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Improving the efficiency of reproduction and breeding in alpacas

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by Jane Vaughan

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Foreword

The aim of this project was to develop a practical protocol that would allow fixed-time mating of alpacas at a known stage of ovarian follicular development, in association with improved pregnancy rates following mating. A successful protocol would allow more efficient dissemination of selected genotypes throughout the national alpaca herd. Follicular growth characteristics in unmated alpacas were not well described in the literature, but this knowledge is essential to developing any exogenous hormone treatment and needed to be described more clearly.

The reproductive physiology of alpacas differs to that of other domestic animals and remains poorly understood. Females exhibit growth of successive large follicles and are induced ovulators. Unmated, non-ovulatory females are sexually receptive most of the time, apparently regardless of the stage of ovarian follicular development. Males mate in sternal recumbency for approximately 20 minutes and ejaculate many times during this period. Each ejaculate consists of low volume, high viscosity semen containing a low sperm concentration. Gestation length is about 11.5 months, twins are rare and males reach puberty from 1 to 3 years of age. Generation intervals are relatively long because males are slow to sexually mature and females exhibit an extended gestation, so conventional breeding results in slower genetic gain in comparison to other fibre-producing domestic species such as sheep and goats.

This report presents new information on ovarian follicular wave characteristics in non-pregnant female alpacas and introduces a practical protocol based on progesterone to control ovarian follicular growth. The protocol allows for fixed-time mating. The Australian alpaca industry will benefit from more efficient utilisation of genetically superior males and females and faster dissemination of improved genotypes throughout the national herd.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 800 research publications, forms part of our Rare and Natural Fibres R&D program, which aims to improve the efficiency of reproduction and breeding in alpacas.

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Simon Hearn
Managing Director
Rural Industries Research and Development Corporation

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The primary research herd of alpacas was generously donated by the Bellarine Veterinary Practice, the Australian Alpaca Association Inc., and Noel and Jan Laity, Park View Alpaca Stud. I am especially appreciative of alpaca breeders Jude Anderson and Alan Cousill of Pucara Alpaca Stud, Janie and Aldous Hicks, Judith and Phillip Street and Neil Parker of Coolaroo Alpaca Stud, and Allan and Carolyn Jinks of Benleigh Alpaca Stud for use of their alpaca herds and generous hospitality. I am indebted to veterinarians Ewen McMillan, David Hopkins and John Quinane of Bellarine Veterinary Practice, Denis Ryan and Mark Duncan of Torquay Animal House Veterinary Clinic and Hospital and Bill Beresford and James Ross of W Beresford and Associates for establishing contact between their alpaca clients and myself, and for unlimited use of equipment in their practices. Farm facilities were generously provided by Geoff and Margie Stanley, Gary and Ivana Stephenson, Max and Nancye Moore and Nathalia Fertiliser. Alpacas were shorn annually by Robert Cuthill. Bob Swinburn and Peter Carah provided assistance with animal husbandry.

Abbreviations

FSH	follicle stimulating hormone
GnRH	gonadotrophin-releasing hormone
hCG	human chorionic gonadotrophin
i.m.	intramuscular
LH	luteinising hormone
RIA	radioimmunoassay

Contents

Executive Summary

The aim of the studies in this report was to develop a treatment protocol that controlled ovarian follicular growth so that the time of optimum fertility could be predicted in female alpacas.

Female alpacas exhibit growth and regression of successive large follicles and typically only ovulate in response to the mating stimulus. Non-pregnant females are sexually receptive most of the time, apparently regardless of the stage of ovarian follicular growth. Conventional breeding results in slow genetic gain because matings occur at random stages of follicular development. Because of the nature of their reproductive physiology, assisted breeding technologies are poorly developed in alpacas and the Australian alpaca industry relies on transport of males and females over relatively large distances to disseminate superior genotypes. The efficiency of this form of genetic improvement would be enhanced if conception rates to a single mating could be increased.

Initial studies in this report aimed to clarify ovarian follicular growth characteristics in non-pregnant females. An inverse relationship between the diameter of the largest follicle and the number of follicles detected supports the hypothesis that follicular growth in camelids occurs in waves. It was established that the growth characteristics of follicular waves varied within and between females. Wide variation in the interval between successive follicular waves made the use of a mean interwave interval value inappropriate. Non-pregnant alpacas had a follicle in the size range potentially capable of ovulating, but of unknown fertility, on either ovary most of the time.

A second objective was to determine the relationships between sexual receptivity, mating behaviour, ovarian follicular state and mating success. It was not possible to correlate mating behaviour or ovarian status with mating success. Matings to optimise pregnancy rates in alpacas need to occur in the presence of an oestrogenic follicle that is capable of ovulation in response to mating. Simple detection of alpacas with follicles in this state was not possible and treatments to control ovarian follicular growth were therefore investigated.

Attempts to control ovarian follicular waves in alpacas were focussed on inducing regression of the existing dominant follicle of unknown age and allowing emergence of a new cohort of follicles at a known time after treatment. The induction of ovulation to remove the existing dominant follicle was not considered in these studies. Single intramuscular (i.m.) injections of 17β-oestradiol (oestradiol) or oestradiol benzoate, at different doses and with and without simultaneous injection of progesterone, were unsuccessful in controlling follicular growth to allow emergence of a new follicular wave at a known time. This finding was unexpected given that oestradiol causes the regression of follicles in cattle and sheep. It was concluded that alpacas, and perhaps camelids in general, have different intra- and/or extra-ovarian mechanisms that control follicular growth and regression compared with ruminants that are spontaneous ovulators.

Subsequent studies examined the effects of different protocols of progesterone treatment on ovarian follicular growth and regression. Twice daily i.m. injection of 25 mg of progesterone for 21 days was effective at inducing regression of the existing dominant follicle and suppressing emergence of a new follicular wave until treatment ceased. To make the treatment more practical and reduce the number of injections required, subcutaneous implants of norgestomet and lower frequency, higher dose progesterone treatments were examined. The most practical and effective protocol for ovarian follicular control in female alpacas was provided by 200 mg progesterone injected i.m. on Days 0, 2 and 4. The majority of females treated with this protocol had a newly-emerged follicle with a diameter

capable of ovulation on Day 16, 12 days after progesterone treatment ceased. Mating trials were performed on three commercial alpaca farms to compare pregnancy rates at Day 60 in females treated with the progesterone protocol and mated on Day 16 and females mated at random. Results showed that treated females were capable of ovulation, fertilisation and pregnancy, however, there was no difference in 60-day pregnancy test percentage between females receiving the Day 0-4 progesterone synchronisation protocol and females treated with oil placebo.

The oocyte contained in the first dominant follicle following progesterone treatment would need to be of normal fertility in order for the progesterone-based protocol to increase pregnancy rates to a single mating. As a first step to examining oocyte integrity, oocytes were retrieved by ultrasound-guided, transvaginal aspiration on Day 17 in females treated with the progesterone protocol described above involving injections on Days 0, 2 and 4. Half of these females had received an injection of luteinising hormone (LH) on Day 16 to simulate a mating-induced LH surge. Oocytes were examined by light and electron microscopy to observe whether the cellular ultrastructure was indicative of normal maturation. Only oocytes from those females that received LH showed changes to the cellular ultrastructure indicative of normal maturation including meiotic progression (nuclear maturation) from Prophase I to Metaphase I, an increase in the width of the perivitelline space and expansion of the cumulus cells surrounding the oocyte. This finding was interpreted to suggest that the oocyte contained in the first ovulatory follicle after progesterone treatment has a normal capacity for fertilisation and embryo development competency.

