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Improving the Response to Synchronisation Programmes of Dairy Cattle

A thesis presented in partial fulfilment of the requirement for the
degree of

Doctor of Philosophy

in

Veterinary Science

at

**Massey University, Palmerston North
New Zealand.**

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2014

Science:

If you don't make mistakes,

you are doing it wrong.

If you don't correct those mistakes,

you are doing it really wrong.

If you can't accept that you're mistaken,

you are not doing it at all.

'Anonymous'

Dedicated to my loving parents

Abstract

A gonadotrophin, prostaglandin, gonadotrophin + progesterone (GPG+P4) programme with fixed-time artificial insemination (FTAI) is the current recommended synchronisation programme for both heifers and anoestrous cows on New Zealand dairy farms. However, it is an expensive programme and a better understanding of the role of all of its components would be very useful in developing alternative cheaper programmes.

The two components of the programme that are the least understood, in terms of their underlying physiological actions and how they influence the outcome of synchronisation, are the Day 0 gonadotrophin-releasing hormone (GnRH) injection and the progesterone device. Additionally it is well known that energy status has a significant impact on fertility but there is little evidence, particularly under New Zealand conditions, of how energy status affects the response to GPG-based treatments in anoestrous postpartum dairy cows.

The effects of a GPG (Day 0: 100 µg GnRH, Day 7: 500 µg PGF_{2α}, Day 9: 100 µg GnRH) programme upon follicular and luteal dynamics, ovulation synchronisation and patterns of oestradiol and progesterone secretion in postpartum anoestrous dairy cows and nulliparous dairy heifers were compared with (i) a GPG programme plus a progesterone insert from Days 0–7 (GPG+P4) and (ii) a GPG+P4 programme from which the first GnRH treatment had been omitted (P+G+P4). Interactions of each treatment with energy balance, as determined by NEFA, IGF-I and insulin concentrations, were also studied in postpartum anoestrus cattle. Finally the conception rate (CR) to fixed time AI of a GPG+P4 programme in which AI was done concurrent with the Day 9 GnRH injection (Cosynch) was compared with a progesterone + prostaglandin programme (P4+PG; Day 0–7: progesterone releasing intravaginal device, morning of Day 6: 500 µg PGF_{2α}, afternoon of Day 9: FTAI) in heifers.

The physiological effects of the GPG and the GPG+P4 programmes were similar in anoestrous dairy cows. The inclusion of the Day 0 GnRH still appeared feasible in a GPG programme for treating anoestrous cows as it led to a higher probability of a corpus luteum (CL) on Day 7. In addition, treatment response was significantly affected by the postpartum duration and negative energy balance as evidenced by the significantly higher NEFA concentrations on Days 0, 7 and 9, and a lower insulin concentration on Day 0, in cows that failed to ovulate in response to the synchronisation protocol compared with cows that did ovulate. A clear and significant relationship between NEFA concentrations and ovulation in response to all synchronisation protocols showed that, regardless of the regimen that was used to treat anoestrus, the response was moderated and limited by the degree of negative energy balance.

In heifers, the removal of the progesterone-releasing device from a GPG+P4 programme had no effect on follicular dynamics or on the proportion of heifers which ovulated after either the GnRH injection on Day 0 or Day 9. Additionally, unlike the anoestrus cows, omitting the GnRH injection on Day 0 did not result in significantly delayed ovulation at the end of the programme, inasmuch as treatment with P+G+P4 was associated with earlier ovulation than GPG. Furthermore, synchronising heifers with a significantly less expensive programme (P4+PG) resulted in similar CR to synchronising with GPG+P4 (54.8% *versus* 52.4%, respectively) further confirming that Day 0 GnRH was not essential in heifer synchrony.

In conclusion, the higher conception rate in cows treated with a GPG+P4 programme rather than a GPG programme reported previously does not seem to be modulated by the actions on follicular dynamics and improved synchronised ovulation in dairy cattle with postpartum anoestrous (or in nulliparous heifers); however, the treatment response in anoestrous cows can be significantly affected by negative energy balance. In contrast, in dairy heifers, no benefit of Day 0 GnRH or the progesterone device in a GPG+P4 programme suggests the possibility of more cost effective options (e.g. P4+PG) which can lead to a CR as high as those synchronised using a GPG+P4 programme.

