, using non-lactating animals. They were kept in indoor pens under natural lighting. On the first day of the experiment, d.0, animals were bled and then bilaterally ovariectomized (see Chapter 2 for surgical procedures). Daily blood samples (5 ml) were taken from d.0 to d.22, the animals being bled as near as possible to the same time every day (08:00). All plasma samples were assayed for LH and FSH and the animals were autopsied on d.44 to confirm that the ovaries had been removed entirely. Between d.22 and d.44, the animals were used in further experiments (diurnal variations and LH-RH studies) which will be described later.
Results

FSH was undetectable in plasma samples taken before and for one day after operation and then began to rise until about d.9-11. It was not, however, a continuous rise: there was considerable fluctuation from day to day in the FSH levels for each animal and this became more marked after d.9-11 (Fig. 6.1(a)). Taking these fluctuations into account, there was also a noticeable variation between animals in the highest FSH levels attained. The FSH levels in 4612 (circular symbols) were considerably higher than in the other two animals. Indeed, in 4115 (triangular symbols) FSH levels remained quite low although higher, overall, than in intact females.

LH levels remained low for 2-3 days after ovariectomy (Fig. 6.1(b)). Thereafter, the general pattern is one of marked fluctuations from day to day. This variation was greatest in 4612, LH levels in her plasma varying from undetectable levels to >40 ng/ml. However, in 4115, LH levels remained low in all samples except that on d.5.

Conclusions

LH and FSH levels in plasma of tammar wallabies rise after ovariectomy, which implies that the ovary is concerned with the negative feedback control of gonadotrophin secretion in the intact animal. However, steady levels are not maintained after these rises have occurred. FSH levels continue to fluctuate about some central level, the magnitude of which varies between animals, while LH levels may vary from <200 pg/ml to >40.0 ng/ml in consecutive daily samples.

These marked fluctuations from day to day suggested that daily blood sampling may not provide adequate information about the pattern of gonadotrophin secretion in the ovariectomized tammar, and implied that
Fig. 6.1. Changes in gonadotrophin levels in plasma of 3 wallabies, 4612(•), 4448(•) and 4115(•) sampled daily for 22 days after bilateral ovariectomy during the non-breeding season.

(a) FSH levels in plasma.

(b) LH levels in plasma.
there may be a diurnal variation in circulating gonadotrophin levels. Thus the gonadotrophin levels measured in each daily sample would depend on the time of sampling. It was decided, therefore, to look for more short-term variations in the LH and FSH levels in plasma of ovariectomized wallabies.

Experimental procedure

The five animals used in this experiment were five ovariectomized animals (4-24 after operation) and two intact, quiescent, non-lactating females, 4504 and 4513. Similar catheters were inserted in all animals prior to the experiment but only in 4504 and 4611, still retained their catheters by the day of the experiment. The other three animals were therefore bled by venipuncture from a cephalic vein. 2 ml blood was taken each time.

The first sample was taken at 07.00 and samples were then taken about every hour until 17.00. Samples were then taken until 07.00 the next morning, with final samples being taken at 07.00. The animals were kept in racks except between 14.00 and 16.00 when they were put into their pens for food and water, being returned in the late afternoon. All plasma samples were assayed for LH and FSH.

Results

In the two intact, quiescent females, LH levels were similar over the 24 hour period, but out of the range listed (Fig. 6.2).
6.3 DIURNAL VARIATIONS IN LH AND FSH LEVELS IN PLASMA OF OVARIECTOMIZED AND INTACT, QUIESCENT FEMALE WALLABIES.

To establish whether or not there was a diurnal variation in plasma gonadotrophin levels, LH and FSH levels in ovariectomized wallabies were measured at one or two hour intervals over a twenty-four hour sampling period. The changes in gonadotrophin levels in intact females were also studied, for comparison.

Experimental procedure

The five animals used in this experiment were the three ovariectomized animals (d.24 after operation) and two intact, seasonally quiescent, non-lactating females, 4504 and 4513. Jugular catheters were inserted in all animals prior to the experiment, but only two, 4115 and 4612, still retained their catheters by the day of the experiment. The other three animals were therefore bled by venipuncture, from the tail vein. 2 ml blood was taken each time.

The first sample was taken at 09.00 and each animal was bled about every hour until 17.00. Samples were then taken every 2 hours until 07.00 the next morning, with final samples at 08.00. Animals were kept in sacks except between 17.00 and 21.00, when they were released into their pens for food and water, being re-caught for the 19.00 sample. All plasma samples were assayed for LH and FSH.

Results

In the two intact, quiescent females, LH levels remained very low except for occasional slight elevations, <3 ng/ml, occurring once or twice over the 24 hour period, but not at the same times of day in both animals (Fig. 6.2).
In the ovariectomized female 4448, LH levels remained fairly steady, between 2 and 4 ng/ml plasma, with no marked elevations observed at any of the sampling times. In the other two ovariectomized animals, however, there were considerable fluctuations in the LH levels measured in successive samples (Fig. 6.2). 4115 had low circulating LH levels, <2 ng/ml, for most of the time, but there were marked elevations, up to 15 ng/ml, at certain times: this was the animal for which daily bleeds had indicated that LH levels remained very low (see Fig. 6.1). The LH levels in 4612 were also highly variable. In this case, however, the levels appeared to fluctuate about a higher base-line than in 4115. No periodicity of LH release was apparent in either animal, and the times of greatest LH release were not coincident.

FSH remained undetectable in all plasma samples from both intact females, but in the three ovariectomized females the levels varied from sample to sample (Fig. 6.3). Again, the variation was greatest in 4612. The pattern of FSH levels appeared to be similar in all three animals, with two peaks occurring during the hours of darkness, but, obviously, no conclusions as to the general diurnal pattern of FSH release can be drawn from this small sample. It is apparent, nevertheless, that there may be significant variations in the FSH levels in plasma samples taken at different times of day.

Conclusions

There is considerable variation from hour to hour in the levels of LH and FSH in plasma of ovariectomized wallabies, which probably explains the large differences in gonadotrophin levels in daily samples. There did not appear to be a regular periodicity of release of either hormone and peaks and troughs occurred at different times of day in different animals, although there was an indication that major FSH releases
occurred during the hours of darkness. Also, the ranges over which the gonadotrophin levels fluctuated varied quite markedly between animals.

In the intact animals, LH levels showed some diurnal variation, but no further information on FSH levels was obtained by the short-term sampling. This suggests that, unlike LH, FSH remains at fairly constant, very low levels in the intact female tammar, at least during seasonal quiescence. A similar differential between the patterns of release of LH and FSH has been observed in eutherian mammals (Lincoln, 1978).

The differences between the intact and ovariectomized females suggest that both LH and FSH can be synthesized and secreted in bursts, but that, as in the rhesus monkey (Dierschke et al., 1970), in the presence of ovaries this is reduced to a fairly stable, low level of secretion. The next problem, therefore, was to discover what part(s) of the ovary is responsible for this stabilization.
Fig. 6.2. Diurnal variations in LH levels in plasma of 3 ovariectomized (ovx) and 3 intact seasonally quiescent wallabies (qu.), in the non-breeding season.
Fig. 6.3. Diurnal variations in FSH levels in plasma of 3 ovariectomized wallabies in the non-breeding season.

The graph illustrates the diurnal variations in FSH levels in the plasma of 3 ovariectomized wallabies during the non-breeding season. The data show a consistent pattern of fluctuations throughout the day, with peaks at certain times.

Key:
- Axis labels: ud ( unspecified units)
- Time intervals: 09.00, 15.00, 21.00, 03.00, 09.00
- Graphs indicate the concentration of FSH in ng/ml plasma over time.

The data suggest a circadian rhythm in FSH levels, with increased levels at specific times of the day, which could be important for understanding the hormonal regulation in these animals.
6.4 ROLES OF THE OVARIAN CORTEX AND INTERSTITIAL TISSUE IN THE FEEDBACK CONTROL OF GONADOTROPHIN SECRETION.

Two experimental approaches have been used to examine the nature of the ovarian suppression of gonadotrophin secretion, both methods being based on the classic endocrinological technique of removal of the endocrine gland in question followed by replacement of the putative active element. First, different ovarian tissues were grafted into ovariectomized females, as described in this section, and, second, steroid hormones were injected in attempts to close the negative feedback control loop broken by ovariectomy.

The ovary of the tammar wallaby is composed of three major tissues (Fig. 6.4). The cortex contains follicles in various stages of development which will be described more fully in Section 6.6. Atretic follicles are also found in the cortex. These do not, however, contribute to the formation of the interstitial tissue which, in the tammar, appears as a discrete mass in the core of the ovary, approximately the same size as a quiescent CL (Renfree and Tyndale-Biscoe, 1973). The cells are arranged in densely packed cords with a rich capillary network around them. Histologically, the cells resemble lutein cells, being polygonal with large, spherical nuclei in which the nucleoli are prominent. The cytoplasm often contains highly refractory, yellow granules and is otherwise eosinophilic.

Alcorn (1976) found that the interstitial tissue of the tammar is of rete cord origin and develops post-natally, being fully differentiated by d.146 of pouch life. He suggests that it is homologous with the interstitial tissue of the Mustellidae, Ursidae and Procyonidae. However, while the tissue shows some morphological variation during the reproductive cycle of some mustelids (Wright, 1963), Renfree and Tyndale-Biscoe (1973) could not detect any change in the size of either the tissue or its component
Fig. 6.4 Section of the ovary of a tammar wallaby 20 days after RPY, during the luteal phase of the oestrous cycle, showing the three major ovarian tissues, the corpus luteum (CL), interstitial tissue (IT) and cortex (C). The cortex contains both non-atretic (NF) and atretic (AF) follicles in various stages of development, including primordial follicles (P).

Magnification: × 25 (Photo by Ivan Fox)
cells throughout the cycle of the tammar. Although it has the appearance of a secretory tissue, nothing is known of the function of the interstitial tissue in the tammar wallaby.

The third major ovarian tissue is the corpus luteum (CL), which will complete a full growth cycle during either a pregnant or a non-pregnant cycle. Development is arrested, however, during lactational or seasonal quiescence.

Each of these three tissues may produce steroids which participate in the regulation of gonadotrophin levels. The role of the CL will be discussed later, in Section 6.7. Here, the contributions of ovarian cortex and interstitial tissue will be considered. Animals were ovariectomized and pieces of either tissue transplanted under the pouch skin. If the pattern of gonadotrophin levels in these animals differed from that seen in ovariectomized animals, then it could be inferred that the grafted portion of the ovary secreted the factor responsible for the depression of gonadotrophin levels.

**Preliminary experiment during the non-breeding season**

This experiment was run concurrently with that described in Section 6.2. Nine non-lactating animals were randomly assigned to groups of three, one group being the ovariectomized animals already discussed. The other six were also bilaterally ovariectomized.

To prepare the grafts, each excised ovary was placed on a sterile petri dish and dissected under a binocular microscope, using sterilized instruments. The corpus luteum, if present, was removed and frozen for other purposes. The ovarian cortex could be peeled away quite easily from the interstitial tissue, leaving the latter as a discrete mass. Except in one case, this was transplanted whole. There always seemed to be more interstitial tissue in the ovary bearing the CL. The cortex was
examined carefully for remnants of interstitial tissue, which could be identified by their red-brown colour, and then divided usually into two pieces.

Three animals received interstitial tissue grafts and three received cortex grafts. Of the latter group, 4452 was found, unexpectedly at that time of the year, to have a new-born young, and laparotomy showed her to be in late proestrus. One ovary carried a medium follicle (diameter about 3 mm), which was included in one of her grafts.

All grafts were autotransplants. Each was placed in a separate pocket under the pouch skin, between it and the body wall, the location being noted carefully. All grafts were positioned as far as possible from the already sutured incision along the linea alba through which the ovariectomies had been performed. The area was dusted lightly with sulphanilamide powder and the skin incision closed with clips. The grafts were visible as small lumps under the thin pouch skin, and during the experiment pouches were checked regularly to monitor the state of the grafts.

The animals were held in indoor pens under natural lighting. They were bled daily (5 ml blood samples) for three weeks after operation, all plasma samples being assayed for LH and FSH. Autopsies were performed on d.44 after operation. All grafts were removed, fixed for histology and serially sectioned. The entire urogenital system (UGS) and each uterus were weighed and the uteri flushed for eggs or blastocysts, a portion of each uterus being fixed for histology.

Results

All grafts were recovered at autopsy, but none of the cortex grafts appeared to have become well-established. In 2/3 animals, no
Fig. 6.5 Histological appearance of:

1. A well-vascularized piece of interstitial tissue grafted under the pouch skin of a bilaterally ovariectomized animal.

2. cross-section of a uterus from the same animal, showing the lack of mitoses in uterine gland cells.

3. a non-functional ovarian cortex graft, with only a few small follicles present.

4. cross-section of a uterus from a bilaterally ovariectomized animal, showing the cuboidal form of the uterine gland cells.

All sections were cut at 10 µ and stained with haematoxylin and eosin.

Magnifications: grafts × 30

uteri × 180
Fig. 6.6 Histological appearance of:

1. ovarian cortex graft from 4452, showing the large atretic follicle (diameter 5 mm) formed from the medium follicle (diameter 3 mm) originally present in the graft.

2. cross-section of a uterus from 4452, showing some mitoses in the uterine glands.

3. ovarian cortex graft from 4473, showing lutein tissue.

4. cross-section of a uterus from 4473, showing hyperplasia (mitoses) of the columnar uterine gland epithelium.

All sections were cut at 10 µ and stained with haematoxylin and eosin.

Magnifications: grafts × 30

uteri × 180
cortex grafts contained more than a few primordial follicles: there had been no development of follicles although the grafts had become vascularized. The uteri of these animals were indistinguishable in weight (see Table 6.1) or histological appearance from those of the ovariectomized animals (see Fig. 6.5).

In 4452, the medium follicle included in one graft had enlarged to a diameter of 5 mm, but had become atretic (i.e. the granulosa had loosened, atretic bodies were present in the granulosa and antrum, and the oocyte had degenerated), as is shown in Fig. 6.6. Compared with those of the ovariectomized and other animals in this experiment, the uteri of 4452 were not enlarged (see Table 6.1), but some mitoses were seen in the uterine glands (Fig. 6.6), which suggests a low level of oestrogenic stimulation.

In 2/3 animals with interstitial tissue grafts, apparently healthy interstitial tissue cells (Fig. 6.5) were found in the grafts although these were surrounded by some atrophic cells. In the third animal, the sole graft had not become vascularized. Again, the uteri of these animals could not be distinguished from those of the ovariectomized controls.

Nevertheless, the weights of the UGS and the uterine weights of the ovariectomized females were not distinguishable from those of intact, seasonally quiescent females at the same time of year (Table 6.1). However, Hearn (1972) found that the uteri of hypophysectomized wallabies regressed to a much greater extent than those of ovariectomized animals, which implies that the adrenal cortex may be capable of supplying some steroid support to the uterus in the absence of the ovaries. I will show in the next experiment that in the presence of functional cortex grafts the UGS does become enlarged.
TABLE 6.1

Weights of the urogenital systems (UGS) and combined weights of the two uteri of intact, seasonally quiescent wallabies, bilaterally ovariectomized (ovaryx) wallabies and bilaterally ovariectomized wallabies with ovarian cortex or interstitial tissue grafts, in October. Italicized figures are the mean values for each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>UGS (g)</th>
<th>combined uteri (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>intact</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4235</td>
<td>9.0</td>
<td>770</td>
</tr>
<tr>
<td></td>
<td>4443</td>
<td>8.4 8.9</td>
<td>552 629</td>
</tr>
<tr>
<td></td>
<td>4516</td>
<td>9.2</td>
<td>564</td>
</tr>
<tr>
<td>ovaryx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4115</td>
<td>11.5</td>
<td>905</td>
</tr>
<tr>
<td></td>
<td>4448</td>
<td>9.2 10.5</td>
<td>563 825</td>
</tr>
<tr>
<td></td>
<td>4612</td>
<td>10.8</td>
<td>1007</td>
</tr>
<tr>
<td>cortex grafts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4328</td>
<td>7.8</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>4452</td>
<td>11.7 9.3</td>
<td>400 473</td>
</tr>
<tr>
<td></td>
<td>4517</td>
<td>8.3</td>
<td>574</td>
</tr>
<tr>
<td>interstitial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tissue grafts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3488</td>
<td>8.0</td>
<td>769</td>
</tr>
<tr>
<td></td>
<td>4323</td>
<td>7.2 7.6</td>
<td>741 716</td>
</tr>
<tr>
<td></td>
<td>4500</td>
<td>7.5</td>
<td>639</td>
</tr>
</tbody>
</table>

1 not included in the experiments described
The LH and FSH levels in plasma of all animals with either type of graft rose after operation in a manner indistinguishable from that seen in the ovariectomized animals, except that in one animal (4323) with interstitial tissue grafts FSH was undetectable in most samples. One hesitates, however, to attach much significance to this in view of the between-animal variations in gonadotrophin levels attained after ovariectomy. There were no obvious depressions of gonadotrophin levels at any stage during the three weeks of daily bleeds. These results will not be presented graphically, but, for reference, the LH and FSH levels in all three groups of animals are given in Appendix 4.