This report presents new information on ovarian follicular wave characteristics in non-pregnant female alpacas and introduces a practical protocol based on progesterone to control ovarian follicular growth. The protocol allows for fixed-time mating. The Australian alpaca industry will benefit from more efficient utilisation of genetically superior males and females and faster dissemination of improved genotypes throughout the national herd. Potential benefits include incorporation of the progesterone protocol into other assisted breeding technologies such as artificial insemination, embryo transfer and possibly the in vivo maturation of oocytes for in vitro fertilisation.

The Research was the basis of a PhD thesis presented by the author. Information has been condensed for this report but more details might be obtained by contacting Dr Vaughan initially by Email –vaughan@ava.com.au.

1. Introduction and objectives

The reproductive physiology of alpacas differs to that of other domestic animals and remains poorly understood. Females exhibit waves of ovarian follicular growth (Adams et al. 1990) and are induced ovulators (San-Martin et al. 1968). Unmated, non-ovulatory females are sexually receptive most of the time, apparently regardless of the stage of ovarian follicular development (Sumar 1983). Males mate in sternal recumbency for approximately 20 minutes and ejaculate many times during this period (Lichtenwalner et al. 1996). Each ejaculate consists of low volume, high viscosity semen containing a low sperm concentration (Sumar 1983, McEvoy et al. 1994). To achieve maximum conception rates, females should be mated when they have a follicle capable of ovulation on either ovary (Bravo et al. 1991b). Gestation length is about 11.5 months, twins are rare and males reach puberty from 1 to 3 years of age (Bravo 1994). Generation intervals are relatively long because males are slow to sexually mature and females exhibit an extended gestation, so conventional breeding results in slower genetic gain in comparison to other fibre-producing domestic species such as sheep and goats.

The Australian alpaca industry is endeavouring to become commercially viable by producing large quantities of high quality fibre. Breeders must place maximum selection pressure on males and combine this with rapid and broad dissemination of appropriate genotypes to achieve this goal. Many alpaca farmers do not own a male suitable for breeding as the Australian alpaca industry is based on hundreds of properties each with less than 20 animals. Added to this, semen is not yet amenable to reliable, cost effective artificial insemination (AI; Burgel et al. 1999). Farmers that do not own a male currently employ the services of other breeders for use of selected males in order to improve the fibre quality of their animals.

Therefore, the Australian alpaca industry relies heavily on ‘mobile’ or ‘trailer matings’ to disseminate throughout the national alpaca herd genotypes perceived to be superior for producing fine fibre. This method involves one alpaca, usually the male, being transported to the farm where mating is to occur. The farm manager determines the time of mating in both mobile matings and on-farm pen-matings. Mating of one male with multiple females in a paddock for a week or more rarely occurs in Australia. As female alpacas are usually receptive but do not always have a follicle capable of ovulation present on one or other ovary, pen mating presents the manager with a problem of determining the optimum time for breeding.

Ideally, the control of folliculogenesis in camelids would allow farm managers to present a female to a male at a time that maximises conception rates. This has been proposed to be when the female has a newly matured follicle of at least 7 mm diameter present on either ovary (Bravo et al. 1991b). Removal of an existing dominant follicle of unknown age, and inducing emergence of a new dominant follicle at a known time, would allow mating to proceed at a time when ovulation would be associated with release of an oocyte of optimal fertility, thereby maximising the likelihood of conception to a single mating.

This research has investigated methods to improve the efficiency of reproduction in alpacas by first gaining a better understanding of their unique reproductive physiology. The primary focus was on ovarian folliculogenesis and the control of ovarian follicular waves in females.

Development of a protocol to control follicular growth will immediately assist the current method of breeding alpacas in Australia. In other fibre industries, such as Merino sheep and Angora goat production, assisted breeding is being used to improve fibre quality more rapidly than would otherwise be possible by natural mating. It is anticipated that basic observations on reproductive physiology, and hormonal treatments to control ovarian follicular growth, examined in this research will facilitate the development of assisted breeding in all camelids.

2. Methodology

2.1 Experimental sites

Site 1

The main research herd was agisted on a 2 hectare property located at Curlewis (38°9'S, 144°30'E), 15 km east-south-east of Geelong on Port Phillip Bay, Victoria. The main soil type found on this property is a hard alkaline duplex soil with a yellow clay subsoil (Northcode 1962). The dominant pasture species is phalaris (*Phalaris spp*) with some cocksfoot (*Dactylus glomerata*), subclover (*Trifolium subterraneum*) and capeweed (*Arctotheca calendula*). This herd was supplemented with mixed-species pasture hay (*Lolium spp, Phalaris spp, Trifolium spp*) and lupins (*Lupinus spp*) when pasture was limiting.

Site 2

The plasma oestradiol and progesterone studies in Chapters 6 and 7, and part of the mating trial in Chapter 10, were carried out on a commercial alpaca farm situated at Curlewis (38°9'S, 144°30'E), 14 km east-south-east of Geelong on Port Phillip Bay, Victoria. The main soil type found on the property is a hard alkaline duplex soil with a yellow clay subsoil (Northcode 1962). The dominant pasture species are annual ryegrass (*Lolium rigidum*), cocksfoot, phalaris and subclover with some capeweed. The herd is routinely supplemented with lucerne hay (*Medicago sativa*), mixed-pasture hay and commercial alpaca pellets.

Site 3

The observational study in Chapter 3 and some of the mating trial in Chapter 10 were carried out on a commercial alpaca farm 30 km south west of Geelong between Torquay and Anglesea, on the south west coast of Victoria (38°24'S, 144°14'E). The main soil type found on the property is a hard alkaline duplex soil with a yellow clay subsoil (Northcode 1962). The dominant pasture species are perennial ryegrass (*Lolium perenne*), cocksfoot and fescue (*Festuca spp*), with some phalaris and capeweed. This herd is also supplemented as necessary with lucerne chaff, oaten chaff and hay (*Avena sativa*), lupins, rolled oats, and ryegrass- or cocksfoot-dominant pasture hay.

Site 4

Some of the studies in Chapters 5 and 8, and the majority of the mating trial in Chapter 10, were carried out on a commercial alpaca farm 100 km south west of Sydney, near Berrima in the southern highlands of New South Wales (34°29'S, 150°20'E). The main soil type found on the property is a Wianamatta shale that produces yellow podzolic soils (G, Van Owen, Dept Land and Water Conservation Mossvale, personal communication). The dominant pasture species are phalaris, perennial ryegrass, cocksfoot, fescue, red, white, strawberry and sub clovers (*Trifolium spp*) and some native grasses. The main weeds are Scotch thistle (*Onopordum acanthium*), capeweed and serrated tussock (*Nassella trichotoma*). The herd is supplemented with a commercial alpaca mix (oaten chaff, cereal grains, minerals) and lucerne hay when pasture is limiting.

2.2 Climate

The climate of the Victorian farms used in the project is characterised by warm, dry summers (December-March) and cool, wet winters (June-August), with an annual average rainfall of 600-700 mm. The farm in New South Wales is similar to the others but can have wetter summers and colder winters.

2.3 Experimental animals

In general, alpacas were mixed-age females, including maidens, mostly Huacaya breed, of Chilean, Peruvian and Australian origin. Because female alpacas may ovulate in the presence of a male, no males were kept at Site 1, and females used for studies on Sites 2, 3 and 4 were not allowed direct contact with any males apart from those participating in the mating trials in Chapters 3 and 10. In each experiment, all alpacas were treated at random stages of ovarian follicular development. No attempts were made to match a female at a known stage of follicular development on a particular day with a particular treatment protocol.

Experiments were performed following the guidelines set out in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 1997 and received approval from the Central Queensland University Animal Experimentation Ethics Panel. Each chapter outlines information detailing animals and treatments involved in that particular experiment.