Acknowledgements

This research would never have been possible without the guidance and the support of several individuals who in one way or another contributed and extended their valuable support in the accomplishment of this thesis.

First, I would like to express my deepest gratitude to my chief supervisor A/Prof. Richard Laven, for his guidance, support, constructive criticism and feedback. I thank him for consistently hammering and churning my brain to extract the best out of me. It was a great privilege to be co-supervised by Prof. Tim Parkinson. His scientific knowledge and experience in the field of farm animal reproduction and excellent problem solving skills have always been an inspiration during the course of my study. This thesis would have never been of this quality without his exceptional scientific and logical comments. I am also grateful to my co-supervisor Prof. John Cockrem for his sustained encouragement and constant advice on reproductive endocrinology and the hormone assays of my samples. I thank him for his thoughtfulness, encouragement and support during my time as a PhD student.

It is a great pleasure to thank Education New Zealand and Massey University for providing the scholarship to undertake my PhD research in New Zealand and making my dream come true to study in such a world class institution. I also gratefully acknowledge Bomac Laboratories Ltd, New Zealand, for providing their products for this research.

My sincere thanks go to Prof. Nicolas Lopez-Villalobos and A/Prof. Geoff Jones for their guidance and support in analysing the data. Many thanks and much appreciation goes to Prof. Kevin Stafford, Director of Postgraduate Studies, for his advice and support during my study. Thanks also to Debbie Hill for all her administrative support during my study.

I owe my deepest gratitude to all those people who were directly or indirectly involved with my research, especially Jane Candy for the hormone assays of the samples, and Hamish Duhan and several BVSc students of Massey University for their help during animal handling, blood collection and ultrasonography. I also acknowledge Dr. Kevin Lawrence for his help during hormone treatment and blood collection on many occasions. Many thanks go to Dr. Kiro Petroski for sharing his research experiences and offering his help at several stages of my research.

I would like to thank Helen Benard for the participation of her dairy cattle in the oestrus synchronisation programme. Many thanks to all the farm managers of Massey owned farms

(Hourongo, Tuapaka, Keebles and Massey No 4 dairy farm). I gratefully acknowledge their help and coordination in making my research hassle-free.

I sincerely thank my several friends and fellow graduate students for their support, kindness and sharing unforgettable times during my research especially: Rashmi, Neha, Gulab, Ashok, Chandradeep, Prateek, Saroj, Kandarp, Doris, Sharini, and Jose. Many thanks to several staff members of the Institute of Veterinary, Animal and Biomedical Sciences of Massey University, especially: Prof. Danny Donaghy, Prof. Peter Davie, Prof. Craig Johnson, Dr. Christine Thompson, Dr. Matthew Perrott, Dr. Sam Peterson, Dr. Preet Singh, Dr. Kavitha Kongana, Dr. Ngaio Beausoleil, Neil Ward and Anthony Jacob. Thanks to all undergraduate Dutch students especially Dorien Eppink, Vera Captein, Ellen Hulls, and Nicole Bos for their support during my experiments. I also thank Prof. U. K. Mishra (Vice Chancellor), Prof. R. P. Tiwari and Prof. O. P. Mishra of C.G. Kamdhenu University, India, for their inspiration and morale boost. A special thank goes to my family friends Sandi Baldwin and Dr. Dave Baldwin for sharing their precious time and for giving valuable help during my study period.

I gratefully thank my family in India for their consistent support and encouragement with special recognition to my mother, brothers and sisters, and my deceased father. Finally, I would like to thank my wonderful wife, Sunita, for her love, patience and many sacrifices – she always stood by me and cheered me up through all the good and bad times of my study – and my two little daughters Lavanya and Sharanya for their jovial smiles.

Last but not least, I thank the pervasive God, for listening to my prayers and for giving me the strength and courage to carry on during the periods of crisis, and for His blessings for the successful completion of this work.