Conclusion

Because the grafts failed to develop adequately, this experiment was inconclusive about the possible roles of ovarian cortex or interstitial tissue in the control of gonadotrophin levels by the ovary. It is clear, however, that while in the cortex grafts no potentially steroidogenic tissue was present because no follicles developed, some of the interstitial tissue grafts did contain apparently healthy cells. In the latter case, then, the results fail to distinguish between two possibilities: either the tissue in the grafts was steroidogenically active but present in insufficient quantities to be effective, or the interstitial tissue does not contribute independently to the feedback control of gonadotrophin secretion by the ovary.

2. Experiment during the breeding season

Although the results of the preliminary experiment were inconclusive, this was due primarily to the technical reason that the grafts failed to become established. However, seasonal factors (perhaps a decline in the number of gonadotrophin receptors on ovarian tissues) may have
affected adversely the ability of the grafts to respond to the changed hormonal environment after ovariectomy. The experiment was therefore repeated during the breeding season, in February-March.

Experimental procedure

The same basic experimental design was followed. Fourteen animals were assigned randomly to three groups (5 + 5 + 4) and bilaterally ovariectomized. Five animals received ovarian cortex grafts under the pouch skin, and five received interstitial tissue grafts, the remaining four serving as the ovariectomized controls. One of the control animals, 4486, carried a pouch young throughout the experiment, and subsequently reared it. (This was a pilot experiment to see if suckling would affect the rises in gonadotrophin levels after ovariectomy. It did not.) All other pouch young were removed at operation. In this experiment, larger and, hence, fewer pieces of tissue were used as grafts, all animals receiving two grafts of the appropriate tissue.

The animals were again held in indoor pens with natural lighting. They were bled daily for the first 21 days after operation and then at longer intervals (2-7 days) until d.62-63 after operation. On the day, all animals with grafts were autopsied as described previously. Special note was taken of the gross appearance of the UGS and a portion of a uterus from each animal was examined histologically. Recovered grafts were fixed and serially sectioned. The ovariectomized animals were not killed. This was felt to be unnecessary because, as the autopsies proceeded, it became clear that the animals with interstitial tissue grafts could effectively be regarded as ovariectomized, due to the failure of grafts to become established.
Graft development and its effects on the urogenital system

Pertinent autopsy data from both groups of animals with grafts are summarized in Table 6.2. The interstitial tissue grafts did not take successfully. In one animal, only remnants, which could not positively be identified as interstitial tissue, were found. In the other four animals, small areas, about 0.5 mm diameter, of recognizable interstitial tissue cells were found, surrounded by connective tissue, in one graft only in each animal. The UGS's of these animals were small, both the UGS weights and the combined weights of the two uteri being similar to those of the animals in the preliminary experiment (Table 6.1). This suggests that the UGS's were not receiving any steroid support from the grafts.

On the basis of the autopsy results, the animals with cortex grafts fell clearly into two categories. In two animals, Sub-group A, the grafts did not contain any non-atretic follicles past the primordial stage, and the urogenital systems were small, similar in weight and appearance to those of the animals with interstitial tissue grafts (Fig. 6.5).

In the three animals designated sub-group B, 4234, 4473 and 4498, it was immediately clear that the UGS's were enlarged considerably, especially the lateral vaginae (Table 6.2). The uteri were about twice the weight of those of the other animals and, when sectioned (Fig. 6.6), numerous mitoses were seen in the columnar epithelium of the uterine glands. These urogenital systems, then, resembled those of normal wallabies during the late proestrous phase of the oestrous cycle.

Large follicles of about 6 mm diameter were found in one graft each from 4234 and 4498, but were atretic. In both animals, however, a smaller luteinized body was present. In 4473 one graft contained a medium
Table 6.2 Results of autopsies on d.62-63 after operation for bilaterally ovariectomized wallabies bearing either ovarian cortex or interstitial tissue grafts under the pouch skin. Animals with cortex grafts have been divided into sub-groups on the basis of the weight and appearance of the urogenital system (UGS). Mean weights of the UGS and combined uteri are given for each group and sub-group. Grafts were fixed, sectioned at 10µ and stained with haematoxylin and eosin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>Body weight (kg)</th>
<th>UGS weight (g)</th>
<th>Lateral vaginae</th>
<th>Combined uterine weight (mg)</th>
<th>Histological appearance of grafts (2 grafts per animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTERSTITIAL TISSUE GRAFTS</td>
<td>4343</td>
<td>4.3</td>
<td>9.2</td>
<td>small</td>
<td>618</td>
<td>No interstitial tissue cells found</td>
</tr>
<tr>
<td></td>
<td>4389</td>
<td>4.4</td>
<td>8.3</td>
<td>small</td>
<td>462</td>
<td>in each animal, in one graft only, a small patch of interstitial tissue cells was found, surrounded by connective tissue.</td>
</tr>
<tr>
<td></td>
<td>4420</td>
<td>3.8</td>
<td>7.9</td>
<td>small</td>
<td>498</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4424</td>
<td>3.9</td>
<td>8.0</td>
<td>small</td>
<td>509</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4444</td>
<td>4.6</td>
<td>9.1</td>
<td>small</td>
<td>723</td>
<td></td>
</tr>
<tr>
<td>Group means</td>
<td></td>
<td></td>
<td>8.5</td>
<td></td>
<td>562</td>
<td></td>
</tr>
<tr>
<td>CORTEX GRAFTS</td>
<td>4394</td>
<td>3.9</td>
<td>9.9</td>
<td>small</td>
<td>553</td>
<td>neither graft contained any non-atretic follicles past the primordial stage.</td>
</tr>
<tr>
<td>Sub-group A</td>
<td>4520</td>
<td>3.5</td>
<td>9.9</td>
<td>small</td>
<td>611</td>
<td></td>
</tr>
<tr>
<td>Sub-group means</td>
<td></td>
<td></td>
<td>9.9</td>
<td></td>
<td>582</td>
<td></td>
</tr>
<tr>
<td>CORTEX GRAFTS</td>
<td>4234</td>
<td>3.8</td>
<td>16.0</td>
<td>enlarged</td>
<td>1040</td>
<td>1 CL and 1 large, atretic follicle</td>
</tr>
<tr>
<td>Sub-group B</td>
<td>4473</td>
<td>5.0</td>
<td>12.0</td>
<td>enlarged</td>
<td>1137</td>
<td>1 CL and 1 medium normal follicle</td>
</tr>
<tr>
<td></td>
<td>4498</td>
<td>3.7</td>
<td>16.0</td>
<td>enlarged</td>
<td>1055</td>
<td>1 CL and 1 large, atretic follicle</td>
</tr>
<tr>
<td>Sub-group means</td>
<td></td>
<td></td>
<td>14.7</td>
<td></td>
<td>1077</td>
<td>(in this sub-group, all grafts had some non-atretic secondary follicles)</td>
</tr>
<tr>
<td>Group means</td>
<td></td>
<td></td>
<td>12.8</td>
<td></td>
<td>879</td>
<td></td>
</tr>
</tbody>
</table>
follicle diameter, 3.1 mm with numerous mitoses in its granulosa and theca interna. The other graft bore a corpus luteum, diameter 4.0 mm, with a large central lacuna (Fig. 6.6).

(Note: it is not clear whether these structures should be classified as corpora lutea or luteinized follicles. In 4234 and 4498, the luteinized body was much smaller than the atretic Graafian follicle and roughly oval, rather than rounded. No oocytes were found in serial sections, which suggest that ovulation had occurred. It is possible that in 4234 and 4498, preovulatory enlargement of the follicle may have been hampered physically by the close apposition of the graft to the body wall. In all cases, the luteinized granulosa cells still formed a distinct layer around the antrum and the thecal layers were intact. However this is also seen in "normal" corpora lutea for a week after ovulation (C.H. Tyndale-Biscoe, unpublished observations).

In animals with cortex grafts, then, an enlarged UGS was associated with the presence of enlarged follicles and lutein tissue in the grafts.

Gonadotrophin levels in plasma

All plasma samples were assayed for LH and for FSH. Each week, the samples collected during that week were assayed to monitor changes in gonadotrophin levels during the course of the experiment. The results presented, however, are from a single subsequent assay for each hormone in which aliquots of all plasma samples were assayed to eliminate any possibility of bias due to inter-assay variation.

Fig. 6.7(a)-(f) illustrates the changes in LH and FSH levels in the circulation of the bilaterally ovariectomized group and the group of wallabies with ovarian cortex transplants, divided, as before, into subgroups A and B. Except for somewhat more variation during the first nine
Fig. 6.7 Changes in LH and FSH levels in plasma of bilaterally ovariectomized wallabies, with or without ovarian cortex grafts under the pouch skin, for d.0–d.47 after operation during the breeding season.

The diagrams on the following three pages are:

(a) LH levels in ovariectomized animals

(b) FSH levels in ovariectomized animals

(c) LH levels in animals with non-functional cortex grafts (Sub-group A)

(d) FSH levels in animals with non-functional cortex grafts

(e) LH levels in animals with functional cortex grafts (Sub-group B)

(f) FSH levels in animals with functional cortex grafts.

Numerical data are given in Appendix 5 with data from ovariectomized animals with interstitial tissue grafts, which has not been presented graphically.
In the ovaries, the results for the selected hormone levels for each animal are given in Appendix A. There was some variation in the LH concentrations. This pattern was also seen in relation to the day of operation. Apart from the initial increase in day 1, they began to decline in day 15 and did not rise again. It is suggested that the levels of 6.5-9.5 ng LH were not due to hyperstimulating drugs but rather to the ovaries. Consequently, no significant level of LH would have been evident by day 20. Their autopsy results were, therefore, not significant.
days after surgery, the results for the animals with interstitial tissue grafts were not distinguishable from those of the ovariectomized group, and have not been presented graphically. However, the numerical data from all groups are given in Appendix 5.

In the ovariectomized animals, LH levels remained undetectable for about 9 days after operation, then rose over the next 5 days to moderate levels, usually <5 ng/ml plasma (Fig. 6.7(a)). Compared with the animals ovariectomized during the non-breeding season (Fig. 6.1(b)), the LH levels for each animal did not fluctuate so markedly from day to day. There was some variation, but the LH concentrations tended to remain at moderate levels.

This pattern was also seen in sub-group A animals with cortex grafts. There was a slow rise in LH levels after about d.9, with fairly steady, moderate levels attained by about d.15 after operation (Fig 6.7 (c))

For the sub-group B animals, the pattern was initially the same (Fig. 6.7(e)). Apart from the single small peaks of 3.6 and 4.8 ng/ml on d.3 and d.5 in different animals, LH levels remained low until about d.9. After this, they began to rise in 4234 and 4473, reaching moderately high levels of 4.5-9.5 ng/ml, and remaining within this range until d.34-40. In 4473, circulating LH then declined to low levels, <3 ng/ml. In 4234, the levels were still moderately high on d.48, but in both animals LH was undetectable in plasma at the time of autopsy on d.63.

Unfortunately, no samples were taken between d.48 and d.63 as it was thought that any effects of the grafts upon LH (or FSH) levels would have been evident by d.48. In 4234 and 4473 this was not the case, and their autopsy results were, therefore, unexpected.
Nevertheless, LH levels in plasma of 4498 did remain low, below
3 ng/ml, throughout the experiment except for the isolated peak of 4.8 ng/ml
on d.5 (Fig. 6.7(e)). The levels did rise somewhat after surgery, but
decayed after d.17, and low or undetectable levels were then maintained
until autopsy. LH was undetectable in plasma of all three sub-group B
animals at the time of autopsy.

The changes in FSH levels in the ovariectomized animals initially
followed a pattern similar to that described for LH: there were small
increases during the first week after operation with more dramatic rises on
d.11-13 (Fig. 6.7(b)). There was little overall change between d.13 and
d.27, but further increases occurred from d.27-29, after which the levels
remained elevated, though fluctuating, with greater variation between
animals.

Similar changes in FSH levels were seen in Sub-group A animals
with cortex grafts (Fig. 6.7(d)) and in those with interstitial tissue
grafts (Appendix 5). In all groups, there appeared to be a biphasic rise
in FSH levels, with substantial increases on about d.11-13 and d.26-28.

In the Sub-group B animals with active cortex grafts, the changes
in FSH levels after surgery (Fig 6.7(f)) presented somewhat different
profiles to that described above. In 4473, the pattern was similar to
that seen in ovariectomized animals for the first 29 days, but between
d.29 and d.41 the levels decreased to below the sensitivity of the assay
and remained so until autopsy. FSH levels in plasma of 4498 also rose
sharply from d.0 to d.11, with a sharp peak on d.13. Thereafter, however,
there was a sustained decline until, by d.27, FSH was undetectable in the
plasma, remaining so until autopsy. In 4234, on the other hand, plasma
FSH levels rose until d.13 after operation and remained elevated except
in the sample taken at autopsy. In all three animals, then, the changes
in FSH levels in each individual followed a pattern similar to that of the LH levels.

In summary, in the ovariectomized animals, in those with interstitial tissue grafts and in the two with underdeveloped cortex grafts, plasma LH and FSH levels rose by about 11 days after operation and thereafter remained elevated, despite some fluctuation. FSH levels appeared to increase further on about d.26. In two of the three animals with cortex grafts containing lutein tissue, LH and FSH levels rose initially, but returned to pre-castrate levels by 4-5 weeks after operation. In the third animal of this sub-group, 4234, gonadotrophin levels increased after operation and remained elevated until d.47, but both LH and FSH were undetectable in plasma collected at autopsy.

Conclusions

First, this experiment has suggested that the responses to ovariectomy during the breeding season are slightly different to those during the non-breeding season, this being most noticeable when the changes in LH levels are compared. During the non-breeding season, LH levels began to fluctuate from undetectable to fairly high levels two days after ovariectomy, but in the breeding season LH remained undetectable for about nine days after operation. Both LH and FSH levels showed less within-animal variation during the breeding season, but the diurnal variations were not measured at this time of year. The results suggested that the hypothalamo-pituitary system may become more sensitive to steroid feedback during the non-breeding season when, presumably, circulating steroid levels are low, so that the system responds more quickly to their withdrawal.

The main purpose of the experiments, however, was to investigate the possible roles of the ovarian cortex and interstitial tissue in the
feedback control of gonadotrophin secretion by the ovary. No conclusions can be drawn about the role of interstitial tissue because the grafts of this tissue were not successful. The reason for this is unknown, but poor vascularization may have been a contributing factor, and it is suggested that in any future experiments grafts should be placed under the kidney capsule, although this has the disadvantages of requiring extra surgery and not allowing grafts to be examined during the experiment. However, Ingram (1957) found that in the rabbit autografts of ovarian medulla, containing primarily interstitial cells, could not survive in the absence of ovarian cortex. It is possible that interstitial tissue of the tammar ovary is also unable to survive autonomously.

Follicular development in cortex grafts was associated with an enlarged urogenital system and proliferation of the uterine epithelium. By analogy with eutherian species, this implies that an oestrogenic hormone(s) was being secreted, presumably by the lutein tissue present in these functional grafts. It is possible, however, that the urogenital systems had remained enlarged due to the effects of oestrogens secreted by the follicle before luteinization (in Table 6.7 it can be seen that the urogenital systems of recently ovulated animals were larger than those of animals in early prooestrus, with enlarging follicles). Large atretic follicles were also present in grafts of two animals, but these are less likely to have been the source of the active steroids.

Both LH and FSH were depressed to pre-castrate levels in the presence of functional cortex grafts. Because the levels were low at autopsy, it is clear that some product of the lutein tissue was inhibiting gonadotrophin secretion, even if the preovulatory follicle had been responsible for oestrogenic stimulation of the urogenital systems. It seems likely, however, that a feedback control system had developed before
luteinization, in order to produce ovulatory surges of LH, although no evidence of such surges was found here. In ovariectomized rats, 4-6 day cycles begin as subcutaneous ovarian autografts become established and start producing oestradiol-17β and progesterone, and normal patterns of LH and FSH secretion have been observed (Chihal et al., 1976). In their experiments also, no positive evidence of ovulation was found although many follicles became luteinized.

In the next section, the possible nature of the ovarian steroid(s) involved in the feedback control of gonadotrophin levels in the tammar wallaby will be considered.
6.5 EFFECTS OF STEROIDS ON GONADOTROPHIN LEVELS IN OVARIECTOMIZED AND SEASONALLY QUIESCENT, INTACT WALLABIES

It has been shown that the ovary exerts a negative feedback control on gonadotrophin secretion in the tammar, and that luteinized granulosa cells are one probable source of the effective agents, which, it may be assumed, are steroid hormones.

Since there was little information available about steroid production by the tammar ovary, the possible identity of the active steroid(s) was uncertain. However, it is known that the tammar CL produces progesterone in vivo (Lemon, 1972; Hinds, unpublished results), while in vitro studies have shown that phenolic steroids, probably oestrogens, are also produced (Renfree and Heap, 1977). Furthermore, the appearance of the urogenital systems, with hyperplasia and hypertrophy of the uterine gland cells, in animals with luteinized cortex grafts suggested that these grafts were producing oestrogenic hormones. In an exploratory study, then, various steroids were tested for their ability to depress gonadotrophin levels in ovariectomized wallabies. The effects of the same steroids on gonadotrophin levels in intact, seasonally quiescent wallabies were also investigated.