Most of the studies were performed in females located at Site 1. All attempts were made to maintain alpacas at a reasonably constant weight to reduce any nutritional effects on reproduction, such as altered metabolism of gonadotrophins (Payne et al. 1991) and steroids (Parr et al. 1993), that have been observed in other species when protein and energy contents of the diet vary. Lupin supplementation was provided as necessary to maintain average dietary crude protein between approximately 8 and 10 % of dry matter intake, and therefore maintain body condition score.

2.4 Ultrasonography

Ovarian follicular dynamics were monitored by trans-rectal ultrasonography by a single operator using an Aloka SSD-500 ultrasound machine (Aloka Co, Japan) equipped with a 7.5 MHz linear array transducer. Typical ultrasonographic findings of ovarian structures in camelids have been described (Adams et al. 1989, Tinson et al. 1992, Skidmore 1994, Tibary et al. 2000). However the validation of ovarian ultrasonography by comparing ultrasonographic images with gross ovarian findings was not possible in this study as the individual value of female alpacas in Australia did not allow for collection of ovaries by ovariectomy or at post-mortem.

The vertical diameter of all follicles, based on the interface between the antral fluid and inner follicular wall, and corpora lutea greater than 2 mm diameter, were estimated and recorded manually on ovarian maps to show relative sizes and locations of ovarian structures every second day. Follicular diameters were generally estimated by comparing to fixed 3 mm and 7 mm markers on the screen to try and maintain objectivity during data collection and to minimise bias (Ginther 1998) but images with structures larger than 12 mm diameter were frozen and measured using internal electronic callipers. Ovaries with structures less than 3 mm diameter were recorded as containing ‘no visible structures’ or ‘multiple structures less than 3 mm’. No attempt was made to track follicles on an every second day basis as described by Ginther (1998) due to difficulty in identifying fixed structures on the ovary such as the ovarian attachment, ovarian poles or a corpus luteum (animals mostly non-ovulatory) and due to the ability of ovaries to rotate freely during examination.

All alpacas undergoing trans-rectal ultrasonography were restrained in sternal recumbency in a purpose-built crate to reduce risk of injury to both animals and operator. The restraint device was portable and allowed easy transport between experimental sites. Faeces were manually removed from the rectum and 20 mL obstetrical lubricant inserted before a gloved, lubricated hand and ultrasound transducer were placed in the rectum.

It has been proposed that rectal palpation and/or ultrasonography may cause induction of ovulation in response to this stimulus (Bourke et al. 1995a, Ratto et al. 1997). Other studies have shown this not necessarily to be the case (Skidmore et al. 1995, Chaves et al. 2002).

It has been observed in the dromedary camel that peritoneal fluid sometimes accumulates in the ovarian bursa around the ovary (Tinson et al. 1992). This phenomenon was observed in some alpacas during the study, making detection of follicles more difficult.

Pregnancy diagnoses in the observational study in Chapter 3 and the mating trial in Chapter 10 were performed trans-abdominally by veterinarians between days 45 and 80 after the last mating date. A Sonoranger II sector scanner with a 5 MHz offset probe (Ausonics, Australia) was used at Site 2 (E. McMillan), a Pie Medical 200 linear array scanner with a 5 MHz probe (Pie Medical, Japan) was used at Site 3 (D. Ryan) and a Microimager 2000 sector scanner with a 5 MHz offset probe (Ausonics, Australia) was used at Site 4 (W. Beresford).

2.5 Treatments used to control ovarian follicular waves

17β- Oestradiol

Oestradiol in sesame seed oil was prepared according to Murray (1997). 100 mg 17β-oestradiol (oestradiol; 1,3,5(10)-estratriene-3,17β-diol; Sigma Chemical Co, USA) in 1.5 g benzyl alcohol (BDH Laboratory Supplies, England) by stirring for 30 minutes. Sesame seed oil (household grade) was then added to make a 0.5, 1 or 2 mg/mL solution. The mixture was stirred continually for 30 minutes while it was heated in a beaker of boiling water. The mixture was placed into sterile 50 mL hypovials, sealed and stored at 4°C until used.

Oestradiol benzoate

Two commercially available products were used. Oestradiol Benzoate® (Intervet, Australia) consists of oestradiol monobenzoate in oily solution and has a concentration of 5 mg/mL. Cidirol® (InterAg, NZ) consists of oestradiol benzoate in oily solution and has a concentration of 0.5 mg/mL. Preparations were stored in the dark, at room temperature.

Progesterone

The commercial product Progesterone® (Jurox Pty Ltd, Australia) was used and has a concentration of 25 mg/mL of progesterone. The placebo used in Chapter 9 contained the same oil and preservative used in the manufacture of Progesterone® and was prepared by Jurox Pty Ltd, Australia. Bottles of progesterone and placebo were stored in the dark, at room temperature.

Norgestomet implants

The commercial product Crestar® (Intervet, Australia) was used. Each silicone implant contains 3 mg norgestomet. Implants were placed subcutaneously at the base of the ear and removed 10 days later using a scalpel blade. Implants were stored at room temperature.

Human chorionic gonadotrophin

Chorulon® (Intervet, Australia) was used. It consists of human chorionic gonadotrophin, and is available as a white, freeze-dried crystalline powder containing either 1500 iu or 5000 iu per vial. This preparation was refrigerated prior to use. It was reconstituted using the solvent provided, immediately before use.

2.6 Administration of intramuscular injections

Intramuscular injections were given in the longissimus lumborum muscles, 5 cm from the midline, and alternated from the right to the left side when consecutive injections were given.

2.7 Blood collection

Blood samples were collected primarily from the right jugular vein with animals lightly restrained manually in a standing position as described by Fowler (1998). All samples were collected from the jugular vein to avoid discrepancy in circulating hormone levels obtained from different body sites (Parr et al. 1993). The left jugular vein was used sparingly as a collection site so as to avoid the oesophagus, which lies close to the jugular vein on the left side of the neck. Alpacas do not have an obvious jugular furrow as the vein is covered by the sternomandibularis muscle but the vein lies just medial to the ventral projection of the transverse process of the cervical vertebrae. Neck fibre was clipped at the start of each experiment to allow easier palpation of landmarks. Samples were collected from high, middle and low sites on the neck using a 0.9 x 25 mm (20 G x 1") needle. When more frequent blood sampling was required in Chapter 5, 2 mL lignocaine was instilled subcutaneously and a 16 G intravenous catheter was placed in the right jugular vein as described by Davis et al. (1996).

Blood was collected into 10 mL lithium heparin evacuated tubes (Vacutainer®, Becton-Dickinson, USA) and stored immediately in crushed ice. All samples were centrifuged at 3000 rpm for 15 minutes when all animals had been bled. Plasmas were removed and stored at -20° C until hormones were assayed. Blood was collected in an anticoagulant so that rapid centrifugation minimised metabolism of any hormones. Unlike progesterone concentrations in bovine whole blood, it is possible to measure plasma progesterone in llama blood samples stored at room temperature for up to 48 hours before centrifugation without appreciable decline in progesterone levels (Moir et al. 1990, Aba et al. 1996). However effect of prolonged storage of other hormones has not been determined in camelids.

2.8 Hormone assays

17β-Oestradiol

Plasma oestradiol concentrations were determined using the Estradiol Maia Kit® (Biochem Immunosystems, Italy). This is a radioimmunoassay (RIA) kit that uses ^{125}I-labelled oestradiol and is based on competitive binding principles. Oestradiol contained in plasma samples, standards and controls competes with ^{125}I-labelled oestradiol (labelled antigen tracer) for a limited number of oestradiol antibody binding sites. The amount of oestradiol in each sample, standard or control is inversely proportional to the amount of ^{125}I-labelled oestradiol tracer.