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CHAPTER 1

Introduction

New Zealand has historically depended on the primary sector for its economic growth. Projections suggest that the pastoral and related food industries will remain at the core of the New Zealand economy through to at least 2020 (Dairy NZ, 2009; Brownlie *et al.*, 2011). Within the primary sector, dairy farming is the single most important economic activity. In the year to June 2008, dairy products accounted for 25.1% (NZ\$9.6 billion) of the nation's total merchandise exports (Statistics NZ, 2010). On the back of continued growth, the dairy exports are forecast to increase 8% to NZ\$14.0 billion in 2013/14, and an average 8% per annum thereafter to reach NZ\$17.7 billion in 2016/17 (Ministry of Primary Industries, 2013).

Because of this important role in the economy, domesticated ruminants have a very special place in New Zealand, hence outnumber the human population (Statistics NZ, 2014). The livestock production systems that have been developed in New Zealand differ from those in most other parts of the world in that grazed pasture provides the basic feeding resource for animal production. This places a number of constraints on animal productivity, the most significant of which is that the dependence on pasture means that the feed supply is seasonal. Productivity in a pasture-based system is dependent on stocking rate and per head performance. Stocking rate can best be maximised by synchronising the time of parturition with the onset of spring pasture growth. Earlier parturition will lead to possible feed deficits in late winter and the need to reduce stocking rate. Delayed parturition may allow additional stock to be carried, but can lead to pasture quality problems and reduced per head performance (McCall and Smith, 1998). Calving cows just prior to the onset of spring growth ensures maximum feed requirements for lactation coincide with the greatest feed supply. This is important to ensure maximum conversion of pasture into milk before restrictions of reduced feed quality occur in summer (Clark *et al.*, 2007). Therefore, efficient reproductive management of dairy cattle is fundamental to successful farming in New Zealand (Brownlie *et al.*, 2011; McDougall *et al.*, 2014).

To make the most efficient use of winter feed in this system, all cows need to be dried off before winter and to have become pregnant so that they calve over a period of 6-8 weeks prior to the onset of spring growth. This is by far the most common system in New Zealand, with more than 96% of dairy cows calving between July and October, and only 2% calving between March and May (usually as an autumn-calving group in a split-calving herd; Rhodes *et al.*, 2003; Dairy NZ, 2007). Cows which calve late in the spring produce less milk during lactation

and hence give a lower return on winter feed consumed which is due to reduced lactation length as all the cows are dried off at the same time. Thus, production of later calving cows declines in the summer at the same time and to the same levels as earlier calving cows (Macmillan, 1979).

Under such a system, reproductive management of replacement heifers can be demanding. Heifers in such herds normally graze separately from lactating cows and, unlike lactating cows that are brought in for milking at least twice a day, special efforts have to be made to access them for oestrus detection and artificial insemination (AI). This has limited the widespread use of AI in heifers in New Zealand. The use of AI in heifers would be facilitated by an oestrus synchronisation programme that allows a single fixed-time AI (FTAI) to achieve an acceptable pregnancy rate.

The need to improve reproductive performance is a high priority for New Zealand's dairy farmers. An industry target of achieving a six week in-calf rate of 78% by 2015 was established in the 'Strategic Framework for Dairy Farming's Future' (Dairy NZ, 2007), though this has now been extended to 2020 (T. Brownlie, personal communication). A mean calving interval of ~365 days is required to maintain a given mean calving date and a concentrated calving. Consequently, cows need to be pregnant within an average of 83 days after calving. Grass-based systems have relatively high oestrus detection rates (>80% within the first 3 weeks; Palmer *et al.*, 2010) and first service conception rates (average 48%; Brownlie *et al.*, 2013), partly because of the large numbers of non-pregnant cycling cows being present at the start of the breeding season (Lucy *et al.*, 2004). Nevertheless, an average of approximately 20% of cows show no oestrus behaviour between calving and the planned start of the seasonal breeding programme (Rhodes *et al.*, 2003). The length of the post-partum anoestrous period is a critical factor determining reproductive efficiency, since the needs of an efficient pastoral system reduce the number of reproductive opportunities for the cows (Rhodes *et al.*, 1998). The main impacts of prolonged anoestrus under New Zealand conditions is delayed pregnancy (and thus reduced lactation length) as well as failure to conceive (McDougall, 2010a). Consequently, induction of premature parturition has been used to overcome this problem and to prolong the lactation period. However, the dairy industry is now trying to minimize the use of this method, as it is increasingly unacceptable to consumers (Holmes *et al.*, 2002).