Experimental procedure

This experiment was carried out during the non-breeding season. Three long-term (one year) ovariectomized wallabies and three intact, non-lactating, seasonally quiescent wallabies were used. All animals were treated with each different steroid, being left for at least five days between treatments. The steroids used and the dose at which each was administered are given below. The doses of oestradiol-17β and progesterone were the same as were used in the lutectomy experiment described in Section 6.7. Doses of the other steroids were decided arbitrarily after consultation of the literature for doses used by workers.
on other species. In each case, the crystalline steroid (Sigma products) was dissolved in the minimum amount of absolute ethanol before being added to the olive oil.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Dose (µg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oestradiol-17β</td>
<td>5</td>
</tr>
<tr>
<td>progesterone</td>
<td>700</td>
</tr>
<tr>
<td>17α-hydroxy progesterone</td>
<td>500</td>
</tr>
<tr>
<td>testosterone</td>
<td>700</td>
</tr>
<tr>
<td>dihydrotestosterone</td>
<td>700</td>
</tr>
<tr>
<td>(oil)</td>
<td>(0.1 ml/kg body weight)</td>
</tr>
</tbody>
</table>

The animals were kept in small, outdoor, grassy pens and were caught whenever a blood sample was required. At 08.00 on the day of each individual experiment, all animals were caught, bled (3 ml blood samples) and injected intra-muscularly with a single dose of the appropriate steroid. They were then bled at 3, 6, 9, 12 and 24 hours after injection. In a control experiment, animals were injected with oil alone, 0.1 ml/kg body weight. Plasma samples were assayed for LH and FSH.

Results

For the ovariectomized wallabies, the results (shown in Fig. 6.8 on two consecutive pages) suggest that, at the doses used here, oestradiol-17β, progesterone, testosterone and dihydrotestosterone all have some inhibitory effect on LH secretion: LH levels were depressed after steroid treatment, and remained low, without the fluctuations normally seen in ovariectomized wallabies (see Fig. 6.2), for up to 24 hours after injection. It is also clear that 17α-hydroxyprogesterone has no such effect.

Because the normal levels of LH in plasma of intact females are so low, it was not possible to determine the effects of the steroid treatments. It does appear that LH levels were depressed after injection of
Fig 6.8 Changes in LH levels in plasma of three ovariectomized and three seasonally quiescent wallabies following single injections (i.m., in oil) of:

Page 1. eestradiol-17β (E) 5 µg/kg body weight
progesterone (P) 700 µg/kg " "
17α-hydroxyprogesterone (OH-P) 500 µg/kg " "

Page 2. oil 0.1 ml/kg " "
dihydrotestosterone (DHT) 700 µg/kg " "
testosterone (T) 700 µg/kg " "

Plasma samples were taken at 0, 3, 6, 9, 12 and 24 hours after injection.
Fig. 6.9 Changes in FSH levels in plasma of three ovariectomized wallabies following single injections (i.m. in oil) of:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>oestradiol-17β (E)</td>
<td>5 µg/kg body weight</td>
</tr>
<tr>
<td>progesterone (P)</td>
<td>700 µg/kg</td>
</tr>
<tr>
<td>17α-hydroxyprogesterone (OH-P)</td>
<td>500 µg/kg</td>
</tr>
<tr>
<td>oil</td>
<td>0.1 ml/kg</td>
</tr>
<tr>
<td>dihydrotestosterone (DHT)</td>
<td>700 µg/kg</td>
</tr>
<tr>
<td>testosterone (T)</td>
<td>700 µg/kg</td>
</tr>
</tbody>
</table>

Plasma samples were taken at 0, 3, 6, 0, 12 and 24 hours after injection.
testosterone, dihydrotestosterone or progesterone (Fig. 6.8), but were also very low after injection of oil alone.

After oestradiol treatment, on the other hand, one intact animal showed a substantial rise in plasma LH levels 6-9 hours after injection. The maximum LH concentration measured, 26.0 ng/ml, was about half the magnitude of peaks recorded during oestrus (see Chapter 5).

Assay of FSH in samples from the intact females demonstrated once again that, with this assay, FSH is normally undetectable in plasma of female tammars, and so these results have not been presented.

In the ovariectomized animals, no steroid treatment caused a consistent, marked depression of plasma FSH levels. Indeed, the levels fell, presumably due to diurnal variation, over the 24 hours after injection of oil alone, and hence it is difficult to assess whether any of the treatments were effective. 17α-hydroxyprogesterone, testosterone and dihydrotestosterone probably did not depress FSH levels, the sharp drop observed in one animal (3669, circular symbols) after dihydrotestosterone treatment being attributable to the large peak of FSH, representing diurnal variation, which was detected by the first sample. Oestradiol-17β and progesterone appeared to have some inhibitory effect on plasma FSH levels (Fig. 6.9).

Conclusions

This experiment has shown that oestradiol-17β, progesterone, dihydrotestosterone and testosterone are potentially capable of contributing to the negative feedback control of LH levels by the tammar ovary. The ability of testosterone to depress plasma LH levels is not attributable solely to its potential as an oestrogen precursor, since dihydrotestosterone, its active metabolite, which cannot be aromatized, is also effective.
Besides its inhibitory effect on LH levels in ovariectomized wallabies, oestradiol-17β may also have a stimulatory, or positive feedback, effect on LH secretion in the intact female. This point will be explored further in the experiment described in Section 6.8.

None of the steroids had a marked effect on plasma FSH levels in the ovariectomized wallabies, which suggests that FSH secretion is not as sensitive to the negative feedback effects of steroids as is LH secretion: the opposite has been reported in women (Yen and Tsai, 1971). Oestradiol-17β and progesterone appear most likely to be involved in the ovarian regulation of plasma FSH levels, but further experiments, using different doses and sampling schedules, will be necessary to clarify this.
6.6 FOLLICULAR GROWTH AFTER HYPOPHYSECTOMY

Introduction

The interaction between the ovary and the pituitary is a two-way relationship: ovarian steroid hormones control gonadotrophin secretion while LH and FSH regulate various aspects of ovarian function.

It is known that, in the tammar, full development of a quiescent CL is initiated by hypophysectomy, and that pregnancy will continue to term. However, oestrus and ovulation do not occur after luteal regression in the hypophysectomized wallaby (Hearn, 1972; 1974), which suggests that follicular maturation ceases after hypophysectomy. A study was made, therefore, of the numbers and classes of follicles present in the ovaries of hypophysectomized wallabies, 21 days after operation, compared to those in intact animals. The aim was to try to determine which stages of follicular maturation were affected by hypophysectomy.

Procedure

Ovaries embedded in paraffin wax were supplied by Dr C.H. Tyndale-Biscoe. Seven ovaries (3 pairs plus 1) were from hypophysectomized wallabies autopsied 21 days after operation during the non-breeding season. Three animals were estimated to be at the d.21 stage of pregnancy. Similar luteal development had occurred in the fourth animal, 4100, but pregnancy had not proceeded further than blastocyst expansion. Control ovaries (3 pairs plus 1) were chosen from sham-operated animals in which, for unknown reasons, the CL had reactivated. Thus it was possible to compare follicular development during cycles induced during the non-breeding season in intact and in hypophysectomized wallabies.

Ovaries were serially sectioned at 10 µ and stained with haematoxylin and eosin. Every section was examined, and all follicles except primordial follicles were assigned to one of six arbitrarily chosen
classes, defined as in Table 6.3. Classification was on the basis of morphological criteria, but the diameters of the follicles within one class tended to fall above or below a certain limit, as given in the table.

Table 6.3. Classification of follicles in tammar wallaby ovaries according to state of maturation, based on morphological criteria.

<table>
<thead>
<tr>
<th>Class</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>single layer of cuboidal to columnar granulosa cells.</td>
</tr>
<tr>
<td>M1</td>
<td>2 layers of granulosa cells, follicle diameter $\leq 50$ $\mu$m.</td>
</tr>
<tr>
<td>M2</td>
<td>2 layers of granulosa cells but no evidence of antrum formation, follicle diameter $&gt; 50$ $\mu$m.</td>
</tr>
<tr>
<td>A1</td>
<td>beginnings of antrum formation and development of theca interna, follicle diameter $\leq 80$ $\mu$m.</td>
</tr>
<tr>
<td>A2</td>
<td>antrum fully formed and theca interna well developed, $80$ $\mu$m &lt; diameter $\leq 100$ $\mu$m.</td>
</tr>
<tr>
<td>L</td>
<td>large follicle, antrum considerably enlarged, follicle diameter $&gt; 100$ $\mu$m. Further classified as normal or atretic, as defined below.</td>
</tr>
<tr>
<td>Early atresia:</td>
<td>A few atretic bodies at the inner edge of the granulosa, in which a few pycnotic nuclei, but also some mitoses, may be seen.</td>
</tr>
<tr>
<td>Advanced atresia:</td>
<td>numerous atretic bodies and granulosa cells showing signs of disintegration, no mitoses. Basement membrane between granulosa and theca interna no longer intact.</td>
</tr>
<tr>
<td>Late atresia:</td>
<td>granulosa almost wholly or completely disintegrated. Oocyte degenerating or already lost.</td>
</tr>
</tbody>
</table>

Also, the three largest follicles in each ovary were examined in detail. Using a micrometer eyepiece, the following measurements were taken from the section in which the area of the follicle was greatest:

i) total diameter of follicle, including thecal layers

ii) diameter across granulosa only.
In each case, the greatest diameter and the diameter at right angles to that were measured. Furthermore, the follicles were classified as normal or atretic (early, advanced or late atresia), as defined in Table 6.3, this classification being based on that of Turnbull, Braden and Mattner (1977).

Also, the numbers of mitoses seen in the granulosa and theca interna were counted for the section on which the measurements were made.

Results

1. Distribution of follicles among the classes defined in Table 6.3 in ovaries of hypophysectomized or sham-operated animals

It was clear that by 21 days after operation no class of follicles had been affected markedly by hypophysectomy (Table 6.4. Data for the single ovary from the sham-operated animal 4128 are not included because some slides were lost). Although there was considerable variation between animals of each group in the actual numbers of follicles counted (the low numbers in 4100 probably reflecting the small size of the ovaries), the relative distribution of follicles among the different classes was similar in the two groups of animals. Furthermore, there were still mitoses occurring in follicles of hypophysectomized wallabies. Mitoses were seen in granulosa cells in follicles of all classes and in both granulosa and theca interna of larger follicles. It should be pointed out, however, that often many mitoses may be seen in the outer layers of the granulosae of large follicles already showing signs of atresia (see examples in Table 6.5).

While studying the follicles, it was also noted that the interstitial tissue of the hypophysectomized animals was not distinguishable from that of sham-operated animals.
Table 6.4 Distribution of follicles among the classes defined in Table 6.3 in ovaries of sham-operated or hypophysectomized wallabies, 21 days after operation during the non-breeding season.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Stages of pregnancy</th>
<th>Ovaries (mg)</th>
<th>CL (mg)</th>
<th>Ovary</th>
<th>CL or LF2</th>
<th>G</th>
<th>M1</th>
<th>M2</th>
<th>A1</th>
<th>A2</th>
<th>L</th>
<th>Atretic</th>
<th>Total no. with mitoses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SHAM-OPERATED</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3299</td>
<td>d.17-20</td>
<td>226</td>
<td>26</td>
<td>left</td>
<td>CL</td>
<td>133</td>
<td>83</td>
<td>27</td>
<td>32</td>
<td>39</td>
<td>12</td>
<td>28</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>right</td>
<td>LF</td>
<td>293</td>
<td>65</td>
<td>119</td>
<td>26</td>
<td>42</td>
<td>35</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>3314</td>
<td>d.21</td>
<td>246</td>
<td>18</td>
<td>left</td>
<td>CL</td>
<td>46</td>
<td>42</td>
<td>96</td>
<td>47</td>
<td>23</td>
<td>20</td>
<td>8</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>right</td>
<td>LF</td>
<td>68</td>
<td>182</td>
<td>100</td>
<td>52</td>
<td>51</td>
<td>29</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td>3851</td>
<td>d.0 (blastocyst)</td>
<td>270</td>
<td>35</td>
<td>left</td>
<td>LF</td>
<td>50</td>
<td>26</td>
<td>34</td>
<td>20</td>
<td>19</td>
<td>24</td>
<td>25</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>right</td>
<td>CL</td>
<td>43</td>
<td>39</td>
<td>48</td>
<td>26</td>
<td>27</td>
<td>15</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td><strong>HYPOPHYSECTOMIZED</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3682</td>
<td>d.21</td>
<td>265</td>
<td>39</td>
<td>left</td>
<td>CL+LF</td>
<td>54</td>
<td>60</td>
<td>125</td>
<td>39</td>
<td>44</td>
<td>28</td>
<td>24</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>right</td>
<td>LF</td>
<td>24</td>
<td>73</td>
<td>108</td>
<td>17</td>
<td>57</td>
<td>23</td>
<td>12</td>
<td>53</td>
</tr>
<tr>
<td>3806</td>
<td>d.21</td>
<td>172</td>
<td>37</td>
<td>left</td>
<td>LF</td>
<td>22</td>
<td>29</td>
<td>65</td>
<td>13</td>
<td>25</td>
<td>11</td>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>right</td>
<td>CL</td>
<td>75</td>
<td>30</td>
<td>69</td>
<td>13</td>
<td>11</td>
<td>3</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>4100</td>
<td>? (expanding blastocyst)</td>
<td>101</td>
<td>34</td>
<td>left</td>
<td>CL+LF</td>
<td>10</td>
<td>5</td>
<td>11</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>right</td>
<td>LF</td>
<td>58</td>
<td>12</td>
<td>15</td>
<td>8</td>
<td>9</td>
<td>1</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>4106</td>
<td>d.21</td>
<td>95</td>
<td>35</td>
<td>left</td>
<td></td>
<td>165</td>
<td>55</td>
<td>64</td>
<td>16</td>
<td>24</td>
<td>8</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>

**NOTES:**
1. combined weight of ovaries after removal of CL
2. indicates which ovary had the CL and which had the largest follicle (LF)
3. the number of follicles of all classes in which at least one mitosis was observed (not recorded for 3299, right ovary).
Table 6.5 Total diameters, diameters across granulosa and number of mitoses in granulosa and theca interna in largest cross-sections of the three largest follicles in ovaries of hypophysectomized or sham-operated wallabies, 21 days after operation during the non-breeding season. Follicles are classified as normal or atretic (at.) as defined in Table 6.3, and the largest follicle in each animal is asterisked.

(a) Hypophysectomized animals

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Ovary (±CL)</th>
<th>Total diameter (mm)</th>
<th>Granulosa diam. (mm)</th>
<th>No. of mitoses in granulosa and theca int.</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>3682</td>
<td>left</td>
<td>*0.70 × 0.53 0.64 × 0.45</td>
<td>4</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.60 × 0.52 0.49 × 0.42</td>
<td>5</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.56 × 0.50 0.49 × 0.52</td>
<td>8</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>0.54 × 0.45 0.44 × 0.40</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.46 × 0.46 0.38 × 0.36</td>
<td>3</td>
<td>3</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.53 × 0.40 0.50 × 0.35</td>
<td>5</td>
<td>1</td>
<td>normal</td>
</tr>
<tr>
<td>3806</td>
<td>left</td>
<td>*0.61 × 0.35 0.48 × 0.28</td>
<td>5</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.46 × 0.46 0.50 × 0.43</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.53 × 0.40 0.50 × 0.35</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>0.54 × 0.47 0.49 × 0.41</td>
<td>10</td>
<td>2</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.54 × 0.38 0.50 × 0.32</td>
<td>0</td>
<td>0</td>
<td>late at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.53 × 0.46 0.47 × 0.43</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td>4100</td>
<td>left</td>
<td>*0.70 × 0.53 0.64 × 0.45</td>
<td>4</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.60 × 0.52 0.49 × 0.42</td>
<td>8</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.56 × 0.50 0.52 × 0.49</td>
<td>5</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>0.54 × 0.45 0.44 × 0.40</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.46 × 0.46 0.38 × 0.36</td>
<td>3</td>
<td>3</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.34 × 0.31 0.33 × 0.30</td>
<td>5</td>
<td>1</td>
<td>normal</td>
</tr>
<tr>
<td>4106</td>
<td>left</td>
<td>*0.50 × 0.46 0.44 × 0.40</td>
<td>4</td>
<td>1</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td>(CL was on right)</td>
<td>0.50 × 0.37 0.45 × 0.34</td>
<td>2</td>
<td>1</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 × 0.43 0.37 × 0.35</td>
<td>3</td>
<td>0</td>
<td>normal</td>
</tr>
</tbody>
</table>

(continued on next page)
Table 6.5 (b) Sham-operated animals

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Ovary (±CL)</th>
<th>Total diameter (mm)</th>
<th>Granulosa diam.(mm)</th>
<th>No. of mitoses</th>
<th>Granulosa theca int.</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>3299</td>
<td>left</td>
<td>0.86 x 0.70</td>
<td>0.79 x 0.64</td>
<td>12</td>
<td>1</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>(+CL)</td>
<td>0.84 x 0.76</td>
<td>0.75 x 0.70</td>
<td>14</td>
<td>1</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>1.97 x 1.89</td>
<td>1.67 x 1.62</td>
<td>0</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.21 x 1.03</td>
<td>1.16 x 0.99</td>
<td>9</td>
<td>2</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.08 x 0.73</td>
<td>0.69 x 0.65</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td>3314</td>
<td>left</td>
<td>0.78 x 0.73</td>
<td>0.69 x 0.65</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td>(+CL)</td>
<td>0.76 x 0.65</td>
<td>0.64 x 0.58</td>
<td>0</td>
<td>0</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>2.54 x 2.03</td>
<td>2.32 x 1.84</td>
<td>0</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.94 x 0.55</td>
<td>0.79 x 0.50</td>
<td>0</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88 x 0.74</td>
<td>0.77 x 0.55</td>
<td>2</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td>3851</td>
<td>left</td>
<td>*1.30 x 1.00</td>
<td>*1.16 x 0.86</td>
<td>0</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.27 x 1.22</td>
<td>1.08 x 1.00</td>
<td>0</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.80 x 0.65</td>
<td>0.70 x 0.54</td>
<td>0</td>
<td>1</td>
<td>advanced at.</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>*0.75 x 0.72</td>
<td>0.64 x 0.63</td>
<td>5</td>
<td>1</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td>(+CL)</td>
<td>0.74 x 0.70</td>
<td>0.65 x 0.58</td>
<td>7</td>
<td>1</td>
<td>early at.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.73 x 0.67</td>
<td>0.64 x 0.62</td>
<td>5</td>
<td>0</td>
<td>early at.</td>
</tr>
<tr>
<td>4128</td>
<td>left</td>
<td>*2.35 x 2.21</td>
<td>*2.19 x 2.16</td>
<td>0</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>(CL was on right)</td>
<td>1.35 x 1.30</td>
<td>1.22 x 1.13</td>
<td>6</td>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.27 x 1.08</td>
<td>1.22 x 1.00</td>
<td>8</td>
<td>2</td>
<td>normal</td>
</tr>
</tbody>
</table>
2. Characteristics of the larger follicles in ovaries of hypophysectomized and sham-operated animals

When the larger follicles were examined in detail, it became clear that here a distinction could be drawn between the ovaries of the hypophysectomized and the sham-operated animals. The dimensions and other morphological characteristics of the largest three follicles in each ovary are given in Table 6.5.