Oestradiol was extracted from duplicate plasma samples prior to assay to improve the sensitivity of the assay. 2 mL diethyl ether was added to each 200 µL plasma sample, vortexed and then the aqueous portion in the bottom of each tube was frozen in an ethanol/dry ice bath. Each ether supernatant, containing the oestradiol, was decanted into labelled assay tubes then dried in a rotary evaporator for 1 hour. 200 µL phosphate-buffered saline-gel (PBS-gel) was then added to each assay tube and left to stand overnight at 4˚ C.

50 µL oestradiol antibody (rabbit antiserum) was added to each assay tube, vortexed and incubated for 1 hour at room temperature. 50 µL tracer was added to each assay tube, vortexed and left for 3 ¼ hours. 300 µL second antibody (goat anti-rabbit IgG covalently bound to magnetic particles) was added to each assay tube, vortexed then placed in a magnetic rack for 20 minutes. Separation of the oestradiol or tracer bound to the double antibody occurred by sedimentation in a magnetic field. Unbound tracer in solution was decanted from each tube, leaving oestradiol or tracer bound to double antibodies in the bottom of each assay tube. The radioactivity in each tube was measured in a gamma scintillation counter for 2 minutes. The oestradiol concentrations and quality control data were determined by the methods and computer program of Burger et al. (1972) using a log-logit transformation.

Cross-reactivity of other naturally-occurring steroids with oestradiol antibody was 2.5 % for 17β-oestradiol-3-benzoate, 1.8 % for oestrone and 0.5 % for oestriol.

The standards of oestradiol were prepared from serial dilutions of stock solution using alpaca wether plasma with undetectable concentrations of oestradiol. The standards of oestradiol used in this study were 0, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 pg/mL. The buffer used was 0.01 M PBS-gel, with a pH of 7. Quality controls were prepared from alpaca wethers treated with oestradiol, then their plasma was diluted to fall in the range of 2.5 (low), 5 (medium) and 10 (high) pg/mL oestradiol. These three different pools of oestradiol were included in each assay to determine the intra- and inter-assay coefficients of variation. Where plasma samples fell outside the range of the standard curve, they were serially diluted in buffer until they fell within the range.

Serially diluted alpaca plasma samples were measured to validate the oestradiol assay for use in alpacas. Results produced a displacement curve that was parallel to the oestradiol standard. The percent recovery for 0, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 pg/mL of oestradiol added to plasma of castrated male alpacas was 82, 88, 89, 92, 89, 99, 99, 103, 101 and 117 % respectively. The sensitivity of the assay was 0.24 pg/tube. The intra-assay coefficients of variation, based on duplicate samples for low, medium and high quality controls, were 8.5 %, 1.3 % and 9.4 %, respectively.

Intra- and inter-assay coefficients of variation and sensitivity for each assay are presented in the relevant chapters. This RIA was performed at the University of Melbourne, Victoria, Australia in the laboratory of Dr PJ Wright by Mrs Fiona Armour.

Progesterone

Plasma progesterone concentrations were determined using the Coat-A-Count Progesterone® kit (Diagnostic Products Corporation, Los Angeles, USA), previously validated for use in alpacas (Sumar et al. 1988) and llamas (Leon et al. 1990). This is an RIA kit that uses a solid phase ^{125}I-labelled progesterone and is based on competitive binding principles.

Duplicate 100 µL aliquots of unextracted plasma samples or standards were pipetted into polypropylene tubes coated with rabbit antibodies to progesterone. Then 1 mL ^{125}I-labelled progesterone tracer was added and each tube mixed using a vortex. Tubes were incubated at room temperature for 3 hours then decanted thoroughly and counted for 1 minute in a gamma scintillation counter.

Cross-reactivity of other naturally-occurring steroids with progesterone antibody was 9.0 % for 5α-pregnan-3,20-dione, 3.4 % for 17α-hydroxyprogesterone and 3.2 % for 5β-pregnan-3,20-dione.

Alpaca quality control internal standards run in the progesterone assay were made by diluting an alpaca plasma sample of high progesterone, with wether plasma. The standards of progesterone provided in the assay kit were not used in this study. Standards were made up in alpaca wether plasma, at dilutions of 0, 0.1, 0.5, 2, 10, 20, 40 and 80 ng/mL. The progesterone concentrations of the plasma samples, intra- and inter-assay coefficients of variation and sensitivity of each assay were determined by the methods and computer program of Burger et al. (1972) using a log-logit transformation and are presented in the relevant chapters. This RIA was performed at the University of Melbourne, Victoria, Australia in the laboratory of Dr PJ Wright, by Mrs Fiona Armour.

2.9 Statistical analyses

Descriptive statistics were obtained using Minitab® for Windows Release 12.1, Microsoft Excel 97 SR-1, SAS/STAT Software Release V 8.0 1999 and Stata® Statistical Software Release 6.0 1999.

Intraclass correlation was used to determine the independence or non-independence of observations made within individual animals in Chapters 3 and 4. The intraclass correlation is the proportion of the total variance of an observation that is associated with the class to which it belongs (Snedecor et al. 1980). In Chapter 3 this involved first and second matings performed by the same male, and in Chapter 4 it involved multiple follicular waves in individual females. The variance inflation factor, or 'design effect' is defined as $VIF = 1 + (m-1)\rho$ where 'm' is a function of the average cluster size (number of observations) made per animal and 'ρ' is the intraclass correlation coefficient (Donner et al. 2000). When $\rho = 0$, members of a cluster are statistically independent. When $\rho = 1$, members of a cluster are totally dependent.

Repeated measures analysis of variance (ANOVA) in Chapters 5, 6, 7 and 8 used the SAS/STAT procedure MIXED with REML estimation and of type autoregressive-1 model (SAS Institute Inc. 1997) unless stated otherwise in a particular chapter. This model assumes a decreasing correlation between time points, the further the time points are from each other. A summary table was generated to examine the overall effects of the main effects (e.g. Treatment, Day) and their interactions (e.g. Treatment*Day). Multiple comparisons were conducted when there was significance in the summary table. Protected least significant difference (LSD) allowed means to be compared when there was a significant effect in the summary table.

Statistical significance was set at $P < 0.05$ and results are presented as means ± standard error of the mean (SEM).

2.10 Definitions of follicular terminology

It was not possible to identify individual follicles on a sequential basis during ultrasonography of ovaries. Alpaca ovaries are mobile and may flip over during ultrasonographic examination. In addition, there is no visible non-follicular reference area such as the ovarian hilus in camelids as there is in cattle (Pierson et al. 1988), or a corpus luteum, as animals are generally anovulatory. The growth and regression profiles of the largest and second largest follicles were made using a non-tracking method in a similar fashion to that described by Adams et al. (1990) and Ginther (1998). There was no problem profiling successive dominant follicles emerging from opposite ovaries. When successive dominant follicles emerged from the same ovary, data on the few days at which crossover occurred were obscured, but profiles on either side, including day of emergence, were not obscured.

Definition of the following terms has been adapted from Ginther et al. (1989) and Adams et al. (1990). 'Follicular waves' involve periodic synchronous emergence of a cohort of follicles, one of which becomes dominant in a few days, while the subordinate follicles in the cohort regress. A 'dominant follicle' has been defined as one that grows to at least 5 mm diameter and exceeds the diameter of all other cohort follicles in that wave. Selection of a dominant follicle of a wave is the point at which it can be distinguished from others in its cohort. Selection is exhibited by 'deviation' in growth between dominant and subordinate follicles and may be retrospectively judged to have begun at the ultrasound examination preceding the first examination with an apparent change in diameter difference between the two largest follicles (Ginther 2000).