Oestrus synchronisation has two main roles in cattle. Firstly, it can be used to get more cows pregnant more quickly and, secondly, to obviate the need for heat detection in systems using AI. In New Zealand, the most common use of synchronisation programmes is in dairy cattle which have not been observed in oestrus by 10 days before the commencement of the breeding season (McDougall, 2010a). In these cows, oestrus synchronisation is used to stimulate ovarian activity

and ovulation. In contrast, the primary reason for synchronising oestrus in dairy heifers is to facilitate use of AI (Xu and Burton, 1999a) and, in such animals, synchronisation programmes which do not require oestrus observation confer significant advantages over methods that rely upon oestrus detection (Laven, 2008). Although it is relatively uncommon to use AI in heifers in New Zealand, there could be several benefits to its use in yearling animals; specifically, it would allow farmers to rear more AI-bred replacement heifers which, in turn would allow them to increase herd size more rapidly and/or increase the rate of genetic gain of the herd (Macmillan, 1998; Dairy NZ, 2007).

A number of systems of oestrous cycle control have been developed but, until the European Union ban (European Union, 2003), oestradiol benzoate (ODB)/progesterone programmes were the mainstay of synchronisation in New Zealand for both postpartum cows (Rhodes *et al.*, 2003; Lucy *et al.*, 2004; McDougall and Compton, 2005; Bo *et al.*, 2007) and nulliparous heifers (Pickering, 2002). The low cost of ODB relative to gonadotrophin releasing hormone (GnRH) underpinned this decision (Lucy *et al.*, 2004). Use of this treatment protocol found improved four-week in-calf rates and reduced the interval from start of breeding to conception compared to untreated controls (Lean *et al.*, 2003; McDougall and Compton, 2005). However, the ban on the use of oestradiol and its esters in food producing animals, which was implemented in New Zealand in 2007, meant that ODB-based synchronisation programmes were no longer allowed. Treatments were revised for heifers and anoestrous cows, with the most obvious change being the substitution of oestradiol with GnRH, while retaining progesterone as the basis of treatment (McDougall, 2010a). The present recommended programme for anoestrous dairy cows is a GnRH-prostaglandin-GnRH + progesterone (GPG+P4) programme that involves two treatments of GnRH 9 days apart starting Day 0, PGF_{2α} injection on Day 7, followed by FTAI, 16-20 h after the second GnRH injection, with a progesterone insert in place between Days 0 to 7 (Laven, 2008; McDougall, 2010a). However for nulliparous dairy heifers the same programme was recommended with a time modification; i.e. FTAI was given at the time of Day 9 GnRH injection (GPG+P4-Cosynch; McDougall *et al.* 2013; Pickering 2008).

The conception rate with a GPG+P4 programme is generally improved by 10 to 20% compared with the GPG alone in both beef (Lamb *et al.*, 2001) and dairy cows (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b). In one large scale field study in New Zealand, McDougall (2010a) reported a higher conception rate in the treatment groups (which included progesterone) than in the control or GPG groups (43%, 56%, 48%, 34% for the GPG, GPG+P4-FTAI, GPG+P4+ AI after heat detection and control groups, respectively; P = 0.48, 0.001, and 0.04 compared to the control group; n = 2200).