The maximum total diameters of the follicles from hypophysectomized animals ranged from 0.34 to 0.70 mm, while in sham-operated animals, follicles reached 0.73 to 2.54 mm in diameter. The mean maximum diameter of the largest follicle per ovary and per animal for the two groups of animals are given in Table 6.6. The figures show quite clearly that greater development of follicles occurred in the sham-operated animals compared to the hypophysectomized animals.

Table 6.6 Mean (±S.E.M.) maximum diameters of the largest follicle, per ovary and per animal, for hypophysectomized (hypox) and sham-operated animals 21 days after operation during the non-breeding season.

<table>
<thead>
<tr>
<th></th>
<th>Diameter of largest follicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypox</td>
</tr>
<tr>
<td>per ovary</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>per animal</td>
<td>0.63 ± 0.05</td>
</tr>
</tbody>
</table>

In the sham-operated animals, two trends were apparent in the data given in Table 6.5. Firstly, in each animal, one follicle (perhaps two in 3851) was considerably larger than any other: this explains the difference between the figures for the mean maximum follicle diameter per ovary and per animal for this group, given in Table 6.6. Also, none of these largest
folicles showed signs of atresia. Secondly, for each pair of ovaries, the follicles in the ovary without the active CL were larger than those on the other ovary.

In the hypophysectomized animals, however, there was little variation either within or between animals in the sizes of the three follicles representing each ovary. Furthermore, in at least two cases, the largest follicle was on the same ovary as the CL, and in every animal the largest follicle was in some stage of atresia. Indeed, 16/21 follicles examined in hypophysectomized animals were classed as atretic compared to 10/21 follicles in sham-operated animals.

Conclusions

The results imply that follicular growth does not cease immediately after hypophysectomy in the tammar wallaby. This could mean that gonadotrophic stimulation is not required for initiation and maintenance of follicular development, or that sufficient stimulation was provided by gonadotrophin already bound to receptors at the time of operation. A further possibility is that the rate of atresia of follicles was decreased: even if recruitment into each class of follicles was slower after hypophysectomy, a decreased rate of loss by atresia could mean, for some time, little change in the overall distribution of follicles among the different classes.

It was clear, however, that the final stages of follicular maturation were slowed, or had ceased, in hypophysectomized animals 21 days after operation. There was increased atresia and decreased development of the large follicles compared to those in the ovaries of sham-operated animals. It would appear, therefore, that these final stages of maturation are particularly dependent upon adequate gonadotrophic stimulation.
similar picture is seen in the ovaries of hypophysectomized rats 21 days after operation: although large antral stages have disappeared, mitotic figures are still seen in granulosa cells of multilaminar follicles (Edwards et al., 1977). The authors suggest that while there is increased atresia of the later antral stages, migration between early stages continues, though the rates of transition may be abnormal.

In the sham-operated animals, the considerably greater development of one follicle in one ovary suggests that this follicle was destined to ovulate about 7 days later (i.e. 28 days after operation and reactivation of the CL). The greater development of follicles in the ovary contralateral to the CL suggests that the CL may have a local, inhibitory effect upon the final stages of follicle growth: ovulation is usually alternate in the tammar (Tyndale-Biscoe, Hearn and Renfree, 1974).

The results obtained here confirm and extend the observations of Hearn (1972) on follicle growth after hypophysectomy. He stated that "Follicular growth and maturation are suspended by hypophysectomy", and gave figures similar to those presented here for the diameters of the largest follicles in ovaries of sham-operated and hypophysectomized wallabies. Furthermore, he has examined ovaries at 21, 30, 40 and 60 days after hypophysectomy and finds that after 40 days there are no antral follicles at all (Hearn, personal communication). This supports the view that the persistence of follicles after hypophysectomy may be due to decreased atresia rather than to continuing growth. In eutherian mammals, it is generally considered that follicle growth to the pre-antral stage with four layers of granulosa cells is independent of gonadotrophin support (Young, 1961).
6.7 EFFECTS OF STEROID REPLACEMENT AFTER LUTEOTOMY ON FOLLICULAR GROWTH AND THE TIMING OF OVULATION.

Besides its central role in the initiation and maintenance of gestation, the corpus luteum (CL) of the tammar also has a major influence on follicular development. Luteectomy experiments have shown that both the young CL of the cycle (up to about d.12) and the quiescent CL exert a strong inhibitory influence on follicular growth and, hence, ovulation, so that a fairly long follicular phase of about 14 days is required for a new crop of follicles to mature after natural or surgical removal of the luteal influence (Tyndale-Biscoe and Hawkins, 1977).

The probable mechanism of this inhibition is a suppression of gonadotrophin secretion by a product of the CL, presumably a steroid. However, the inhibitory agent is unlikely to be progesterone because its maximal production occurs during the second half of the cycle, about d.21, when the CL is no longer inhibiting follicular growth, and the quiescent CL produces relatively little progesterone (Lemon, 1972; Renfree and Heap, 1977; Hinds, unpublished results). Baird et al. (1975) have postulated that in the woman, where the follicular phase is also about 14 days long, oestradiol secreted by the CL during the mid-luteal phase inhibits gonadotrophin secretion and, hence, blocks follicular development. An experiment was designed, therefore, to test the hypothesis that, in the tammar, oestradiol is the agent responsible for the suppression of follicular growth by the CL.

Animals were luteotomized on d.0 and given daily injections of oil, oestradiol-17β, progesterone or both steroids from d.0 to d.6. Oil-injected control animals were expected to return to oestrus and ovulate 12-18 days after operation. However in animals receiving a steroid treatment that mimicked the luteal inhibition of follicular growth, it was expected that ovulation would be delayed about 7 days.
Experimental procedure

This experiment was done in June, which is the beginning of the non-breeding season. An earlier experiment in March-April was inconclusive because no animal returned to oestrus or ovulated after lutectomy. A contributing factor to this failure may have been that the animals were kept in small cages in the Animal House during the 7 days of injections, and that these conditions were unduly stressful. In the June experiment, the animals were kept in large indoor pens, about $2.5 \times 1.5 \times 2$ m, each containing four females. The pens were under artificial lighting, with time-clocks adjusted to the natural lighting regime for that time of year. In a trial experiment, 2/3 lutectomized animals had returned to oestrus when kept in these pens.

Sixteen lactating females were assigned randomly to groups of four which were, in turn, assigned randomly to treatments and days of operation as follows:

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Dose/day</th>
<th>Day of operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>oil</td>
<td>0.1 ml/day</td>
<td>31.5.77</td>
</tr>
<tr>
<td>2</td>
<td>progesterone(P)</td>
<td>700 µg/kg body wt</td>
<td>1.6.77</td>
</tr>
<tr>
<td>3</td>
<td>oestradiol-17β(E)</td>
<td>5 µg/kg body wt.</td>
<td>30.5.77</td>
</tr>
<tr>
<td>4</td>
<td>P + E</td>
<td>700 µgP + 5 µgE/kg</td>
<td>2.6.77</td>
</tr>
</tbody>
</table>

Steroids were dissolved in the minimum amount of alcohol and then diluted with olive oil for intra-muscular injection. The oestradiol dose was reduced compared to that used in the earlier experiment, where there were two cases of pyometria (pus-filled uteri) and nil recovery of eggs or blastocysts in animals given oestradiol-17β at 20 µg/kg/day with or without progesterone. Bolliger (1946) observed similar pyovagina in other marsupials given high doses of oestrogen.
Animals were lutectomized (see Section 2.3) on d.0, all CL's being fixed for histological confirmation that the active CL had been removed. The first injection was given immediately after operation and daily doses were given for the next 6 days (d.1-d.6). On d.8, a male was introduced into each pen, and from d.10 and d.12, respectively, daily vaginal smears and 5 ml blood samples were taken.

Although control animals were expected to come into oestrus between d.12 and d.18, none had mated by d.21, so all animals were autopsied on d.22 to see if silent ovulations had occurred. The weights of the urogenital systems, each uterus and each ovary were recorded and the uteri flushed for eggs or blastocysts. Ovaries were fixed and serially sectioned at 10\(\mu\) to be examined for new CL's and the degree of follicular development. Plasma samples were assayed for LH, as high LH levels would indicate the approximate times of any ovulations.

**Results**

Although no matings occurred, autopsies showed that 2/4 animals in both the control and the progesterone-treated group had ovulated by d.22 (Table 6.7), presumably with silent oestrus. Elevations of plasma LH levels suggested that in at least three of these animals ovulation occurred between d.13 and d.18, when it was predicted that the controls would ovulate. No evidence of an LH surge was seen in 4421: it was probably missed by daily sampling, as discussed in Chapter 5.

Of the other progesterone-treated animals, one had a large follicle and an enlarged urogenital system, indicating that she was in late proestrus and likely to ovulate in 24-48 hours. The fourth animal in Group 2 and the two remaining controls all had an enlarging, non-atretic follicle, diameter >2 mm, but their urogenital systems showed no signs of preovulatory enlargement and LH levels remained low.
There were no ovulations in any of the animals given oestradiol-17β alone or with progesterone. 1/4 and 2/4 animals in Groups 3 and 4 respectively were classed as preovulatory on d.22 (plasma LH being slightly elevated on d.22 in one of these), and another animal in each group had an enlarging follicle. However, the remaining three had only small to medium follicles, implying that follicular growth had been delayed relative to that in control and progesterone-treated animals.

Newly ovulated unfertilized eggs were recovered from two of the four animals with new corpora lutea (Table 6.7). One of these, 4599, also carried an old egg in the other uterus. A quiescent blastocyst found in the right uterus of 4204 was the result of a previous ovulation and mating: the newly ovulated eggs in this animal and in 4421 may have been still in the oviducts, which were not flushed.

In all, two quiescent blastocysts and two old unfertilized eggs were recovered from the eight animals in Groups 1 and 2. Also, in 4173, from Group 2, extensively developed embryonic membranes, but no sign of an embryo, were found in the enlarged uterus ipsilateral to the removed CL. This phenomenon has previously been reported in progesterone-treated tammars by Renfree and Tyndale-Biscoe (1973). No unfertilized eggs or blastocysts were found in any of the animals given oestradiol-17β (Groups 3 and 4) except in 4229, which had an old egg in the right uterus. The CL had been removed from her right ovary.

Fourteen animals carried their pouch young to the end of the experiment. The young of 4290 and 4214, both in Group 4, were found dead on d.14 and d.17 respectively, but losses at that stage are unlikely to have affected the responses to luteectomy and steroid treatment.
Table 6.7  Weights and contents (if any) of ovaries and uteri, and weight of the urogenital system (UGS) at autopsy (d.22) of lutectomized wallabies given oil, progesterone (prog.) oestradiol-17β (oest.) or both steroids for 7 days after operation (see text for doses). Days when plasma LH levels were above 2 ng/ml are indicated, and diagnoses made from the results are presented.

<table>
<thead>
<tr>
<th>Animal No. and Group</th>
<th>CLx&lt;sup&gt;1&lt;/sup&gt; ovary</th>
<th>Left ovary wt(mg)</th>
<th>Content</th>
<th>Right ovary wt(mg)</th>
<th>Content</th>
<th>UGS wt(g)</th>
<th>Left uterus wt(g)</th>
<th>Content</th>
<th>Right uterus wt(g)</th>
<th>Content</th>
<th>Day of high LH</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4204</td>
<td>R</td>
<td>111.9</td>
<td>new CL</td>
<td>158.4</td>
<td></td>
<td>17.2</td>
<td>0.8</td>
<td></td>
<td>0.8</td>
<td>quiescent blastocyst</td>
<td>d.13</td>
<td>ovulated</td>
</tr>
<tr>
<td>4421</td>
<td>R</td>
<td>160.3</td>
<td>new CL</td>
<td>160.3</td>
<td></td>
<td>16.2</td>
<td>0.5</td>
<td></td>
<td>0.4</td>
<td>quiescent blastocyst</td>
<td></td>
<td>ovulated</td>
</tr>
<tr>
<td>4605</td>
<td>L</td>
<td>155.8</td>
<td></td>
<td>151.7</td>
<td>Follicle</td>
<td>14.2</td>
<td>0.4</td>
<td>quiescent blastocyst</td>
<td>0.4</td>
<td></td>
<td>enlarging follicle</td>
<td></td>
</tr>
<tr>
<td>4590</td>
<td>R</td>
<td>95.7</td>
<td>Follicle</td>
<td>143.6</td>
<td>2.3 mm</td>
<td>11.2</td>
<td>0.5</td>
<td></td>
<td>0.5</td>
<td>old UFE&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>enlarging follicle</td>
</tr>
<tr>
<td>2. Prog.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3949</td>
<td>L</td>
<td>170.2</td>
<td></td>
<td>104.5</td>
<td>new CL</td>
<td>20.1</td>
<td>0.7</td>
<td></td>
<td>0.7</td>
<td>new UFE</td>
<td>d.16</td>
<td>ovulated</td>
</tr>
<tr>
<td>4599</td>
<td>R</td>
<td>139.8</td>
<td>new CL</td>
<td>160.4</td>
<td></td>
<td>20.5</td>
<td>1.0</td>
<td>new UFE</td>
<td>1.0</td>
<td>old UFE</td>
<td>d.18</td>
<td>ovulated</td>
</tr>
<tr>
<td>3807</td>
<td>R</td>
<td>218.4</td>
<td>Follicle</td>
<td>155.5</td>
<td>3.5 mm</td>
<td>23.2</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td>pre-ovulatory</td>
</tr>
<tr>
<td>4173</td>
<td>L</td>
<td>132.0</td>
<td>Follicle</td>
<td>137.8</td>
<td>2.1 mm</td>
<td>12.8</td>
<td>4.0 embryos</td>
<td>membranes</td>
<td>0.5</td>
<td></td>
<td>enlarging follicle</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>i.e. ovary from which the original CL was removed.