The ‘growing phase’ of a follicular wave has been defined as the period between emergence and the day at which the dominant follicle appeared to stop its progressive increase in diameter. The ‘static phase’ was the period from the last day of the growing phase to the first day the dominant follicle appeared to begin a progressive decrease in diameter. The ‘regressing phase’ extended from the last day of the static phase to the day the previously dominant follicle was no longer detectable.

‘Interwave interval’ has been defined as the number of days between emergence of two successive follicles that were destined to become the largest for consecutive waves (Evans et al. 1994). Emergence of follicular waves has been defined as the ‘earliest ultrasonic detection of follicles that are compatible with retrospective tracking’ (Ginther 2000). The ‘day of emergence’ or day of first detection of successive dominant follicles in alpacas was determined retrospectively to the day when the particular follicle had progressed from being less than 3 mm to a diameter of 3-5 mm.

Spontaneous ovulation was observed by ultrasonography when the sudden disappearance of a dominant follicle was associated with appearance of a corpus luteum on the same ovary 2 to 4 days later. Results were analysed only from data obtained from follicles where there was no corpus luteum visible on either ovary. Adams et al. (1990) showed that follicular waves were shorter and the maximum follicle diameter smaller in the presence of a corpus luteum. Information relating to follicular wave characteristics, such as site and pattern of follicular emergence, was sourced from whole waves or part waves where there was no ambiguity.

3. Effects of sexual receptivity, mating behaviour and ovarian follicular state on pregnancy in female alpacas

The objectives in this chapter were to determine relationships between sexual receptivity, mating behaviour, ovarian follicular state and pregnancy in female alpacas. Observations were included for breeding duration and pregnancy rate achieved by male alpacas. Seventy pen matings were observed at a commercial alpaca stud in south-western Victoria, Australia. The parameters observed were time taken to assume sternal recumbency, mating duration, and evidence of non-receptive behaviour such as spitting, kicking and vocalisation. Ovarian follicular state was determined by ultrasonography and was complemented by measuring plasma concentrations of oestradiol and progesterone. Pregnancies were confirmed by transabdominal ultrasound between 45 and 80 days after mating. There were no significant differences between females that became pregnant and those that failed to become pregnant in the time taken to adopt the copulation position of sternal recumbency, mating duration, or maximum follicle diameter. However, there was a significant quadratic relationship between plasma oestradiol concentration (log_{10}) and follicle diameter, and the probability of pregnancy increased as the plasma concentration of oestradiol (log_{10}) at the time of mating increased. Females were sexually receptive most of the time in the absence of a corpus luteum, and regardless of size of the largest follicle or plasma concentration of oestradiol. Follicles with a diameter less than 7 mm were able to ovulate in response to mating which was smaller than previously reported. The pregnancy rate for first or second matings of the day by males did not differ even though the first mating was longer in duration than the second. Behaviour and ovarian state were not correlated with mating success. Matings to optimise pregnancy rates in alpacas need to occur in the presence of an oestrogenic follicle that is capable of ovulation in response to mating. Simple detection of alpacas with follicles in this state is not possible and treatments that control ovarian follicular growth should therefore be investigated.

4. Ovarian follicular wave characteristics of unmated alpacas

The objectives in this chapter were to describe ovarian follicular growth characteristics and establish the interval between successive follicular waves in unmated alpacas as a first step in the development of protocols to control the emergence of a new ovarian follicular wave. The ovaries of 16 non-pregnant, non-lactating mature female alpacas were observed by ultrasound every second day for at least 46 days and up to 100 days. Individual follicular profiles are described for 8 animals. Follicular growth patterns of waves with differing interwave intervals, and plasma oestradiol concentrations during follicular growth, were examined. From these data, an inverse relationship between the diameter of the largest follicle and the number of follicles detected supported the hypothesis that follicular growth in camelids occurs in waves ($P < 0.001$). The interwave interval in the total group of non-ovulatory follicular waves examined ($n = 38$) was 15.4 ± 0.5 days. The interwave interval had a distribution of 15/38 (39 %) intervals of 12 days and 12/38 (32 %) intervals of 16 days. The maximum follicular diameter of each follicular wave averaged 8.8 ± 0.3 mm ($n = 38$). Interwave intervals of longer duration were associated with a larger maximum follicle diameter ($P < 0.001$). Interwave interval was poorly described by differences in live weight among animals ($P = 0.39$). The diameter of the largest follicle averaged 7.8 ± 0.1 mm throughout the observation period, achieved by substantial overlapping of follicular waves. This study showed that the growth rate of dominant follicles was consistent over the first 10 days after emergence, allowing the dominant follicle to reach a diameter capable of ovulation by this time regardless of subsequent interwave interval. This would suggest that it should be possible to synchronise new wave emergence and predict optimum mating time. The optimum time of mating was predicted to be 6 to 8 days after new wave emergence.

5. Plasma concentrations of 17β-oestradiol and ovarian follicular growth in alpacas treated with 17β-oestradiol

The objectives in the study reported in this chapter were (i) to characterise plasma concentrations of injected 17β-oestradiol (oestradiol) in female alpacas, and (ii) to determine the effects on patterns of ovarian follicular development of injected oestradiol administered alone, or in combination with progesterone. Five non-pregnant, non-lactating mature females were injected with 1 mg oestradiol i.m. and blood was sampled at regular intervals. The plasma concentration of oestradiol at 0 h was 8.0 ± 0.8 pg/mL. The concentration had peaked at 193.6 ± 17.7 pg/mL by 1.5 h after injection after which it declined steadily to 5.5 ± 0.4 pg/mL 24 h after treatment. In the second experiment, 8 non-pregnant, non-lactating females received 1 mg oestradiol, while 8 control females received 1 mL of sesame oil intramuscularly on Day 0. Animals in both groups were bled and ovaries observed by ultrasonography every second day from Days 0 to 16. All females received 750 iu hCG on Day 10 to induce ovulation. There were no differences on any day in diameter of the largest follicle ($P > 0.05$) or plasma oestradiol concentrations ($P > 0.05$) between treated and control groups from Day 0 to Day 10 inclusive. There was also no difference between groups in the ability to ovulate on Day 10 (control 8/8, treated 7/8; $P > 0.05$). In the third experiment, 100 mg progesterone was combined with 1 mg oestradiol and administered i.m. to 16 non-pregnant females and 1 mL of sesame oil was administered to 18 control females on Day 0. Animals were bled and ovaries observed by ultrasonography on Days 0, 5 and 10. They all received 750 iu hCG i.m. on Day 10 and were bled again on Day 17. There were no differences between treatment and control groups in diameter of the largest follicle ($P > 0.05$) or plasma oestradiol concentration ($P > 0.05$) 5 and 10 days after hormone administration. A greater ($P = 0.045$) proportion of control females (17/17) ovulated on Day 10 than did treated females (12/16). It was concluded from the above findings, that a single injection of 1 mg oestradiol (with or without progesterone) did not induce regression of the dominant follicle in alpacas.