Nonetheless the exact mechanism underlying this increase in conception rate due to inclusion of a progesterone device in a standard GPG programme is unclear in both postpartum cows and maiden heifers – it may be due to the inhibition of premature oestrus (between the first GnRH and PGF_{2α} injection; Roy & Twagiramungu 1999; DeJarnette *et al.* 2001), better synchronisation of ovulation (reducing the period of time over which treated animals ovulate) after the first GnRH or a reduction in the proportion of short cycles after the induced oestrus. Better identification of how progesterone treatment improves the response to synchronisation could be valuable in identifying alternatives to progesterone, as the progesterone device accounts for almost half the cost of synchronisation programme. Thus, the identification of cheaper equally effective alternatives to progesterone could markedly reduce costs and, the economic benefits of synchronisation programmes. To identify alternatives, the mechanism by which progesterone improves the response to synchronisation needs to be clarified. However, there is only limited evidence of its mechanism of action, particularly whether it improves the synchronisation of ovulation (McDougall, 2010a; McDougall *et al.*, 2013).

In contrast, the benefits of using GnRH at the start of such programmes have not been evaluated in either anoestrous cattle or maiden heifers. The aim of GnRH treatment on Day 0 is to stimulate the ovulation of large dominant follicles. In cyclic dairy cattle, a lower response rate to the first GnRH may result in reduced pregnancy rates following FTAI due to asynchronous ovulation (Vasconcelos *et al.*, 1999). Moreover, a high proportion of treated anoestrous cattle may not have dominant follicles that are responsive to GnRH. If this is the case then removal of the first GnRH could save cost without affecting follicular dynamics (i.e. changes in dominant follicle size in relation to time) and conception rates. Similarly, the benefit of using the initial GnRH injection in heifers is also less certain as the pattern of follicular development of dairy heifers is different from that of lactating dairy cows (Sartori *et al.*, 2004). Moreover, Day 0 GnRH injection has been shown not to affect the recruitment of a new follicular wave and conception rate in a GPG+P4-Cosynch programme in dairy heifers (Colazo and Ambrose, 2011). Heifer follicles thus seem to be responsive to GnRH for a lower proportion of their time, resulting in a low follicle turnover success and failure to induce a new follicular wave (Haughian and Wiltbank, 2002). Therefore, the benefit of using GnRH at the start of such programmes needs to be evaluated in both the maiden heifers and postpartum anoestrous cows. These findings suggest the need for a more careful understanding of the design and effect of any oestrus synchronisation programmes in dairy animals.

Additionally, across all dairy farming systems, negative energy balance is a consistent finding in the early lactation dairy cow, because during the early postpartum period the energy requirements for milk production and maintenance exceed energy intake. The key impact of

negative energy balance in the New Zealand system is to delay the return to cyclicity and thereby increase the intervals from calving to first service, and from calving to conception (McDougall *et al.*, 1993). The adverse effect of negative energy balance on reproduction is exaggerated in New Zealand's seasonal calving system, because the planned start of mating, to ensure a 12 months calving pattern, coincides with peak lactation, when many cows are still in negative energy balance (Clark *et al.*, 2000; Kay *et al.*, 2009). Most anoestrous cows are not cycling due to a combination of being in negative energy balance (i.e. when their nutritional intake is insufficient to meet the metabolic demands of lactation) and having too short an interval between calving and the planned start of mating for oestrous cycles to have resumed (Parkinson, 2007). Cows in negative energy balance may preferentially divert nutrients away from reproduction, thereby limiting the number of ovarian follicles, growth, and maximum size of the dominant follicle, delaying the first ovulation and hindering the expression of oestrus (Rhodes *et al.*, 2003; Peter *et al.*, 2009).

When extreme, negative energy balance can result in impaired reproductive as well as productive performance (Clark *et al.*, 2000; Harris and Kolver, 2001). The mechanisms that regulate energy and nutrient distribution in the somatotropic system may also affect reproduction at different levels of the hypothalamo-pituitary-ovarian axis (Roche, 2006; Chagas *et al.*, 2007). Several studies have shown that postpartum high concentrations of non-esterified free-fatty acids (NEFAs), and low concentrations of insulin and insulin-like growth factor-I (IGF-I), negatively affect the ovarian activity of lactating dairy cows (Lucy *et al.*, 1991; Lucy *et al.*, 1992; Beam and Butler, 1997; Butler *et al.*, 2004; Leroy *et al.*, 2005; Vanholder *et al.*, 2006; Kawashima *et al.*, 2007; Ortega *et al.*, 2008). It is thus likely that the metabolic status of anoestrous cows will affect their response to treatment. These findings suggest that influence of energy balance on reproductive outcomes in post-partum lactating dairy cattle might coincide with the optimum response to any oestrus synchronisation programme. Due to this, the maximal response to any oestrus synchronisation programme in the early post-partum period might also depend upon the level of negative energy balance during this period and might be directly related to several indicators of the energy balance (NEFAs, IGF-I, insulin, body weight and milk production). The role of these factors in affecting and gaining the prime response to any oestrus synchronisation programmes has not been studied in greater depth.