<sup>2</sup>diameter of largest follicle/animal

<sup>3</sup>unfertilized egg

(continued on next page)
### Table 6.7 (continued)

<table>
<thead>
<tr>
<th>Animal No. and Group</th>
<th>CLX ovary wt(mg)</th>
<th>Left ovary Content</th>
<th>Right ovary wt(mg)</th>
<th>Right ovary Content</th>
<th>UGS wt(g)</th>
<th>Left uterus wt(g)</th>
<th>Right uterus wt(g)</th>
<th>Day of High LH</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3. Oest.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4061</td>
<td>L 171.4</td>
<td>221.8 Follicle 3.8 mm</td>
<td>24.2 1.2</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pre-ovulatory</td>
</tr>
<tr>
<td>4341</td>
<td>L 189.8</td>
<td>147.8 Follicle 2.7 mm</td>
<td>12.0 0.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>enlarging follicle</td>
</tr>
<tr>
<td>3968</td>
<td>L 160.0</td>
<td>122.3 Follicle 1.2 mm</td>
<td>11.8 0.8</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no large follicle</td>
</tr>
<tr>
<td>3168</td>
<td>L 196.0</td>
<td>no follicle &gt;1 mm</td>
<td>91.4 no follicle &gt;1 mm</td>
<td>11.4 1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td>no large follicle</td>
</tr>
<tr>
<td><strong>4. Oest. + Prog.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4214</td>
<td>L 222.0</td>
<td>162.0 Follicle 3.3 mm</td>
<td>23.0 1.0</td>
<td>1.0</td>
<td>d.21</td>
<td></td>
<td></td>
<td></td>
<td>pre-ovulatory</td>
</tr>
<tr>
<td>3860</td>
<td>L 255.8</td>
<td>Follicle 2.7 mm</td>
<td>216.0 20.6 1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pre-ovulatory</td>
</tr>
<tr>
<td>4229</td>
<td>R 114.4</td>
<td>Follicle 2.2 mm</td>
<td>101.8 10.2 0.6</td>
<td>0.6 old UFE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>enlarging follicle</td>
</tr>
<tr>
<td>4290</td>
<td>L 91.8</td>
<td>115.0 Follicle 1.6 mm</td>
<td>11.2 0.6</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no large follicle</td>
</tr>
</tbody>
</table>
Conclusions

Although only about 75% of lactating female tammars may be expected to be carrying blastocysts (Renfree and Tyndale-Biscoe, 1973), the recovery of only one unfertilized egg from the eight oestradiol-treated animals suggests that the oestradiol adversely affected the survival of blastocysts or eggs. Smith and Sharman (1969) found that, in ovariectomized tammars, survival of blastocysts induced to develop by injections of oestradiol benzoate, 50 or 100µg/day for 3 days, was poor compared to other experiments where progesterone was the inducing agent. These doses are 2-4 times greater than the 5 µg/kg/day used here.

Although there was some overlap between the stages of follicular and UGS development reached by d.22 after lutectomy in the different groups, the results suggest that while the responses of the progesterone-treated animals (Group 2) did not differ from those of controls, oestradiol treatment, with or without progesterone, delayed follicular maturation by several days. Thus, as predicted, the inhibition of follicular growth by the CL does not appear to be effected by progesterone, but oestradiol-17β may be the inhibitory agent. The combination of the two steroids was not more effective an inhibitor than oestradiol alone.

It is suggested, therefore that the quiescent CL (as in this experiment) or the young CL of the cycle secretes an oestrogen such as oestradiol-17β which, through a negative feedback effect on gonadotrophin secretion, suppresses follicular maturation. After about d.12 of the cycle, luteal oestrogen production presumably declines, with progesterone secretion increasing as the CL reaches full maturity by d.18-21. This hypothesis will be discussed later in the light of other results presented in this chapter.
6.8 INDUCTION OF LH RELEASE BY A SINGLE INJECTION OF OESTRADIOL-17\(^\beta\)

The previous experiment suggested that the inhibition of follicle development by the CL may be due to a depression of gonadotrophin levels by oestradiol. This steroid did indeed have a negative feedback effect on gonadotrophin secretion in ovariectomized tammars, but in 1/3 intact, quiescent animals there was a large release of LH 6-9 hours after injection. This implies that, as in eutherian mammals (see Chapter 1), oestradiol-17\(^\beta\) may have both positive and negative feedback effects on LH secretion in the tammar, which has important implications for the control of the LH surge and ovulation in this species.

The nature of the response is probably dependent on the dose of oestradiol, high doses being stimulatory and low doses inhibitory. Since 5 \(\mu g/kg\) body weight induced LH release in 1/3 animals, this appears to be an intermediate dose. In the following experiment, then, the responses of intact, quiescent females to a lower and a higher dose of oestradiol, 1 \(\mu g/kg\) and 10 \(\mu g/kg\) respectively, were investigated, the same basic experimental design being used as in the previous experiments on the effects of steroids (Section 6.5).

Experimental procedure

The experiment was performed in November to early December, during the non-breeding season, the same six non-lactating, quiescent females being used throughout. All animals received the same treatment on the same day and were left for a week between treatments. They were maintained in outdoor, grassy pens and were re-caught for every blood sample.

The three treatments were:

1) 0.1 ml oil/kg body weight, i.m.

2) 1 \(\mu g\) oestradiol-17\(^\beta\)/kg body weight, in oil, i.m.
3) 10 μg oestradiol-17β/kg body weight, in oil, i.m.

20 mg oestradiol-17β (Sigma Biochemicals) was dissolved in the minimum amount of ethanol and diluted with olive oil to give a stock solution of 2 mg oestradiol/ml oil, from which the above doses were prepared by dilution.

On the day of each treatment, the animals were injected with the appropriate dose immediately after the first blood sample (3 ml) at 08.00. They were also bled at 3, 6, 9, 12 and 24 hours after injection. Plasmas were separated as soon as possible and stored frozen until assayed for LH and FSH.

Results

When oil alone was injected, both LH and FSH remained undetectable in all samples from all animals, which fits the usual pattern for quiescent females. FSH also remained undetectable in all animals after both doses of oestradiol.

One animal responded to the lower dose of oestradiol with a moderate release of LH, up to 12.5 ng/ml plasma by 12 hours after injection, and in another, LH levels were slightly elevated (i.e. over 2 ng/ml) 12 hours after treatment (Fig. 6.10). In the other four animals, LH remained low or undetectable.

With the higher dose of oestradiol, 3/6 animals responded with small elevations of plasma LH levels, 3-4 ng/ml, 9-12 hours after injection (Fig. 6.10). In another two animals, plasma LH levels rose to 1.8 and 1.9 ng/ml 9 and 12 hours, respectively, after the oestradiol treatment. Such levels are within the normal range for quiescent females (see Chapter 5), but LH was very low or undetectable in all plasma samples taken at 0, 3, 6 and 24 hours after injection. In the sixth animal, it remained low throughout.
Changes in LH concentration in plasma of 6 wallabies after single injections of 1µg or 10µg Estradiol/kg body weight in oil, i.m.

Oil-injected controls: LH was undetectable in all samples.

This experiment has, therefore, provided further evidence that estrogens have a negative feedback effect on LH release in the wallaby, although the dose required to produce the effect was much higher than in similar experiments by Kodicek et al. (1966).
Conclusions

Neither dose of oestradiol-17β stimulated FSH release, and in only two animals given the lower dose was there clear evidence of a positive feedback effect of the steroid upon LH secretion. Contrary to the original hypothesis, there were no large releases of LH after the higher dose, although in at least 3 animals LH levels 9-12 hours after injection were elevated compared to those in normal quiescent females. It is of course possible that further releases of LH occurred between 12 and 24 hours after injection, and future experiments should take this into account. The design of this exploratory experiment was based on the occurrence of an LH peak 6-9 hours after oestradiol injection in the previous experiment, Section 6.5, which was an interval comparable to that seen in sheep in similar experiments by Goding et al. (1969).

This experiment has, therefore, provided further evidence of a positive feedback effect of oestradiol-17β in the tammar, but little information on the doses required to produce such effects. It was carried out, however, late in the non-breeding season, when pituitary sensitivity to steroid feedback may be depressed. It would be interesting to repeat the experiment in the breeding season to see if the animals are then more responsive.
6.9 HYPOTHALAMIC CONTROL OF LH SECRETION: RESPONSES TO LH-RH

Although modulated by the action of steroids such as oestradiol acting at the hypothalamic or pituitary levels, the immediate cause of gonadotrophin release is the stimulation of pituitary gonadotrophs by LH-RH. Either the natural or the synthetic decapeptide will induce LH release in a wide variety of eutherian mammals, although the FSH responses are less consistent. However, there have not been any reports of the action of LH-RH in marsupials, presumably because of the previous lack of LH and FSH assays. Neither has it been confirmed that the structure of the LH-RH molecule in marsupials is the same as in eutherians.

The following experiments were performed, therefore, to see if eutherian LH-RH would stimulate LH and/or FSH release in tammar wallabies in various physiological conditions. Since pituitary responsiveness to LH-RH may vary during the year in seasonal breeders, experiments were carried out during both the breeding and the non-breeding seasons. Also, there have been numerous reports of a self-potentiating effect of LH-RH, i.e. a greater response to a second stimulus given an appropriate time after the first (eg. Crighton and Foster, 1977; ter Haar, 1978). Thus in some experiments here, a second injection of LH-RH was given one hour after the first to see if this would induce a greater release of LH-RH.

Experimental procedure

The same basic experimental design was used throughout. Animals were fitted with either chronically indwelling jugular catheters or disposable winged infusion sets (Surflo, Terumo) inserted into a tail vein. In each case, the catheter tube was kept filled with dilute heparin, 250 U/ml in sterile 0.9% saline, which was removed, with the first 0.5 ml blood, before each blood sample.
Initial experiments used natural LH-RH (source unknown) donated to Dr. J. Hawkins by Dr. M. Radford, C.S.I.R.O., Division of Animal Physiology, Prospect, NSW. Later, synthetic commercial preparations from Hoechst and Calbiochem were used. In each case, the powdered preparation was dissolved in sterile 0.9% saline to give 10 µg LH-RH/ml. A 1 ml dose was injected via the catheter and flushed through with 1 ml sterile saline before the tube was refilled with dilute heparin.

At least two blood samples, 1.5 or 2 ml, were taken before administration of the LH-RH. Samples were taken every 10-15 min for the first hour after injection, then at increasing intervals over the next 2-3 hours. Where a second LH-RH stimulus was given 1 hour after the first, the sampling schedule was adjusted accordingly. The animals were kept in sacks throughout the experiment. When using the winged infusion sets, the animal was laid on its side on a trolley, lightly restrained by cords, with its tail protruding from a hole in the sack.

All plasma samples were assayed for LH and in certain experiments FSH was also measured.

Experiments during the breeding season

a) Response of an intact female to a single injection of LH-RH

Several experiments were performed using the natural LH-RH, but most samples were assayed using the solid-phase, rather than the double-antibody, RIA for LH, and so only a single set of results will be presented here.

The response of the intact female 4102 to 10 µg LH-RH i.v. was tested on d.22 RPY. (She had a jugular catheter.) LH was low in the three samples taken before injection, rising to a peak of 19 ng/ml by 30 min after LH-RH and declining more slowly to regain basal levels by 150 min
Fig. 6.11.
Changes in LH levels in plasma of an intact female wallaby after one injection of LH-RH (10 µg, i.v.).
after injection (Fig. 6.11). Assuming that this decline, or decay curve, represents the clearance of LH from the circulation, then, from the graph, the half-life of LH in the circulation is about 30 min.

FSH remained undetectable in all plasma samples.

b) Responses of ovariectomized females to a single injection of LH-RH

A series of experiments were carried out using twelve of the animals from the ovarian tissue graft experiment, Section 6.4, 34-36 days after surgery. At this stage, gonadotrophin levels were elevated in all twelve animals used, which will, therefore, be regarded as an effectively ovariectomized group. Each animal was used once only, and winged infusion sets were used for sampling. 10 μg LH-RH (Calbiochem) in 1 ml 0.9% saline was given i.v. and samples taken over the next 3 hours. Only LH was assayed.

As the response patterns were very uniform, the data for the 12 animals were combined (Fig. 6.12). In these animals, the basal level of LH was about 5 ng/ml plasma. The levels rose rapidly in response to LH-RH, reaching a mean peak of 16.9 ± S.E.M. 1.4 ng/ml 25 min after injection. Again, the decline back to basal levels was slower than the rise to the peak, taking another 90-150 min. From the graph, the half-life of LH in the circulation of ovariectomized wallabies is about 40 min.

c) Responses to two injections of LH-RH, one hour apart

Three animals in different physiological conditions were tested for their responses to two injections of LH-RH (Calbiochem, 10 μg/dose) given one hour apart. They were the lactating female 4528, the non-lactating intact female 3855 and the ovariectomized female 4124, all fitted with winged infusion sets. Samples were taken every 10-15 min during the two hours after the first injection and at 30-60 min intervals over
Fig. 6. 12

Changes in LH concentration in plasma of ovariectomized wallabies following administration of LH-RH (Calbiochem), 10μg i.v.

(Mean ± S.E.M., n=12)
Fig.6.13. Changes in LH and FSH levels in plasma of female wallabies given 2 injections of LH-RH (10 µg/dose, i.v.) 1 hour apart: experiments in the breeding season. Arrows indicate times of LH-RH injection.

(a) 3855, cycling
 FS H remained undetectable.

(b) 4528, lactating
 FS H remained undetectable.

(c) 4124, ovary

LH ———
FSH ———

Hours since 1st LH-RH injection

Hours since 1st LH-RH injection (note scale change)
the next 1\(\frac{1}{2}\) hours. For the ovariectomized animal only, hourly samples were taken for 6 hours before the first injection to check that responses to LH-RH could be differentiated from peaks due to diurnal variations in LH levels. All samples were assayed both for LH and for FSH.

All three animals responded to the first injection of LH-RH with a rapid rise in LH levels, peaks occurring 20-30 min after injection (Fig. 6.13). The peak was greatest in the ovariectomized animal 4124 (Table 6.8), but her basal LH levels were, of course, much higher (4-8 ng/ml) than in the intact animals. In 4124, peaks due to diurnal variation (i.e. in samples taken before LH-RH injection) did not approach the magnitude of the responses to LH-RH (Fig. 6.13(c)). The differences in peak values between the intact animals are probably not significant.

Table 6.8 Time intervals between LH-RH stimulus and the associated peak, and peak LH levels in plasma of 3 female wallabies given 2 injections of LH-RH (10 \(\mu g\), i.v.) 1 hour apart.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Basal LH ng/ml</th>
<th>First peak</th>
<th>Second peak</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Min from peak</td>
<td>ng LH/ml</td>
<td>Min from peak</td>
</tr>
<tr>
<td>3855 cycling</td>
<td>(&lt;0.2-0.5)*</td>
<td>30</td>
<td>9.5</td>
<td>10</td>
</tr>
<tr>
<td>4528 lactating</td>
<td>(&lt;0.2-1.0)</td>
<td>20</td>
<td>12.0</td>
<td>20</td>
</tr>
<tr>
<td>4124 ovaryx</td>
<td>4-8</td>
<td>20</td>
<td>21.5</td>
<td>30</td>
</tr>
</tbody>
</table>

* undetectable by the RIA.

After the first peak, LH levels began to decline, but did not reach basal levels by the time of the second LH-RH stimulus. Although all animals responded to this with a further release of LH, in no case was there a greater response to the second injection than to the first. Again, the peak was greatest in 4124. In 3855, the peak occurred only 10 min after the second LH-RH stimulus and LH levels remained high for about
another 35 min before beginning to decline significantly (Fig. 6.13 (a)).

In both intact animals, FSH remained undetectable in all plasma samples. In the ovariectomized animal, there was a small rise in FSH levels 10 min after each LH-RH injection and during the experimental period they remained somewhat higher than in the previous 6 hours. It is doubtful, however, that this slight rise is significant compared to the diurnal variations.

Experiments during the non-breeding season

a) Responses of intact animals to LH-RH

During the non-breeding season, the responses of female tammars to LH-RH were unpredictable. In only 3 of 9 experiments carried out in late October to December, using 4 different animals, did the peak LH level after LH-RH exceed 4 ng/ml plasma. Some representative results are given in Fig. 6.14. All animals were non-lactating and had jugular catheters.

In 4513, there was a small rise in LH levels 10 min after a single i.v. injection of 10 µg LH-RH (Hoechst) in 1 ml 0.9% saline (Fig. 6.14(a)). LH then returned to undetectable levels but rose again slightly to 2.9 ng/ml plasma 50 min after LH-RH, though undetectable thereafter. This animal unexpectedly gave birth on 22.11.77, implying that reactivation had occurred at the time of this experiment, 26 days before.

The other three examples are different experiments performed on 9th, 12th and 19th December, 1977, using the same animal, 4522. In these experiments only, each dose of LH-RH was 2 µg, i.e. 0.2 ml, of the Hoechst solution for veterinary use, Hoe 766. There was little response to a single injection of LH-RH (Fig. 6.14(b)). LH levels rose to 0.5 ng/ml by 20 min after injection and remained at this level, or slightly higher (<1 ng/ml plasma) to the end of the sampling period. In four other experiments with
Fig. 6.14.

Changes in LH levels in plasma of non-lactating intact wallabies after 1 or 2 injections, 1 hour apart, of LH-RH (10 µg/dose, i.v.): experiments in the non-breeding season.

Arrows indicate times of LH-RH injection.

(a) 4513 (28-10-77) (b) 4522 (19-12-77)

(Gave birth 26 days later)

(c) 4522 (9-12-77) (d) 4522 (12-12-77)

Hours since 1st LH-RH injection
4522, a second injection of LH-RH was given 1 hour after the first. Only in one trial (Fig. 6.14(c)) was there a significant rise in LH levels after either injection: LH levels reached a peak of 4.7 ng/ml 55 min after the first injection, then declined to about 1 ng/ml before rising to a second peak of 6.3 ng/ml 60 min after the second LH-RH dose. Thus the interval between each injection and its associated LH peak was about twice as long as in the experiments during the breeding season (but there was no sample between 30 and 55 min after the first injection, due to problems with the catheter).