6. Plasma concentrations of oestradiol and ovarian follicular growth in alpacas treated with oestradiol benzoate

The objectives in this chapter were to (i) characterise plasma concentrations of oestradiol following injection of oestradiol benzoate and (ii) observe the effects of oestradiol benzoate on follicular wave turnover in nonovulatory alpacas. In the first experiment, 8 non-pregnant mature female alpacas were injected intramuscularly at 0 h with 0.2 (n = 4) or 2 (n = 4) mg oestradiol benzoate and blood was sampled at regular intervals for the next 120 h. Females treated with 0.2 mg oestradiol benzoate had an oestradiol plasma concentration at 0 h of 3.85 ± 0.23 pg/mL. Plasma oestradiol had peaked at 14.75 ± 1.91 pg/mL by 24 h and declined to basal levels again by 60 h (6.02 ± 1.01 pg/mL). Females treated with 2 mg oestradiol benzoate had a plasma oestradiol concentration at 0 h of 3.90 ± 0.98 pg/mL. Plasma oestradiol peaked at 56.12 ± 14.19 pg/mL by 24 h then declined to levels of 9.72 ± 3.08 pg/mL by 120 h. In the second experiment, 6 non-pregnant, non-lactating females received 2 mg oestradiol benzoate, 5 females received 5 mg oestradiol benzoate while 6 control females received no treatment on Day 0. Animals in all groups were bled on Days 2 and 4 and ovaries were observed by ultrasonography every second day from Day 0 to Day 18. There were no differences in diameter of the largest follicle among control and treated groups on Days 0 to 18 inclusive. There were relative changes in follicle diameter between Day 0 and subsequent days within each treatment group. In the third experiment, 12 non-pregnant, non-lactating females received 5 mg oestradiol benzoate and 5 females received no treatment on Day 0 in an attempt to repeat the findings of Experiment 2. There was no difference ($P > 0.05$) in diameter of the largest follicle between control and treated groups from Day 0 to Day 16 inclusive but there were relative changes in follicle diameter between Day 0 and subsequent days within each treatment group. It was concluded from the observations in these experiments that 2 or 5 mg of oestradiol benzoate could not be used to manipulate follicular growth to allow emergence of a new follicular wave at a known time.

7. Plasma concentrations of progesterone and ovarian follicular growth in alpacas treated with progesterone

The objectives of this study were to (i) characterise plasma progesterone concentrations following twice daily injections of progesterone and (ii) observe the effects of progesterone, in the absence and presence of oestradiol benzoate on follicular wave turnover in nonovulatory alpacas. In the first experiment, 9 non-pregnant mature females were injected intramuscularly with 10 mg (n = 5) or 100 mg (n = 4) progesterone at 12 hourly intervals over a period of 96 hours. Blood was sampled at regular intervals for 228 h (9.5 days). Plasma progesterone levels at 0 h were below assay sensitivity in all females in both groups. Plasma progesterone concentrations took several days to plateau in response to treatment and by 96 hours, females that received 10 mg or 100 mg had plasma progesterone concentration of 3.3 ± 0.5 ng/mL and 35.0 ± 3.5 ng/mL, respectively. Females that received 10 mg progesterone had less than 1 ng/mL plasma progesterone 48 hours after ceasing progesterone treatment, while females that received 100 mg had plasma progesterone of 2.2 ± 0.3 ng/mL ($P < 0.001$), 132 hours (5.5 days) after ceasing progesterone treatment. In the second experiment, 8 non-pregnant, non-lactating females received 25 mg progesterone intramuscularly twice daily for 21 days (Days 46 to 66), while 8 control females received no treatment. Animals in both groups were bled and ovaries observed by ultrasound every second day from Day 0 to Day 100. Three of 8 treated females ceased new follicular wave emergence until progesterone treatment ended on Day 66. The other 5 females exhibited follicular waves of shorter duration (9.0 ± 1.7 days, n = 6 waves, $P < 0.001$) and smaller maximum follicular diameter (5.0 ± 0.6 mm, n = 6 waves, $P < 0.001$) during progesterone treatment compared with control females (15.4 ± 0.5 days, 8.8 ± 0.3 mm, n = 38 waves). All females exhibited at least a 7 mm follicle by Day 76 (10 days after progesterone ceased. In the third experiment, 19 non-pregnant, non-lactating females received 2 mg oestradiol benzoate intramuscularly on Day 0 (n = 7), 2 mg oestradiol benzoate intramuscularly on Day 0 and 25 mg progesterone i.m. twice daily on Days 0-9 (n = 6) or 25 mg progesterone i.m. twice daily on Days 0-9 (n = 6). Ultrasonography of ovarian structures was performed on alternate days in all animals for 6 days prior to the start of hormone treatments and for a further 22 days. Diameter of the largest follicle in females that received oestradiol benzoate and progesterone or progesterone was smaller than females treated with oestradiol benzoate on Day 4 ($P = 0.048$) and Day 2 ($P = 0.018$), respectively. There were no differences on Day 16 ($P = 0.055$) and Day 20 ($P = 0.065$), respectively. It was concluded that exogenous progesterone induced regression of the existing dominant follicle and suppressed subsequent follicular development in alpacas. The inclusion of oestradiol benzoate with progesterone resulted in similar follicular responses as progesterone alone.

8. Effects of different routes, doses and frequencies of progestagens on follicular activity in alpacas

The objective in this chapter was to develop a practical method of progestagen delivery that would induce follicular regression and allow synchronous emergence of a new follicular wave in alpacas. In the first experiment, 27 non-pregnant alpacas received 0 (n = 10), 1 (n = 9) or 2 (n = 8) 3 mg norgestomet implants each subcutaneously at the base of the ear(s) on Day 0. Implants were removed on Day 10. Ovaries were observed by ultrasonography on Days 0, 5, 10, 15 and 20. The diameter of the largest follicle was greater for control females than those treated with 2 implants on Day 10 ($P = 0.031$) and Day 15 ($P = 0.004$), but not on Days 0, 5 or 20 ($P > 0.05$). There was no difference between control females and females receiving 2 norgestomet implants in number of follicles less than 6 mm or follicles 6 mm or greater on Days 10 ($P = 0.56$) and 15 ($P = 0.27$). In the second experiment, 17 non-pregnant, non-lactating alpacas received no treatment (n = 5), 50 mg progesterone intramuscularly once daily on Days 0 to 9 (n = 6) or 100 mg progesterone intramuscularly on Days 0, 2, 4, 6 and 8 (n = 6). Ovaries were monitored by ultrasonography every second day from Day –8 to Day 20. The largest follicle in the two treatment groups was smaller on Day 4 ($P = 0.003$) and Day 0 ($P = 0.031$) compared with controls. Maximum follicular diameter in females treated with 100 mg progesterone every other day was not different to controls on Day 20 ($P = 0.13$) while diameter of the largest follicle in females treated with 50 mg progesterone daily remained smaller than control females on Day 20 ($P = 0.02$). In the third experiment, 18 non-pregnant, non-lactating alpacas received 100 mg progesterone intramuscularly on Days 0, 2, 4, 6 and 8 (n = 6), 200 mg progesterone intramuscularly on Days 0, 2, 4, 6 and 8 (n = 6), or 200 mg progesterone intramuscularly on Days 0, 2 and 4 (n = 6). Ovaries were monitored by ultrasonography every second day from Day –4 to Day 18. The diameter of the largest follicle in each treatment group was first observed to be smaller than the diameter on Day 0 by Day 4 ($P = 0.001$) in females receiving 100 mg progesterone every other day, by Day 6 ($P < 0.001$) in females receiving 200 mg progesterone for 5 treatments, or by Day 2 ($P = 0.018$) in females receiving progesterone for 3 treatments. Eighty-three percent of females receiving 200 mg progesterone for 3 treatments had a 6 mm follicle by 10 days, and 100 % by 12 days, following progesterone cessation. In the fourth experiment, 19 non-pregnant, non-lactating alpaca females received 200 mg progesterone intramuscularly on Days 0, 2 and 4. Follicular activity was monitored by ultrasound every second day from Day –2 to Day 16. All females exhibited follicular regression followed by emergence of a new follicular wave, and on Day 16, 16 females (84 %) possessed at least one follicle $\geq$ 6 mm diameter. The most practical protocol for ovarian follicular control was provided by injecting 200 mg progesterone intramuscularly on Days 0, 2 and 4.