The research described in this thesis was designed to assess the efficacy of using a GPG+P4-FTAI programme either without the use of the progesterone device or without the initial GnRH treatment in nulliparous dairy heifers and postpartum anoestrous dairy cows under New Zealand conditions. Because of great variation in response to treatments and the cost of the

programme, the usefulness of these protocols in heifers and dairy cows was investigated under the following headings.

- Can the use of current heifer and cow synchrony programmes in New Zealand be justified?
- How do changes in dominant follicle size in relation to time (follicular dynamics) and the response to synchronisation in heifers and post-partum cows relate to each other, and can this relationship be used to improve the synchronisation of ovulation?
- How does the response to synchronisation relate to overall pregnancy rates in heifers?
- What are the relationships between energy status (insulin, IGF-I and NEFA), concentrations of progesterone, oestradiol, and the response to synchronisation in postpartum dairy cows?
- Are there any other cheaper but equally effective options to that of a GPG+P4 programme?

The present study was undertaken to study these questions, as follows:

1. Optimise synchronisation protocols for FTAI in post-partum anoestrous dairy cows and heifers in New Zealand, by studying
 - i. The effect of removing the first GnRH treatment of a regular GPG+P4 programme on follicular dynamics and synchronisation of ovulation in post-partum anoestrous cows and heifers.
 - ii. The effect of removing the progesterone treatment of a regular GPG+P4 programme on follicular dynamics and synchronisation of ovulation in post-partum cows and heifers.
2. Improve understanding of the effect of energy balance (insulin, IGF-I and NEFA) on response to GPG+P4 protocols and its modifications (one without Day 0 GnRH injection and one without progesterone device) for FTAI in post-partum anoestrous New Zealand dairy cows, by studying
 - i. Corpus luteum and dominant follicle development by Day 7 in relation to energy balance.
 - ii. Ovarian follicular dynamics in synchronised cows in relation to concentrations of reproductive (progesterone, oestradiol,) and metabolic (IGF-I and insulin) hormones and metabolites (NEFAs).
3. Investigate a cost-effective field-applicable synchronisation protocol for dairy heifers and compare the conception rate to that of GPG+P4 programme.

CHAPTER 2

Literature Review

The basic principle of any oestrus synchronisation programme is to achieve predictable control of an animal's reproductive physiology. This requires a significant level of understanding of the underlying physiological processes and the factors affecting the actions of the reproductive hormones. The products used in synchronisation programmes for cattle are usually (or are derived from) the hormones that are responsible for regulating ovarian activity. Therefore, a review of the basic endocrinology and physiology of the female reproductive system is important for an effective understanding of the mechanisms involved in successful synchronisation.