In all other experiments, however, there was very little response to either injection of LH-RH (example in Fig. 6.14(d)). The general pattern was a small increase in LH levels, to about 1 ng/ml, after the first injection, these levels being maintained until the end of the sampling period with no increase after the second LH-RH injection.

In all experiments, FSH remained undetectable in every plasma sample.

b) Responses of ovariectomized animals to LH-RH

The responses of two ovariectomized females, 4115 and 4448, to a single injection of LH-RH (Hoechst, 10 μg, i.v.) were tested first. In 4115, basal LH levels before LH-RH injection were about 2 ng/ml (Fig. 6.15(a)). LH remained at such levels for 40 min after LH-RH and then rose to 36 ng/ml at 50 min, declining back to basal levels by 120 min after injection. The second peak of 5.2 ng/ml at 150 min was probably due to endogenous diurnal variation.

In 4448, on the other hand, LH levels were moderately high, 5.6 ng/ml, 20 min before LH-RH injection, then decreasing so as to be undetectable 10 min after LH-RH (Fig. 6.15(c)). They remained fairly low until rising
Fig. 6.15.

Changes in LH levels in plasma of ovariectomized wallabies after 1 or 2 injections, 1 hour apart, of LH-RH (10µg/dose): experiments in the non-breeding season.

Arrows indicate times of LH-RH injection.

(a) 4115(24-10-77): 1 injection.
(b) 4115(27-10-77): 2 injections.
(c) 4448(28-10-77): 1 injection.
to 4.2 ng/ml 60 min after injection. Two further peaks, both 6.6 ng/ml, were seen at 120 and 180 min. On the basis of the time intervals between LH-RH injections and response peaks, it is likely that these two peaks at least were unrelated to the LH-RH stimulus, probably representing endogenous variation in LH secretion.

In the experiments where two injections of LH-RH, each 10 µg, i.v. were given 1 hour apart, both animals showed fairly high LH levels in samples taken before the first injection and only slight increases, to about 3 ng/ml, 10 min after this. In 4115, LH levels continued to decline until the second injection was given (Fig. 6.15(b)). As before, there was a slight increase 10 min after the injection and the levels then declined until 150 min, but increased sharply to 8.2 ng/ml at 180 min. Again, this rise was probably not due to the LH-RH stimulus.

In 4448 (Fig. 6.15(d)), on the other hand, LH levels rose to a peak of 4.7 ng/ml at 50 min but then declined, even after the second LH-RH injection. There was another moderate peak of 4.8 ng/ml at 120 min probably due to diurnal variation.

In these ovariectomized animals, then, the degree of response to LH-RH seemed to vary according to whether there had been a recent release of LH due to endogenous stimuli. In no case was there any evidence of a rise in FSH levels in response to either a first or a second injection of LH-RH.

Conclusion

During the breeding season, intact and ovariectomized female tammars respond to LH-RH in a uniform manner. LH levels begin to rise immediately after i.v. injection of LH-RH and reach a peak 20-30 minutes later, then declining more slowly to regain basal levels 1½-2 hours later.
From such decay curves, the half-life of LH in the circulation may be estimated at about 30 minutes, which agrees well with half-lives for LH reported in ovariectomized ewes (Butler *et al.*, 1972) and castrate rats (Gay and Sheth, 1972), but is about one-third of that in rhesus monkeys (Dierschke *et al.*, 1970).

The peak LH levels attained in response to LH-RH were greater in ovariectomized than in intact animals, but this may be due to the higher basal levels in the former. In no case, however, did the magnitude of the responses to LH-RH approach those of ovulatory LH peaks. This, together with the relatively short duration of the response compared to the ovulatory LH surge, suggests that the surge is not triggered by a single release of LH-RH, but by a series of such stimuli.

Although peak levels of LH released in response to oestradiol (Sections 6.5 and 6.8) were also not as great as ovulatory LH peaks, these responses were of much longer duration (several hours) than those to LH-RH and, in this respect, mimic more closely the LH surge. This is probably due to slow release of oestradiol from the injection site, but illustrates once again that sustained stimulation of the pituitary, presumably mediated by an effect of oestradiol on the hypothalamus, is necessary to induce the LH surge.

A second injection of LH-RH given one hour after the first did not invoke a greater release of LH: in the animals treated during the breeding season, the second response was similar to the first. This may mean that LH-RH does not have a self-potentiating effect in the tammar, or, more likely, that the latent period for this to become effective is longer than one hour. More experiments should be performed with the LH-RH injections spaced at different intervals to clarify this point.
During the non-breeding season, the responses to LH-RH were unpredictable. The poor responses of intact females suggested that either less LH is synthesized and stored by the pituitary during this season, or that it is less readily available for release.

In the ovariectomized animals, on the other hand, it appears that the ability to respond to LH-RH depends upon whether a large release of LH occurred shortly before the LH-RH challenge. This, together with the marked diurnal variation in LH levels at this time of year, suggests that LH is continually being synthesized and secreted in bursts. After such a release, the pituitary stores are depleted until resynthesis, or movement of LH into a readily-releaseable pool, has occurred. During this refractory period, an LH-RH stimulus would be ineffective.

In none of the experiments, using either intact or ovariectomized females, did it appear that FSH levels increased in response to the LH-RH stimulus.
6.10 SUMMARY

1. Plasma LH and FSH levels rise after ovariectomy in the tammar wallaby.

2. Plasma LH and FSH levels in ovariectomized wallabies fluctuate markedly from hour to hour. In intact, quiescent animals, occasional small peaks of LH, up to 2 ng/ml plasma, are observed, but FSH remains undetectable.

3. Grafts of ovarian cortex in which lutein tissue develops are effective in depressing plasma gonadotrophin levels in bilaterally ovariectomized wallabies. The urogenital systems of such animals appear to be under oestrogenic stimulation.

4. Oestradiol-17β, progesterone, dihydrotestosterone and testosterone will all depress LH levels in ovariectomized females. FSH secretion appears to be less sensitive to such steroid feedback, but the levels may be depressed by oestradiol-17β.

5. Follicular maturation has ceased by 21 days after hypophysectomy. The persistence of small follicles suggests a decreased rate of atresia.

6. Inhibition of follicular growth by the corpus luteum is probably due to a depression of plasma gonadotrophin levels by luteal oestrogen.

7. There is some evidence that oestradiol-17β also has a stimulatory, or positive feedback, effect on LH levels in intact females.

8. LH-RH will induce LH, but not FSH, release in intact and ovariectomized females, but responses are poor during the non-breeding season. There is no greater response to a second injection of LH-RH given one hour after the first.
CHAPTER 7

GENERAL DISCUSSION

In this thesis, I have been concerned with two aspects of pituitary-ovarian interactions in the tammar, those involved in the control of the oestrous cycle and those concerned with the annual cycle. Any study of this nature must consider the pituitary gonadotrophins, and the development of specific RIA's for the measurement of LH and FSH in wallaby plasma was an essential prerequisite for the endocrinological studies discussed in Chapters 5 and 6 of this thesis: besides Hearn's measurements of "total gonadotrophin" levels in tammar plasma, there was no information available on the patterns of gonadotrophin secretion in any marsupial.

First, however, it was confirmed that, as in eutherians, the tammar pituitary produces two distinct gonadotrophic hormones. The RIA work reported here, together with that of Stewart and Sutherland (manuscript in preparation) on the behaviour of highly purified kangaroo LH's and FSH's in the same RIA's, has shown that macropod marsupials have separate LH and FSH molecules, immunologically similar to the corresponding hormones of eutherians. Furthermore, the results of receptor binding assays (Gallo et al., in press; Stewart and Sutherland, manuscript in preparation) have demonstrated that in macropods there are specific testis receptors for each hormone, and suggest further homologies of structure and biological activity with eutherian gonadotrophins.
This emphasizes that the RIA's developed here for the measurement of tammar LH and FSH are potentially applicable to the study of other marsupials. Indeed, recent work by Dr. Sutherland has shown that homogenates of pituitaries from 7 species of macropod and one dasyurid marsupial, *Antechinus stuartii*, all gave displacement curves parallel to the ovine LH standard curve in the RIA for LH (Evans and Sutherland, manuscript in preparation). The RIA's would, however, require validation for use in each new species.

The existence of two structurally distinct gonadotrophins implies that they fulfil different physiological functions, and a major aim of this work was to provide some understanding of the roles of LH and FSH in the female tammar. However, the effects of the gonadotrophins on the ovary are but one aspect of the control of reproduction by the hypothalamo-pituitary-ovarian system. By consideration of data from various experiments, a working model of the control of the ovarian cycle by both gonadotrophic and steroid hormones has been constructed (Fig. 7.1), although its validation, or otherwise, must await the development of more sensitive assays for several key hormones in tammar plasma.

Except for the surges of LH associated with ovulation, the levels of LH and FSH are so low that measurements of gonadotrophin levels at different times of the cycle have not been very informative. The pattern of progesterone secretion has been fairly well established (Lemon, 1972; Hinds, unpublished results) but attempts to measure oestrogens in tammar plasma by RIA have, so far, been unsuccessful (Sutherland, unpublished results). However, *in vitro* studies have shown that the tammar CL produces both progesterone and phenolic steroids, presumably oestrogens (Renfree and Heap, 1977). Since the model presented below assigns oestradiol to a key role in the control of the ovarian cycle in the tammar, it must be emphasized, therefore, that this hypothesis is based primarily
Fig. 7.1 A model of the endocrine control of the oestrous cycle of the tammar wallaby

Moderate levels of oestradiol are assumed to have a negative feedback effect on both LH and FSH secretion, while rising levels of oestradiol induce LH release by positive feedback.

Progesterone is assumed to have a negative feedback effect on the secretion of LH only.

**KEY**

Solid lines: hormone levels based on measurement of hormones in plasma

- LH (this thesis)
- progesterone (Lemon, 1972; Hinds unpublished results)

Dotted lines: change in hormone levels hypothesized from experimental evidence.

- FSH
- oestradiol ($E_2$)

The thick bars indicate the approximate duration of oestrus.
Day of cycle:
0 — ovulation
7
14
21
28
maximal luteal development

E2 from CL

E2 from follicles

young CL inhibits follicular growth beyond the gonadotrophin-independent phase

follicles mature
upon the limited data available from experiments described in this thesis.

The control of the ovarian cycle fundamentally involves the control of follicular growth and maturation. During the first half of the cycle in the tammar, follicular development is inhibited by the CL. This is demonstrated by the observation that removal of the CL (luteectomy) during this period induces a premature ovulation 12-18 days later (Tyndale-Biscoe and Hawkins, 1977). This relatively long interval implies that the CL is inhibiting the whole of the gonadotrophin-dependent phase of follicular growth which, the effects of hypophysectomy imply, includes antrum formation and subsequent maturation (Hearn, 1972; this thesis).

In this respect, then, the tammar resembles the woman, in which the follicular phase is fourteen days long, rather than the sheep, in which ovulation occurs only two to three days after luteectomy (Baird, et al., 1975). Even so, the analogy with the woman is not complete because, in the tammar, luteectomy is not effective at advancing ovulation if performed during the second half of the cycle (Tyndale-Biscoe and Hawkins, 1977).

Baird et al. (1975) have ascribed this difference between the sheep and the woman to differences in steroid production by their respective corpora lutea. While the only major product of the sheep CL is progesterone, which has little effect on gonadotrophin levels, the human CL also produces over 90% of the total ovarian output of oestradiol, which is a potent inhibitor of gonadotrophin secretion. In the latter case, then, the gonadotrophin-dependent stages of follicular development are inhibited during the luteal phase.

In the tammar, the length of the follicular phase and the observation that progesterone levels are low during the first half of the cycle (Lemon, 1972; Hinds, unpublished results) suggested that here,
also, luteal oestrogen, rather than progesterone, may be involved in the suppression of follicular development. The effects of steroid replacement after luteectomy (Section 6.7) appear to substantiate this theory: treatment with oestradiol-17β delayed follicular maturation and ovulation in response to luteectomy, while progesterone did not. Furthermore, oestradiol-17β has a negative feedback effect on gonadotrophin secretion in ovariectomized wallabies, and may be inferred to have similar effects in intact females, though this is not demonstrable due to the low levels of the hormones in the circulation. It appears, therefore, that the inhibition of follicular growth during the early luteal phase may be attributed to a depression of gonadotrophin levels by oestrogen secreted by the CL. Since the CL itself is independent of gonadotrophin support [Hearn, 1972; 1974], luteal steroidogenesis would be unimpeded by the low gonadotrophin levels.

During the second half of the cycle, the CL reaches maturity and is secreting maximal levels of progesterone by about day 21 (Lemon, 1972; Hinds, unpublished results). However, although the CL is dominating ovarian steroid output, the block on follicular development is now lifted, and this continues unimpeded to produce a preovulatory follicle by about day 28. It appears, therefore, that the steroidogenic capacity of the CL must alter as it matures, oestrogen production ceasing, or being proportionately diminished, as progesterone production increases (Fig. 7.1).

In ovariectomized females, progesterone has little effect upon the elevated FSH secretion, though it does depress plasma LH levels. By analogy, it is suggested that the high progesterone levels in the intact female during the luteal phase inhibit only LH secretion, thus preventing premature ovulation, while follicular maturation can proceed under the influence of FSH. Nevertheless, the mature CL does appear to have a local inhibitory effect on the final stages of follicular maturation,
as ovulation almost invariably occurs from the contralateral ovary, this effect already being evident by day 21 of the cycle (Section 6.6).

This model therefore requires differential effects of oestrogen and progesterone upon tonic gonadotrophin secretion: the former must have a negative feedback effect on LH and FSH secretion, while the latter depresses LH levels only. While there is good evidence that both steroids can depress LH levels in ovariectomized wallabies (Section 6.5), their effects on FSH levels were not clear. However, if it is assumed that follicular growth is an indication of FSH secretion, then the delayed responses to luteectomy in oestradiol- but not progesterone-treated animals is indirect evidence that oestradiol inhibits FSH secretion. Furthermore, in ovariectomized animals bearing ovarian cortex grafts under the pouch skin, depressed plasma levels of FSH (and LH) were associated with enlargement of the lateral vaginæ, which suggests oestrogenic activity.

Whether the interstitial tissue plays any part in the control of the oestrous cycle is as yet uncertain. Interstitial tissue is very prominent in tammar ovaries, but grafts with apparently healthy cells did not depress gonadotrophin levels in ovariectomized animals. This could have been because there was not enough tissue present or because the tissue may not function autonomously in the regulation of gonadotrophin levels, but may act either as a source of steroid precursors for, or as a regulator of, another ovarian tissue. In the pregnant rat, androgen produced by thecal cells of the follicles is aromatized within the CL and the oestradiol thus formed has a local luteotrophic effect, stimulating progesterone production (Gibori, Keyes and Richards, 1978). A similar relationship could exist between the interstitial tissue and the CL in the tammar: when dissecting ovaries to prepare tissues for grafting, it was noticed that the ovary with the CL always seemed to have more interstitial tissue.
From work on eutherian species, reviewed in Chapter 1, it is probable that as the follicles reach preovulatory maturity they become the major ovarian source of oestrogen, probably secreted as oestradiol. Indeed, evidence that certain doses of oestradiol-17β have a positive feedback effect on LH secretion suggests that in the tammar, as in eutherians, the ovulatory surge of LH is triggered by rising levels of oestradiol during late proestrus, acting at the hypothalamic level.

The notion that oestradiol controls both tonic and cyclic gonadotrophin secretion agrees with prevailing ideas, reviewed in Chapter 1, of the endocrinology of eutherians with spontaneous ovulation and luteal development, although Hauger, Karsch and Foster (1977) have taken the alternative view that, in the sheep, oestradiol secretion is controlled by LH levels, rather than vice versa, with progesterone being the important ovarian negative feedback agent. It seems, however, that the latter cannot be true in the tammar, as follicular maturation, which may be regarded as an indication of gonadotrophin secretion, occurs when progesterone levels are highest.

The changing levels of oestradiol (or another oestrogen) are thus seen as the central coordinating influence in the control of the oestrous cycle in the tammar. However, while it is probably luteal oestradiol that controls tonic gonadotrophin secretion and, hence, follicular development during the first half of the cycle, it is oestrogen produced by the enlarging follicles that probably triggers the ovulatory LH surge by positive feedback (Fig. 7.1).

The LH surge, as defined in Section 5.2, lasts about 12 hours, and peak levels of LH may be over 50 ng/ml plasma. Certainly, sustained hypothalamic stimulation would be necessary to induce an LH discharge of this magnitude, since after a single injection of LH-RH, peak LH levels, rarely over 20 ng/ml, are reached within 30 minutes and then decay to
basal levels in about 2 hours. Radioimmunoassay of LH and LH-RH in plasma of sheep during oestrus (Crighton et al., 1973) suggested that to mimic the natural LH surge in anoestrous sheep, multiple injections of LH-RH would be more effective than a single injection of the same dose.