9. Preliminary observations on the morphology of oocytes obtained from newly emerged dominant follicles after ovarian follicular wave synchronisation in alpacas

The objectives in this chapter were to undertake a preliminary investigation on oocyte morphology after ovarian follicular wave synchronisation in alpacas. Following treatment with 200 mg progesterone on Days 0, 2 and 4, 8 females selected at random were injected with 12.5 mg luteinising hormone (LH) i.m. on Day 16, 17-19 hours prior to ultrasound-guided transvaginal oocyte aspiration. Changes indicative of oocyte maturation following LH treatment included expansion of cumulus cells, meiotic progression (nuclear maturation) from Prophase I to Metaphase I, an increase in the perivitelline space and a tendency for increased numbers of cortical granules to be positioned along the oolemma. The findings indicated that oocytes contained in newly emerged dominant follicles after follicular wave synchronisation can respond to LH and show morphological changes consistent with final oocyte maturation before ovulation.

10. Use of a progesterone synchronisation protocol in fixed-time mating trials

Fixed-time mating trials were conducted on 3 commercial alpaca farms to compare pregnancy rates in female alpacas treated with the Day 0-4 progesterone synchronisation protocol and mated on Day 16 with pregnancy rates in placebo-treated females. Results indicate that the newly emerged follicle from synchronised females is capable of ovulation and fertilisation, but there was no difference in 60-day pregnancy rates between synchronised females (19/57, 33 %) and placebo-treated females (15/54, 28 %; P = 0.526).

11. General discussion

This report presents new data on ovarian follicular wave characteristics in non-pregnant female alpacas and introduces a protocol based on progesterone to control ovarian follicular growth, and the emergence of a new follicular wave.

Conclusions made during the studies conducted in this thesis may be summarised as follows:

- An inverse relationship between the diameter of the largest follicle and the number of follicles detected supports the hypothesis that follicular growth in camelids occurs in waves.
- Wide variation in the interval between successive follicular waves, within and between alpacas, made the calculation of a mean interwave interval inappropriate.
- The growth rate of the follicle destined to become the dominant follicle was consistent for the first 10 days after new wave emergence, and was not influenced by the interwave interval.
- Matings to optimise pregnancy rates in alpacas need to occur in the presence of an oestrogenic follicle that is capable of ovulating in response to mating; unfortunately, simple detection of alpacas with follicles in this state is not currently possible.
- A single i.m. injection of oestradiol or oestradiol benzoate, of varied doses and with and without simultaneous injection of progesterone, did not induce follicular atresia that was followed by the emergence of a new follicular wave at a predictable time.
- The most practical and effective protocol for ovarian follicular control in female alpacas was provided by injecting 200 mg progesterone i.m. on Days 0, 2 and 4 (The Day 0-4 progesterone protocol). The majority of females treated with this protocol had a newly-emerged follicle with a diameter capable of ovulation on Day 16, 12 days after progesterone treatment ceased.
- Following treatment with the Day 0-4 progesterone protocol, females that received LH 17-19 hours prior to oocyte aspiration had an oocyte morphology indicative of normal maturation in preparation for ovulation.
- Females treated with the Day 0-4 progesterone synchronisation protocol and subsequently mated on Day 16 were capable of ovulation, fertilisation and pregnancy.

The apparent lack of response to oestradiol treatment by the dominant follicle in alpacas was unexpected given that oestradiol induces regression of follicles in cattle and sheep. It was concluded that alpacas, and perhaps camelids in general, have different intra- and/or extra-ovarian mechanisms that control follicular growth and regression compared with ruminants. The differences between camelids and ruminants may be related, in part, to the fact that camelids are induced ovulators and ruminants spontaneous ovulators.

The mechanism(s) by which progesterone induced follicular atresia and suppressed the emergence of a new follicular wave in alpacas (Chapters 7, 8 and 9) were not elucidated in this research nor have they been explored in detail in other camelids. One mechanism could be that progesterone exerts feedback on the hypothalamus to suppress GnRH release in a dose-dependent manner, resulting in suppression of FSH and LH secretion. It may be that at physiological doses of progesterone, LH pulsatility is suppressed with no effect on FSH secretion, but at pharmacological doses of progesterone, both LH and FSH are suppressed. Continuous exogenous oestradiol and/or progestogen treatment in rabbits showed that both these hormones could act alone and possibly synergistically to exert negative feedback on mean levels, pulse amplitudes and pulse frequencies of hypothalamic production of GnRH (Pau et al. 1986). Progesterone depressed FSH secretion in pony mares and was influenced by dose of progesterone (Gastal et al. 1999).

An alternative mechanism to explain why progesterone induced follicular atresia and suppressed the emergence of a new follicular wave in alpacas is that progesterone may reduce LH pulsatility alone in alpacas, thereby inducing regression of the existing dominant follicle in a similar manner to cattle, a species in which progesterone reduces LH pulsatility and inhibits the dominant follicle in a dose-dependent manner, but has no effect on FSH secretion (Adams et al. 1992a). FSH secretion would

not be affected by progesterone treatment and new wave emergence would still occur. However, if the FSH-dependent follicular diameter was $\leq$ 3 mm and LH-dependent follicular diameter $\geq$ 3mm, then FSH-dependent follicles would be unlikely to be detected by ultrasonography. Therefore, emergence of new waves would not be observed even though it may be still occurring.

Implants of the synthetic progestagen, norgestomet, did not adequately control ovarian follicular waves in alpacas (Chapter 8, Experiment 1). This was disappointing given their relative ease of administration and their use would be unlikely to induce ovulation during insertion or removal. However, these implants, along with intravaginal progestagen-containing devices such as CIDRs, PRIDs and sponges, were designed to prevent ovulation in spontaneously ovulating species such as sheep, cattle and goats, and not to completely inhibit follicular waves.

It was established in this research that alpacas have a follicle with a diameter capable of ovulation present on either ovary most of the time (Chapters 3 and 4) and that single random matings can achieve a pregnancy rate of 50 % (Chapter 3). Cancino et al. (1999) described 18 of 23 female llamas ovulating after use of 6 mg norgestomet implants, while Aba et al. (1999) used MPA sponges in llamas with 100 % ovulation rate and a 50 % conception rate. The findings by these two authors are not different to observations made in untreated females. Unless a method is developed to release more progesterone from each device to enable synchronisation of new wave emergence in combination with improved pregnancy rates, the side-effects of the devices in camelids (ovulation induction, vaginal discharge, expulsion of device) will preclude further use.

Future research on ovarian function in alpacas should focus on investigating the dynamics of gonadotrophin secretion during follicular development as more reliable FSH and LH assays become available. Elucidating LH and FSH patterns of secretion in relation to oestradiol production and follicle growth and regression in camelids will allow a better understanding of the control of folliculogenesis. In conjunction with these studies, an investigation into the competence of oocytes in relation to age and diameter of the dominant follicle will assist in determining optimum mating times. Practical applications of this knowledge will allow continued development and increasing application of artificial breeding technologies.

The Day 0-4 progesterone synchronisation protocol did not improve conception rates in comparison with random matings in alpacas. However, the protocol should be further evaluated in a larger number of females to identify why some females refused to mate following progesterone treatment.

The increased understanding of follicular growth characteristics and the ability to control new wave emergence will assist with developing artificial breeding technology in alpacas such as optimum timing of gonadotrophin treatment during multiple ovulation and embryo transfer programs. This will benefit the Australian alpaca industry with more efficient utilisation of genetically superior males and faster dissemination of improved genotypes throughout the national herd.

A comprehensive literature review, full experimental results and bibliography appear in the Doctor of Philosophy thesis generated from these studies, entitled *Control of ovarian follicular growth in the alpaca, Lama pacos*.

12 Bibliography

Aba MA, Ghezzi M, Quiroga M, Solana H, Auza N. Concentrations of progesterone during storage of whole blood from llama (*Lama glama*): Effects of anticoagulants, storage time and temperature. *Acta Veterinaria Scandinavica* 1996; 37:123-125.