The bovine oestrous cycle

Puberty

There are reports of puberty occurring any time between six (Waldmann *et al.*, 2001) and 24 (Ghanem *et al.*, 2006) months after birth. The key factor responsible for puberty in cattle appears to be liveweight rather than age, as although considerable variation exists in age at first oestrus, there is considerably less variation in live weight at first oestrus (McMillan *et al.*, 1998). Live weight and age at puberty were determined by McNaughton (2003) in three strains of Holstein-Friesian dairy cattle (i. overseas-origin high-breeding-worth genetics [OS], ii. New Zealand-origin high breeding-worth genetics [NZH] and iii. New Zealand-origin low-breeding-worth genetics [NZL]) during two different years. Overseas heifers reached puberty at higher body weight compared to the NZ origin heifers, and NZL animals attained puberty at lighter body weight than both NZH and OS heifers (Year 1: OS, 274 ± 4.4 kg; NZH, 253 ± 4.9 kg; NZL, 230 ± 4.9 kg; Year 2: OS, 271 ± 6.0 kg; NZH, 258 ± 5.9 kg; NZL, 237 ± 7.3 kg; $P<0.05$). Age at puberty was also significantly different between the strain during Year 1 (OS, 373 ± 6.0 days; NZH, 356 ± 6.9 days; NZL, 329 ± 6.7 days $P = 0.05-0.07$) but not during Year 2 (OS, 374 ± 6.5 days; NZH, 380 ± 6.5 days; NZL, 381 ± 8.1 days) which was caused by inadequate feeding due to drought in Year 2.

The strategy used to develop liveweight targets by Dairy New Zealand in the "InCalf" programme (Dairy NZ, 2007), was to base live weight targets on proportions of expected mature weights (Table 1.2). Martinez *et al.* (1999) proposed a similar but more advanced method for calculating heifer liveweight targets using the estimated breeding value for liveweight of the individual. Nevertheless, despite the importance of live weight in determining the timing of puberty, there is still significant individual variation; within a group of

contemporary heifers, such that the lightest individual heifer at puberty may be 70–90 kg lighter than the heaviest heifer (McMillan *et al.*, 1998).

Table 1.2 Target live weights of New Zealand dairy heifers for high reproductive performance based on 90% of a mature cow liveweight at 22 months (Dairy NZ, 2007).

	Liveweight for typical heifers (kg)				
Mature cow LWT	400	450	500	550	600
LWT breeding value	-103	-53	-3	+47	+97
3 months (fully weaned)	70	80	90	100	110
6 months (30% of mature liveweight)	120	135	150	165	180
9 months	160	180	200	220	240
12 months	200	225	250	275	300
15 months (60% of mature liveweight)	240	270	300	330	360
18 months	290	330	365	400	440
22 months (90% of mature liveweight)	360	405	450	495	540

The phases and control of the oestrous cycle

Cattle are polyoestrus in nature and typically show oestrus around once every 21 days (range 18–24 days: Wishart 1972; Sirois & Fortune 1988; Ginther *et al.* 1989a). Recent data also suggest that, at least in Holstein cows, the average inter-oestrus interval has increased; Ginther *et al.* (1996) reported that average oestrous cycle length was 24–28 days in the USA, while Royal *et al.* (2000) reported that the length of the interovulatory interval had increased by 2.1 days (mean 22.3 days) based on 20 years of data in two UK dairy herds. This increase in mean cycle length may be related to reduced fertility; Royal *et al.* (2000) also reported that cows with an oestrous cycle length of 19–24 days before insemination had a higher pregnancy rate (54.4%) than those with cycles of <19 or >24 days (29.4%). In comparisons of New Zealand and Overseas strains of Holstein-Friesian cows and heifers (Fahey *et al.*, 2003; McNaughton, 2003), New Zealand strains have shorter oestrous cycles (Cow: range, 20–25 days [Fahey *et al.*, 2003]; Heifer: range, 19–20 days [McNaughton, 2003]).

The hormonal regulation of the oestrous cycle is principally dependent on gonadotrophin releasing hormone (GnRH) from the hypothalamus, follicle stimulating hormone (FSH) from the anterior pituitary, steroids (e.g. progesterone and oestradiol) and peptides (e.g. inhibin) from the ovaries and prostaglandin F_{2α} (PGF_{2α}) from the uterus (Forde *et al.*, 2011). These hormones function through positive and negative feedback mechanisms (Roche, 1996). The oestrous cycle involves two main phases: follicular and luteal (see Noakes, 2009, for review). The follicular phase is characterised by follicular growth in the absence of a functional corpus luteum (CL), with oestradiol being the main circulating ovarian hormone. The luteal phase is characterised by the presence or the development of a functional CL, with progesterone being the main circulating ovarian hormone (Noakes, 2009).