This was confirmed by Crighton et al. (1975), who found that there was an accelerated response to the second injection, 1.5 hours after the first. In the tammar, I found that there were similar responses to two injections of LH-RH one hour apart, but further investigation would be worthwhile, as Yen (1977) has proposed that the induction of a self-priming effect of LH-RH, plus a large increase in the readily-releasable pool of pituitary LH, by rising levels of oestradiol is the mechanism whereby the ovulatory LH surge is induced.

The proestrous peak of oestradiol is also responsible for the essential synchronization of oestrus and the LH surge in the sheep, although progesterone priming is also necessary for normal oestrous behaviour (Scaramuzzi et al., 1971). In the tammar, the LH surge reaches a peak about 8 hours after mating, and precedes ovulation by 20-40 hours, probably about 24 hours. This is very similar to the sheep, in which the LH peak occurs between the 5th and 12th hours of oestrus (Hansel, Concannon and Lukaszewska, 1973) and 24-28 hours before ovulation (Cumming et al., 1973), the LH surge here also lasting about 12 hours (Goding et al., 1969). However, both the interval between the LH peak and ovulation and the duration of the surge appear to be longer in primates (Weick et al., 1973, Karsch et al., 1973).

The model of the endocrine control of the oestrous cycle which I have developed during this discussion assigns LH and FSH to their classic roles of ovulating hormone and stimulator of follicular growth respectively. Many studies of eutherians have found a peak of FSH in the
circulation coincident with the LH surge, but in tammars sampled frequently through the time of oestrus, FSH levels were undetectable except in single plasma samples from certain animals only. Because the levels are so low, the variations in FSH secretion during the cycle and the role of this hormone in the female tammar remains, at present, a matter of supposition. Indeed, any further elucidation of the role of either gonadotrophin must await the development of more sensitive assays, perhaps RIA's based on purified macropod LH and FSH preparations or improved RIA's using macropod testis receptors, with which changes in tonic gonadotrophin levels may be monitored.

Nevertheless, it is already clear that in many respects the endocrine control of the oestrous cycle in the tammar conforms to eutherian models. However, it is more difficult to find parallels for other aspects of its reproductive biology, the most striking of which being its strict seasonality and the associated seasonal quiescence.

The breeding season of the tammar is initiated at the summer solstice with remarkable synchrony among the females. Although the proximate stimulus causing reactivation appears to be the decreasing day-length (Sadleir and Tyndale-Biscoe, 1977), this is presumably mediated by some endocrine mechanism, originally thought to involve an increase in gonadotrophin levels. However, Hearn's experiments on the effects of hypophysectomy first suggested that seasonal quiescence is ended by removal of a pituitary inhibition rather than by gonadotrophic stimulation (Hearn, 1972; 1973; 1974).

Tyndale-Biscoe's group have now provided convincing evidence that prolactin is the agent of the pituitary inhibition of the CL during lactational quiescence. Prolactin injections postponed CL reactivation after hypophysectomy (Tyndale-Biscoe and Hawkins, 1977), while single i.m.
injections of bromocryptine (0.2, 1.0 or 5.0 mg/kg body weight), a substance which reduces, but does not abolish, prolactin secretion, induced luteal reactivation in lactating females during the breeding season (Tyndale-Biscoe and Hinds, manuscript in preparation). In June, non-lactating females which had entered seasonal quiescence reactivated after a single dose of 1.0 or 5.0 mg/kg of bromocryptine, but in September to late December, even the higher dose was ineffective. This suggests that either prolactin levels are very high during seasonal quiescence or that another pituitary factor, not affected by bromocryptine, is involved.

However, recent measurements by Dr. A.S. McNeilly of prolactin in plasma of tammar wallabies by heterologous RIA (McNeilly and Friesen, 1978) have suggested that the end of the non-breeding season may be associated with an abrupt drop in prolactin levels. In the absence of further evidence, therefore, it could be postulated that both lactational and seasonal quiescence are governed by changes in prolactin levels.

During my own study, hints began to emerge that the non-breeding season is also characterized by subtle changes in the hypothalamo-pituitary-ovarian axis which reinforce, or interact with, the prolactin-centred control system. The rest of this discussion, therefore, will be concerned with the annual cycle of the tammar.

The control of lactational quiescence by prolactin is obviously correlated with its primary role in lactogenesis, but, in the tammar, prolactin secretion must continue after the young is weaned (usually in late September), and quiescence is maintained until the end of the non-breeding season is signalled by a photoperiodic stimulus (Sadleir and Tyndale-Biscoe, 1977). Here, analogies with eutherian species may be found.

The endocrine control of seasonality has been most thoroughly studied in the sheep, as this is an economically important species.
Prolactin has previously been implicated in the maintenance of lactational anoestrus in the sheep (Kann and Martinet, 1975), and Walton et al. (1977) have extended this to suggest that it is also important in the control of seasonal anoestrus. They propose that anoestrus is maintained by an antigonadotrophic effect of high prolactin levels, the resumption of oestrous cycles being brought about by the drop in prolactin levels which occurs in response to decreasing daylength at the beginning of the breeding season, this being comparable to recent findings in the tammar (Tyndale-Biscoe and Hawkins, 1977; Tyndale-Biscoe and Hinds, manuscript in preparation; McNeilly et al., manuscript in preparation).

The finding that female tammars show elevated plasma LH levels at the time of reactivation after the summer solstice was unexpected, as there is no rise in total gonadotrophin levels following reactivation after removal of pouch young during the breeding season (Hearn, 1972; 1974). I did not investigate this point specifically, but examination of data from several experiments failed to provide any evidence for a rise in LH levels at such times, though it is possible that short-lived elevations were missed by daily blood sampling.

It appears, however, that LH levels do rise during reactivation from seasonal quiescence, presumably shortly after the drop in prolactin levels, although simultaneous measurement of both hormones will be necessary to clarify this. On the other hand, one non-lactating animal given a single injection of LH-RH in October, the middle of the non-breeding season, gave birth 26 days later (see Section 6.9), i.e. the quiescent CL and blastocyst had reactivated, apparently in response to the rise in plasma LH. The peak LH level was only 2.9 ng/ml plasma, but this is similar to the levels measured in plasma of wallabies shot during the period of reactivation. Of course, it is also possible that reactivation
was a more indirect effect of the experiment and not related to the pulse of LH.

Indeed, because prolactin has a direct effect on the CL and because luteal development is independent of gonadotrophin support, it is unlikely that the elevations of plasma LH are a necessary stimulus for reactivation. A close inverse relationship between gonadotrophin and prolactin secretion has been observed in rams (Lincoln, McNeilly and Cameron, 1978) and the same may be true in the tammar. Thus the LH peaks may represent simply a major readjustment of this system after the sharp drop in prolactin levels. Since prolactin levels are probably higher during the last quarter of the year than at any time during lactation (Tyndale-Biscoe and Hinds, manuscript in preparation), reactivation from lactational quiescence would not involve such a marked change in the endocrine balance.

Although the patterns of hormone secretion associated with the seasonality of reproduction appear to be very similar in the tammar and the sheep, different endocrine control systems are involved in the suppression of ovulation. In the quiescent tammar, blastocyst development is arrested at a stage equivalent to day 7-8 of pregnancy (Tyndale-Biscoe, 1978), and luteal development presumably ceases at or before this stage. I envisage, therefore, that the inhibition of follicular development occurs by the same mechanism as appears to operate during the first half of the oestrous cycle, i.e. gonadotrophin secretion is inhibited by the negative feedback effect of luteal oestradiol. It should be recalled that lutectomy during the non-breeding season can induce ovulation (Tyndale-Biscoe and Hawkins, 1977). In addition, though, hypothalamic sensitivity to steroid feedback is probably increased. The latter was also proposed by Hearn, Short and Baird (1977), although they implicated the low levels of progesterone produced by the quiescent CL as being
responsible for the inhibition of gonadotrophin secretion.

The ovaries of an anoestrous sheep, however, lack corpora lutea, and a different explanation must be found for the antigonadotrophic effect of prolactin. There is evidence that it has a direct action on the hypothalamus, altering its sensitivity to steroids (Kann, Martinet and Schirar, 1976), but Walton et al. (1977) speculated that prolactin directly influences ovarian, i.e. follicular, activity, as has been found in the woman (McNatty, Sawers and McNeilly, 1974) and the mouse (McNatty, Neal and Baker, 1976). If the latter theory is correct, it would form an interesting analogy with the tammar, as in each species prolactin would have a direct inhibitory effect on the ovarian tissue which controls follicular development during the oestrous cycle.

Being an opportunistic, rather than a seasonal, breeder, the red kangaroo, Megaleia rufa, does not exhibit seasonal quiescence. However, it is mentioned here because it differs from the tammar in that the interval between lutectomy and ovulation is longer in lactating than in non-lactating females (Sharman and Clark, 1967). This suggests that, besides maintaining luteal quiescence, the suckling stimulus also has some inhibitory effect on follicular growth. In this macropod, then, prolactin may have a direct effect upon both the CL and the follicles.

However, the macropod marsupials which offer the best parallel to the anoestrous sheep are the grey kangaroos, Macropus fuli ginosus and M. giganteus. Unlike other macropods such as the tammar, these species, which are seasonal breeders, do not carry a quiescent CL through lactation. Instead, the female enters a true anoestrus, the proestrous phase of the cycle being suppressed by suckling. Here then, as in the anoestrous sheep, follicular, rather than luteal, development is suppressed, presumably by a prolactin-mediated mechanism. Loss of the pouch young during the
breeding season induces ovulation about 10 days later (Poole and Pilton, 1964; Poole and Catling, 1974), suggesting that most, or all, of the gonadotrophin-dependent phase of follicular growth is suppressed by suckling. However, if this occurs later in the year, the female does not re-ovulate. This implies that anoestrus is then being maintained by a different mechanism, probably a reduction in gonadotrophin levels.

Endocrinological studies of anoestrous grey kangaroos may therefore provide useful comparisons to the seasonally quiescent tammar. Although the inhibition of luteal development by prolactin is the primary cause of lactational quiescence in the tammar, it appears that seasonal quiescence is also characterized by changes in the hypothalamo-pituitary-ovarian axis.

In quiescent tammars, ovariectomy causes a rise in LH levels and exogenous oestradiol will induce LH release, which suggests that the hypothalamo-pituitary system is potentially responsive to both negative and positive feedback effects of steroids. I have also suggested that the sensitivity of the hypothalamus to negative feedback is increased during quiescence: this has been clearly demonstrated in the anoestrous sheep (Scaramuzzi and Baird, 1977; Legan, Karsch and Foster, 1971).

Martensz et al. (1976) have suggested that ovarian androstenedione modulates the feedback effects of oestradiol in the anoestrous sheep: there was an increase in the mean LH level and in the frequency of spontaneous LH discharges in sheep after active immunization against androstenedione.

Although in sheep and women, androstenedione is secreted almost entirely by the follicles (Baird, Swanston and Scaramuzzi, 1976; Baird et al., 1974), it is tempting to speculate that in the tammar a steroid with similar effects may be secreted by the interstitial tissue. As
already mentioned, the inability of apparently healthy interstitial tissue
grafts to depress gonadotrophin levels in ovariectomized wallabies leaves
open the suggestion that the tissue has an indirect, or modulatory,
effect on gonadotrophin secretion.

There is evidence that gonadotrophin secretion is altered
during quiescence. Tyndale-Biscoe and Hawkins (1977) found that ovula-
tion occurred in only 9/16 females luteectomized in the non-breeding season,
and in some of these, ovulation occurred without oestrus (presumably
because of the lack of progesterone priming), and without vaginal corni-
ication. The latter suggests that oestrogen levels were below normal,
which in turn implies that gonadotrophin secretion was inadequate and that
the hypothalamus was hypersensitive to the negative feedback effect of
oestrogen.

Conversely, though, the failure of ovarian cortex grafts in
ovariectomized animals to become functional during the non-breeding season
suggests that ovarian receptivity (or the number of receptors) may also
be decreased, as gonadotrophin levels rose in these animals. However,
this may also have been due to a too hasty termination of the experi-
ment, as even in the breeding season, similar cortex grafts did not
become functional (i.e. depress gonadotrophin levels) until several weeks
after operation. Hearn and Tyndale-Biscoe (unpublished results) found
that ovarian cortex grafts made in the non-breeding season were able to
support pregnancies in the following January, and there is evidence that
ovulations did not occur until early in that month.

However, the best evidence for a change in the hypothalamo-
pituitary system during seasonal quiescence comes from a comparison of
the effects of ovariectomy and of LH-RH on plasma gonadotrophin levels
at different times of year. The greatly diminished responses of intact
females to LH-RH during the non-breeding season, even a series of 3 LH-RH
injections at 1 hour intervals failing to evoke a response comparable
to those seen in the breeding season, suggests that pituitary stores of
LH are low. Indeed, Hearn (1972) found that the gonadotrophin content of
the pituitaries of male wallabies was 4 times greater in January than in
August.

Furthermore, in ovariectomized wallabies, the fluctuations in
LH levels in daily blood samples were greater in the non-breeding season,
implying a more markedly pulsatile secretion pattern, and the ability
to respond to an LH-RH stimulus seemed to depend upon whether a spontaneous
discharge of LH had occurred recently (within about 2 hours). Again
this suggests that LH is not being stored by the pituitary, but is
synthesized and then released in a continuous series of large pulses,
which in the quiescent intact animals is suppressed by ovarian steroids
to a constant, very low level of secretion.

The differences in the patterns of gonadotrophin secretion in
ovariectomized wallabies at different times of year suggest that a
non-ovarian control factor is also involved. As the non-breeding season
is governed ultimately by photoperiod, the pineal is a likely candidate.
 Acting as a neuroendocrine transducer of such environmental stimuli
(Axelrod, 1974), it modulates gonadotrophin release in eutherian mammals,
its proposed effectors being melatonin or serotonin (Kamberi, Mical and
Porter, 1971), a gonadotrophin-inhibiting factor, probably arginine-
vasotocin (Cheesman and Fariss, 1970) or a polypeptide gonadotrophin-
releasing factor (Kotaras, McIntosh and Seamark, 1977). Thus the endocrinology of the pineal in the tammar is another area for future research.

Although the marsupial mode of reproduction is the primary
feature distinguishing them from eutherian mammals, there has emerged
during this discussion the basic similarity of hypothalamo-pituitary-
oviductal relationships in the tammar and in eutherians. The model presented
here of the control of the tammar oestrous cycle is based mainly upon deduced, rather than experimentally verified, patterns of hormone secretion, but it is clear that the endocrine interactions are comparable to those regulating ovarian events in eutherians with spontaneous ovulation and luteal development.

The one feature which clearly distinguishes the reproductive endocrinology of the tammar from that of other mammals studied so far is the tonic inhibition of the CL by prolactin. This is the central mechanism controlling lactational and seasonal quiescence, but, as the work presented here has shown, the latter is also characterized by subtle changes in the hypothalamo-pituitary-ovarian axis, and here again close eutherian parallels can be found.

Thus although the general reproductive biology may differ markedly between species due to variations in the finer details of the endocrine interactions involved in its regulation, there is an underlying basic pattern of hormonal control which now appears to be common to both eutherian and marsupial mammals. This study, therefore, has illustrated once again that intriguing principle of mammalian reproductive endocrinology, similarity with diversity.


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ADDENDUM


A SOLID PHASE RADIOIMMUNOASSAY FOR LUTEINIZING HORMONE

The basis of the solid phase radioimmunoassay is the ability of certain polymeric surfaces to adsorb antibodies. Thus the inner surfaces of polypropylene or polystyrene assay tubes can be coated uniformly with antibody simply by allowing diluted antiserum to stand in the tubes for a short while. After the antiserum solution is removed, the entire reaction is carried out in the coated tubes and the antibody-bound and free fractions are finally separated by aspirating the reaction mixture.

The main advantage of the coated tube RIA method is its technical simplicity. When I began my study, I did not have access to a refrigerated centrifuge suitable for spinning large numbers of small tubes: this precluded use of a double-antibody separation. A coated tube assay therefore seemed ideal. This Appendix summarizes my experiments with such an assay system based upon an anti-ovine LH and using ovine LH as tracer.

MATERIALS AND METHODS

Antiserum: this was a rabbit anti-ovine LH raised against NIH-LH-S12 by Dr. A.A. Gidley-Baird, University of Sydney (Gidley-Baird and Bindon, 1977).

The standard was NIH-LH-S19. All dilutions of the standard were prepared in HWP.
The tracer was also NIH-LH-S19. This was iodinated to a specific activity of 100 - 250 µCi/µg by the method of Greenwood, Hunter and Glover (1963), using 1 mCi Na$^{125}$, 100 µg chloramine-T and 120 µg sodium metabisulphite for iodination of 5 µg LH. The iodinated hormone was separated by adsorption chromatography on Whatman's CF-11 cellulose.

The assay procedure followed that of Gidley-Baird and Bindon (1977). Tubes were coated with antiserum by allowing them to stand containing 0.8 ml of a 1:40,000 dilution of A-oLH for 2 hours at room temperature. After the coating solution was sucked off, the tubes were rinsed with saline. The standard was diluted in HWP to give 0.0625 - 8.0 ng NIH-LH-S19/100 µl. 100 µl plasma samples were usually assayed, but if necessary HWP was added to keep the volume of plasma constant at 100 µl/tube. Each tube also received 700 µl diluent. Tracer (200 µl of $^{125}$I-LH, 30,000 - 40,000 cpm/tube) was added after incubation for 24 hours at 37°C, and the tubes were incubated for a further 24 hours at 37°C before the unbound fraction was sucked off and the tubes rinsed twice before radioactive counting.