Aba MA, Quiroga MA, Auza N, Forsberg M, Kindahl H. Control of ovarian activity in llamas (*Lama glama*) with medroxyprogesterone acetate. *Reproduction of Domestic Animals* 1999; 34:471-476.

Adams GP, Griffin PG, Ginther OJ. In situ morphologic dynamics of ovaries, uterus and cervix in llamas. *Biology of Reproduction* 1989; 41:551-558.

Adams GP, Sumar J, Ginther OJ. Effects of lactational and reproductive status on ovarian follicular waves in llamas (*Lama glama*). *Journal of Reproduction and Fertility* 1990; 90:535-545.

Adams GP, Matteri RL, Ginther OJ. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *Journal of Reproduction and Fertility* 1992a; 96:627-640.

Bourke DA, Kyle CE, McEvoy TG, Young P, Adam CL. Superovulatory responses to eCG in llamas (*Lama glama*). *Theriogenology* 1995a; 44:255-268.

Bravo PW. Reproductive endocrinology of llamas and alpacas. In: Johnson LW, editor. *Veterinary Clinics of North America Food Animal Practice* WB Saunders, Philadelphia, USA 1994; 10:265-279.

Bravo PW, Stabenfeldt GH, Lasley BL, Fowler ME. The effect of ovarian follicular size on pituitary and ovarian responses to copulation in domesticated South American camelids. *Biology of Reproduction* 1991b; 45:553-559.

Burgel H, Erhardt G, Gauly M. Cryopreservation of llama (*Lama glama*) semen. *Libro de Resumenes II Congreso Mundial sobre Camelidos,* Cusco, Peru 1999; p82.

Burger HG, Lee VWK, Rennie GC. A generalised computer program for the treatment of data from competitive protein binding assays including radioimmunoassays. *Journal of Laboratory Clinical Medicine* 1972; 80:302-312.

Cancino AK, Aller JF, Rebuffi G, Alberio RH. El uso del norgestomet para la sincronizacion de la onda folicular en llamas en dos epocas del año (invierno y verano). *Libro de Resumenes II Congreso Mundial sobre Camelidos,* Cusco, Peru 1999; 93 abstr.

Chaves MG, Aba MA, Agüero A, Egey J, Berestin V, Rutter B. Ovarian follicular wave pattern and the effect of exogenous progesterone on follicular activity in non-mated llamas. *Animal Reproduction Science* 2002; 69:37-46.

Davis IA, McGaffin JR, Kuchinka GD. Intravenous catheterization of the external jugular vein in llamas. *Compendium* 1996; 18:330-335.

Donner A, Klar N. *Design and Analysis of Cluster Randomization Trials in Health Research*. Arnold, London, UK 2000; pp8-9.

Evans ACO, Adams GP, Rawlings NC. Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. *Journal of Reproduction and Fertility* 1994; 100:187-194.

Fowler ME. *Medicine and surgery of South American camelids*. 2nd edition. Iowa State University Press, Ames, USA 1998; pp1-11.

Gastal EL, Bergfelt DR, Nogueira GP, Gastal MO, Ginther OJ. Role of luteinizing hormone in follicle deviation based on manipulating progesterone concentrations in mares. *Biology of Reproduction* 1999; 61:1492-1498.

Ginther OJ. *Ultrasonic Imaging and Animal Reproduction: Cattle.* Book 3. Equiservices Publishing, Cross Plains, Wisconsin, USA. 1998 pp 29-58.

Ginther OJ. Selection of the dominant follicle in cattle and horses. *Animal Reproduction Science* 2000; 60-61:61-79.

Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. *Journal of Reproduction and Fertility* 1989; 87:223-230.

Leon JB, Smith BB, Timm KI, LeCren G. Endocrine changes during pregnancy, parturition and the early post-partum period in the llama (*Lama glama*). *Journal of Reproduction and Fertility* 1990; 88:503-511.
Lichtenwalner AB, Woods GL, Weber JA. Ejaculatory pattern of llamas during copulation. *Theriogenology* 1996; 46:285-291.
McEvoy TG, Bourke DA, Adam CL. Recent advances in understanding and controlling the reproductive biology of South American camelids. In: *Proceedings of Assisted Reproductive Technology and Andrology Meeting*, Spain 1994; 5:277-298.
Moir CE, Young P, Adam CL. Plasma progesterone concentrations in llamas. *Veterinary Record* 1990; 126:561.
Murray AJ. A novel approach to synchronization of oestrus and artificial insemination of *Bos indicus* heifers in northern Australia. Master of Science Thesis, James Cook University 1997. p60.
Northcode KH. *Atlas of Australian Soils*. Division of Soils, Commonwealth Scientific and Industrial Research Organisation, Australia, 1962; sheets 1-2.
Parr RA, Davis IF, Miles MA, Squires TJ. Liver blood flow and metabolic clearance rate of progesterone in sheep. *Research in Veterinary Science* 1993; 55:311-316.
Pau KYF, Orstead KM, Hess DL, Spies HG. Feedback effects of ovarian steroids on the hypothalamic-hypophyseal axis in the rabbit. *Biology of Reproduction* 1986; 35:1009-1023.
Payne E, Smith JF, Cope BC, McGowan LT. Studies on the role of liver cytochrome P-450 and oestradiol metabolism in the effects of nutrition and phenobarbital on ovulation rate in the ewe. *Reproduction, Fertility and Development* 1991; 3:725-736.
Pierson RA, Ginther OJ. Ultrasonic imaging of the ovaries and uterus in cattle. *Theriogenology* 1988; 29:21-37.
Ratto MH, Gatica R, Correa JE. Timing of mating and ovarian response in llamas (*Lama glama*) treated with pFSH. *Animal Reproduction Science* 1997; 48:325-330.
San-Martin M, Copaira M, Zuniga J, Rodreguez R, Bustinza G, Acosta L. Aspects of reproduction in the alpaca. *Journal of Reproduction and Fertility* 1968; 16:395-399.
SAS Institute Inc. SAS/STAT Software: Changes and enhancements through release 6.12. SAS Institute Inc, Cary, NC, 1997:571-701.
Skidmore JA. *Reproduction in the Dromedary Camel PhD Thesis*, University of Cambridge. 1994.
Skidmore JA, Billah M, Allen WR. The ovarian follicular wave pattern in the mated and non-mated dromedary camel (*Camelus dromedarius*). *Journal of Reproduction and Fertility Supplement* 1995; 49:545-548.
Snedecor GW, Cochran WG. *Statistical Methods*. 7th edition. Iowa State University Press, Ames, Iowa, USA 1980; pp243-244.
Sumar J. Studies on reproductive pathology in alpacas. Masters Thesis. Swedish University of Agricultural Sciences, Uppsala and Universidad Nacional Mayor de San Marcos 1983; 9-103.
Sumar J, Fredriksson G, Alarcon V, Kindahl H, Edqvist L. Levels of 15-keto-13,14-dihydro-$PGF_{2\alpha}$, progesterone and estradiol-17β after induced ovulations in llamas and alpacas. *Acta Veterinaria Scandinavica* 1988; 29:339-346.
Tibary A, Anouassi A. Ultrasonography of the genital tract in camels (*Camelus dromedarius* and *C bactrianus*). In: TK Gahlot, editor. *Selected Topics on Camelids.* The Camelid Publishers, Bikaner, 2000, p431-465.
Tinson AH, McKinnon AO. Ultrasonography of the reproductive tract of the female dromedary camel. In: Allen WR, Higgins AJ, Mayhew IG, Snow D, Wade JF, editors. *Proceedings of the First International Camel Conference.* R&W Publications, Newmarket, UK 1992; 129-135.