EXPERIMENTAL PROCEDURES AND RESULTS

A typical standard curve is shown in Fig. A.1. Complete displacement of tracer was effected by 1 µg LH, and the standard curve, plotted as log dose versus B/B$_0$ (as defined in Chapter 3), was linear over the range 0.25 - 8.0 ng LH/tube, which corresponds to 20 - 80% displacement of tracer from the antibody.

To investigate some factors affecting the sensitivity of the assay, several experiments were performed. Results are given in brief:

1. More tracer was bound when the diluent pH was 5.6 rather than 7.5 Diluent of pH 5.6 was used routinely.
2. EDTA is normally included in diluent to minimize possible interference in the antibody-antigen reaction by complement factors present in plasma (Raiti and Davis, 1968). Comparison of standard curves run in diluent with or without EDTA showed that the absence of EDTA had little effect upon the amount of tracer bound to antibody. However, EDTA continued to be included in the diluent.

3. Since assay sensitivity may be affected by tracer purity, a more highly purified ovine LH preparation (G3-222B, Papkoff) was iodinated to similar specific activity as the NIH-LH-S19. Antibody titration curves and standard curves at three different dilutions of A-oLH showed that there was little difference in the % binding of the two tracers and no increase in sensitivity when G3-222B was used as tracer. (Note: it was later found that there was little difference in the potencies of G3-222B and NIH-LH-S19 in the double antibody RIA - see Fig. 3.3).

4. To investigate the effects of plasma, serum and protein concentration on tracer binding, standard curves were set up using standards diluted in HWP, plasma from a normal male wallaby (NWP), serum from the same bleeding as the NWP and diluent containing 0, 2.5, 5.0, 10.0 or 20.0 g% BSA. There was less tracer bound in the presence of increasing BSA concentration, but in HWP and NWP there was significantly more tracer bound than with any BSA concentration although the protein concentrations of the plasmas (5.9 g% for HWP and 6.5 g% for NWP) fall in the middle of the range of BSA concentrations used. Slightly more tracer was bound in the presence of serum ($B_0 = 20\%$) than NWP ($B_0 = 19\%$).
Dose-response curves for dilutions of plasma from an oestrous female (o) and a pool of 1974 plasma with (v) and without (\wedge) 80ng NIH-LH-S19/ml plasma compared with the NIH-LH-S19 standard curve (•).
Some factor present in plasma may protect the tracer from incubation damage (or, conversely, BSA may induce damage), as non-specific binding was lowest in HWP and NWP (0.28 and 0.22%B) and significantly higher in the absence of protein (3.5%B).

Dose-response curves for wallaby plasmas are shown in Fig. A.1.

The plasmas were diluted in HWP to keep the total volume of plasma in each tube constant, and 100 µl of each dilution were assayed in triplicate. Regression coefficients for the standard curve and the dose-response curve for 3894 plasma, calculated using 0.25, 0.5, 1.0, 2.0 and 4.0 for the x axis and raw data in cpm for the y axis, were 604.4 (variance 3510.3) and 595.6 (variance 3510) respectively. Analysis of variance showed that these were not significantly different. LH levels in plasma varied with physiological condition in a predictable manner, being 10 ng/ml in an ovariectomized female, 36 ng/ml in an oestrous wallaby and undetectable in a seasonally quiescent wallaby before, during and after hypophysectomy.

However, later experiments found that dose-response curves for plasmas did not always appear to be parallel to the standard curve except when NIH-LH-S19 was added to the undiluted plasma (Fig A.1) and recovery of added LH was good.

Dose-response curves for pituitary preparations are shown in Fig. A.2. A crude homogenate of wallaby anterior pituitary lobe caused strong displacement of tracer, suggesting high LH content, but posterior lobe homogenate also displaced tracer and in this case, but not the first, the dose-response curve appeared parallel to the standard curve. The dose-response curve for the crude gonadotrophin fraction J.H 74.9(1) also was not parallel to the standard curve, the deviation being more marked at higher concentrations.
Dose-response curves for dilutions of wallaby gonadotrophin fraction J.H. 74.9(1) (••••••) and homogenates of wallaby anterior pituitary (•) and posterior pituitary (▲) compared with the NIH-LH-S19 standard curve (●).
Measurement of LH in wallaby plasmas: since the assay was believed to be suitable for the purpose, it was used to investigate the changes in plasma LH after luteectomy in two series of samples, one series collected in 1974 (i.e. two years old) and the others recently collected in 1976. Some 1974 samples were pooled to form a "standard plasma", aliquots of which were included in every assay. When LH concentrations were calculated, it was found that:

1. LH levels generally appeared to be higher (2.0 - 80.0 ng/ml) in 1974 samples than in 1976 samples (0 - 37.0 ng/ml).

2. In a series of jugular vein plasma samples collected from 2 catheterized wallabies during an early experiment with LH-RH, LH was undetectable in 25/32 samples, and B/B for these 25 samples ranged from 1.0 to 2.55. Also, the LH levels in plasma did not appear to be correlated with time after LH-RH as was found in later experiments using the GDN #15 assay.

3. The interassay variation of the standard plasma was unacceptably high at 57% (mean LH concentration = 33.9 ± S.D. 19.3 ng/ml plasma, n = 9).

4. One possible source of interassay variation was a shifting of the standard curve. Originally, the standards were prepared by adding 8 µl (i.e. 80 ng) of stock solution to 1 ml HWP and then serially diluting in HWP. The 8 µl was measured by marking off an appropriate length on a 100 µl microcap: in one assay, the mean B/B for triplicate tubes containing 8 ng/tube measured using 5 different microcaps was 0.39 ± S.D. 0.03.

Use of a 10 µl Hamilton microsyringe for measuring the standard stock solution did not improve the interassay variation.
of the standard plasma ($\bar{x} = 15.6 \pm S.D. 12.4$ ng/ml, $n = 8$: coefficient of variation = 79%). However, there did appear to be better correlation between the values obtained from 3 different assays for a series of 1976 samples, as shown in Table 1.

Table 1: LH content (ng/ml plasma) of 1976 plasmas measured in three assays

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* undetectable LH

DISCUSSION

Although it had originally appeared that this solid-phase assay was suitable for measuring LH in wallaby plasma, further experiments showed it to be unreliable. Contributing factors may have been a lack of sensitivity for wallaby LH, aggravated by the use of only 100 $\mu l$ plasma in the incubation mixture, and the use of the relatively impure NIH-LH-S19 as tracer. The behaviour of posterior lobe homogenate in the assay system suggested that the antiserum may have contained antibodies to structural proteins and/or a non-specific inhibition of tracer binding by high protein concentrations. Other experiments supported the idea that varying protein
concentrations in samples, especially those stored for extended periods, was the major problem with this assay: coated-tube assays are known to be sensitive to variations in protein concentration (Daughaday and Jacobs, 1971).

After about 18 months' unsuccessful work with the solid-phase assay, some preliminary experiments with a double-antibody separation method were performed using facilities kindly provided by Dr. C. Eastman, Woden Valley Hospital, Canberra. Using the antiserum at a dilution of 1:300,000 with Goat anti-rabbit gamma globulin (Calbiochem) as second antibody, diluted 1:10, it was found that in most plasma samples from female wallabies, including the standard plasma, LH was undetectable. In three ovariectomized wallabies, LH levels were 3.0, 5.2 and 7.5 ng/ml plasma. These values are more comparable to those obtained with the GDN #15 assay, described in Chapter 3.

At about this time, Dr. R.L. Sutherland joined Dr. Tyndale-Biscoe's group at CSIRO, Division of Wildlife Research as a radioimmunoassayist. A refrigerated centrifuge and gamma counter were installed in their laboratory so I was able to continue work under the guidance of Dr. Sutherland. With the acquisition of some of Niswender's GDN#15 antiserum, my original antiserum was abandoned in its favour.

REFERENCES


## Appendix 2

### LH Levels in Plasma of Female Wallabies During the Periovulatory Period

(see Section 5.3)

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3797 (RPY between 1-3 and 8.3.78)

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### APPENDIX 3

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LH and FSH concentrations in plasma of bilaterally ovariectomized wallabies, and bilaterally ovariectomized wallabies bearing ovarian cortex or interstitial tissue grafts under the pouch skin from d.0 to d.22-23 after operation (see Sections 6.2 and 6.4).

1. LH concentrations (ng NIH-LH-S19/ml plasma)

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<th>Int. tissue grafts</th>
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Note: ud = undetectable.
2. **FSH concentrations** (ng NIH-FSH-S12/ml plasma)

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1. **Level of hormone below the limit of detection of the assay**

2. **No sample for that day.**
APPENDIX 5

LH and FSH concentrations in plasma of

1. animals bilaterally ovariectomized during the breeding season
2. ovariectomized animals bearing ovarian cortex grafts under the pouch skin
3. ovariectomized animals bearing interstitial tissue grafts under the pouch skin

for 0-43 days after operation (see Section 6.4).

1(a) Ovariectomized animals: LH (ng NIH-LH-S19/ml plasma)

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1 The operations were performed over 3 consecutive days. For the first 21 days, animals were bled at set intervals after operation. After d.21, all animals were bled on certain days, so the intervals after operation differed accordingly.

2 This animal suckled a pouch young throughout the experiment.

3 LH level undetectable, i.e. <0.2 ng/ml plasma.
1(b) Ovariectomized animals: FSH (ng NIH-FSH-S12/ml plasma)

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APPENDIX 6

Publications arising wholly or partly from the work presented in this thesis.


Abstracts a, b and c are given in full.
A HETEROLOGOUS RADIOIMMUNOASSAY FOR
TAMMAR WALLABY LUTEINIZING HORMONE

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A heterologous double antibody radioimmunoassay for tammar wallaby LH has been developed using a rabbit anti-ovine LH antiserum and highly purified ovine LH (Papkoff G3-222B) as the radioligand. This antiserum (GND#15) has previously been shown to cross-react specifically with a large number of mammalian Lh4s including that of a marsupial, the American opossum (1). The assay procedure followed that of Niswender et al. (2). At an antiserum dilution of 1:40,000, 32-40% of the 125I-ovine LH was specifically bound, while the non-specific binding was never greater than 5% of the total bound radioactivity. At this dilution of antiserum, the minimum amount of ovine LH standard (NIH-LH-S19) which resulted in significant displacement of the radioligand from the antiserum was 40 pg/tube (200 pg/ml plasma), while complete displacement was effected by 10 ng/tube. The between-assay coefficient of variability was 12%.

Highly purified human (LER-960), ovine (Papkoff G3-222B, LER-1374A) and rat (NIAMDD rat LH-1-4) LH preparations showed parallel dose-response curves to the ovine LH standard while highly purified human (NIH-FSH-HS10, ovine (Papkoff G4-150C) and rat (NIAMDD rat FSH-I-3) FSH preparations demonstrated less than 1% cross-reactivity. Serial dilutions of crude tammar wallaby pituitary homogenates, partially purified wallaby gonadotrophin fractions, a highly purified wallaby LH preparation and plasma samples from ovariectomized and oestrous female gave dose-response curves which were parallel to the ovine LH standard.

Preliminary results suggest that the basal levels of LH in non-oestrous female tammar wallabies are in the range 0.20 - 1.50 ng NIH-LH-S19/ml (n = 26) with many animals having values at or near the limit of detection of the assay. LH concentrations were markedly increased after ovariectomy (3.5 - 9.6 ng/ml, n = 6), on the day of oestrus (4.6 - 10.0 ng/ml, n = 4) and following administration of LH-RH when peak values of 10.2 - 12.0 ng/ml (n = 3) were observed. The levels in 14 adult males were in the range 0.20 - 6.0 ng/ml with only 2 animals having values near the limit of detection of the assay. Plasma from hypophysectomized animals had no detectable LH activity.

It is concluded that the anti-ovine LH antiserum, GDN#15, cross-reacts with tammar wallaby LH and a radioimmunoassay based on this antiserum should be useful in monitoring changes in plasma LH levels in this species.

REFERENCES
A heterologous double antibody radioimmunoassay for tammar wallaby FSH was developed using a rabbit anti-ovine FSH antiserum (1) and highly purified human FSH (NIH-FSH-S11) as the radioligand. Tammar wallaby pituitary extracts and highly purified FSH preparations from the human (NIH-FSH-S11), ovine (Papkoff G4-150C) and rat (NIAMDD rat FSH-I-3) displaced labelled FSH from the antiserum while ovine LH, and prolactin showed negligible cross-reactivity. Serial dilutions of serum from ovariectomized wallabies gave dose response curves which were parallel to partially (NIH-FSH-S11) and highly (Papkoff G4-150C) purified ovine FSH. When the final dilution of antiserum was 1:40,000, 37-41% of the radioligand was specifically bound while non-specific binding was less than 2.5%. Preliminary data indicate that basal levels of plasma FSH in non-oestrous female tammar wallabies are in the range 0 - 120 ng NIH-FSH-S11/ml (n = 20) with 50% of the animals having values below the limit of detection of the assay (50 ng/ml plasma). Ovariectomy resulted in a significant increase in FSH concentration (350 - 530 ng/ml, n = 8). FSH levels in 14 adult male wallabies were in the range 160-1, 332 ng/ml while serum from hypophysectomized animals showed no detectable FSH activity.

Radio-receptor assays using rat and kangaroo testicular FSH receptors and 125I-labelled human FSH (NIH-FSH-S11) have also been developed. Crude preparations of seminiferous tubules bound 4-5% of the added radioligand and 80 - 85% of this label was bound specifically. Highly purified human, ovine and rat FSH preparations and a partially purified wallaby pituitary gonadotrophin fraction displaced the radioligand from both testicular preparations while ovine LH, TSH and prolactin showed less than 1% cross-reactivity. The minimal detectable amount of highly purified human FSH was 2 and 5 ng/tube in the rat and kangaroo assay respectively. All FSH preparations and the wallaby gonadotrophin fraction gave parallel dose response curves when either rat or kangaroo testes were employed. However, the relative ability of these preparations to displace the radioligand differed depending on the source of seminiferous tubules eg. the wallaby gonadotrophin fraction was more potent than NIH-FSH-S11 in the kangaroo assay but less potent in the rat system. Similar techniques employing crude Leydig cell preparations and radio-iodinated human LH have also been developed for the measurement of pituitary LH. These results illustrate that the testes of macropod marsupials possess FSH and LH receptors which are specific for mammalian FSHs and LHS respectively. This has facilitated the development of radio-receptor assays for wallaby FSH and LH which will be valuable in monitoring the purification of wallaby FSH and LH. Their application to the measurement of gonadotrophins in wallaby serum is under investigation.

REFERENCE
LUTEBINIZING HORMONE AND OVULATION IN THE TAMMAR WALLABY

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Hearn (1), using a homologous radioimmunassay (RIA) based upon an antiserum to a crude wallaby gonadotrophin fraction with the same gonadotrophin fraction as the labelled hormone, reported a rise in gonadotrophin in plasma of tammar wallabies on the day of oestrus. Development of a sensitive and specific ovine-ovine RIA for wallaby LH (2) and more intensive blood sampling around the time of oestrus have allowed a fuller investigation of the temporal relationships of mating, the LH peak and ovulation in this species.

Pouch young were removed from 9 wallabies on d.0. to induce resumption of synchronous pregnant (3 animals) or non-pregnant (6 animals) cycles. From d.25 all animals were observed closely to detect as accurately as possible the times of parturition and/or first mating, and were bled every 12-24 h. After mating, they were bled every 4 h until autopsied at 24-48 h post-coitum.

LH assay showed that the LH peak was of approximately 12 h total duration, with mean maximum height 44 ng NIH-LH-S19/ml plasma (n = 8). The timing of the LH peak relative to mating was highly variable (-12 h to 24 h) but correlation between time after LH peak maximum and ovarian events was good. 0 - 20 h after the LH peak, follicles of increasing sizes, 3.5 - 4.3 mm, were observed, but by 40 h ovulation had occurred and oviducal eggs were found. By 48 h, the egg had reached the uterus.

To investigate whether LH release is triggered by similar stimuli as in eutherians, 12 female wallabies were given 10 µg LH-RH (Calbiochem) i.v. In all cases, LH levels rose sharply, from a mean pre-injection level of 3.3 ng/ml plasma to a mean peak height of 21 µg/ml, attained 25 min after LH-RH. In 3 animals given a second 10 ng LH-RH i.v., 1 h after the first, the second response was of similar magnitude to the first. Preliminary experiments using seasonally quiescent females showed that a single i.m. injection of estradiol-17β in oil at doses of 1 µg, 5 µg or 10 µg/kg body weight induced LH release after 9-12 h in 2/6, 1/3 and 5/6 wallabies respectively. However only in 2 cases did the magnitude of the response approach that of the oestrous LH peak or the response to LH-RH.

It appears, therefore, that endocrine mechanisms inducing LH release, and correlation between the LH peak and ovulation in the tammar are similar to the eutherian pattern.

REFERENCES