Follicular phase

Pro-oestrus

This is the phase immediately preceding oestrus, during which there is follicular growth and regression of the CL of the previous cycle. Regression of the CL is a two-step process which involves both functional luteolysis (reduction in progesterone production) and structural luteolysis (by cell death and degeneration of luteal tissues) (Knickerbocker *et al.*, 1988; Juengel *et al.*, 1993; McCracken *et al.*, 1999). During this period, there is a decline in circulating progesterone concentrations due to the regression of the CL, which is in turn brought about by PGF_{2α} secretion from the uterus (Lamothe *et al.*, 1977; Hansel and Convey, 1983). If there is no conceptus in the uterus, the CL regresses after Day 18 of the cycle in response to PGF_{2α}, resulting in a pronounced decrease in progesterone concentration and, subsequently, the start of the follicular phase (Goff, 2004). If there is a viable conceptus, luteolysis is prevented by interferon-τ (IFNT) produced by the conceptus. This means that the CL continues to produce the progesterone which is required for fetal implantation, growth and development (Spencer *et al.*, 2004).

The follicular phase is characterised by the presence of a rapidly growing dominant follicle, which produces increasingly large amounts of oestradiol. The increased circulating oestradiol concentration is responsible for an increased GnRH secretion from the hypothalamus, an effect mediated by the positive feedback effect of the oestradiol on the hypothalamus (Reeves *et al.*, 1971; Kesner *et al.*, 1981). During the latter stages of its development, the growth of the follicle is increasingly reliant upon luteinising hormone (LH) rather than the follicle stimulating hormone (FSH) which regulated the earlier stages of growth; hormonal changes that occur in parallel with declining FSH concentrations are due to the negative feedback effects of follicular oestrogens and inhibins, and increasing LH concentrations as progesterone concentrations decline.

Oestrus

Oestrus is the period during which cows are sexually receptive and will stand to be mounted. Behavioural oestrus develops due to rising oestradiol concentrations, secreted by the rapidly growing preovulatory follicle (Robertson, 1969; Smith *et al.*, 1975). A preovulatory surge release of GnRH further leads to the surge release of LH and FSH (Schally *et al.*, 1971; Redding, 1972; Zolman *et al.*, 1973). In cattle, peak LH concentrations occur 3–10 h after the onset of standing oestrus, with ovulation occurring from 21–30 h after the LH peak (Henricks *et al.*, 1970; Swanson and Hafs, 1971; Schams *et al.*, 1977; Rajamahendran *et al.*, 1989). Thus, ovulation occurs about 25–35 h after the onset of oestrus, which, for the majority of cattle, is around 10–14 h after the end of standing oestrus (McMillan *et al.*, 1998). The preovulatory LH surge also triggers the onset of luteinisation of the follicle, transforming it into the progesterone-producing CL.

Behavioural oestrus typically lasts for 10–18 h (Wiltbank *et al.*, 2012), although some cattle show signs for as short a period as 3 h (Cerri *et al.*, 2011; Giuliodori *et al.*, 2011). It has recently been reported that declining intensity and duration of oestrus is an important component of the worldwide decline of reproductive performance of high producing dairy cows (Butler, 2000a; Lucy, 2001; Xu and Burton, 2003; Macdonald *et al.*, 2008; Walsh *et al.*, 2011). In this context, in the past 50 years there have been reports of a decline in the percentage of cows that stand to be mounted (50% *versus* 80%) as well as in the mean oestrus duration (5 *versus* 15 h) (Baratta *et al.*, 1994).

Compared with lower producing dairy cows (< 39.5 kg per day), high-producing dairy cows (≥ 39.5 kg per day) have a shorter oestrus (6.2 h *versus* 10.9 h), reduced total time per mount (21.7 sec *versus* 28.2 sec) and lower serum oestradiol concentrations (6.8 pg/mL *versus* 8.6 pg/mL) (Lopez *et al.*, 2004). The average duration of standing oestrus for dairy cows was reported to be 8.1 h, whereas in heifers it is relatively longer (12–14 h) (Souza *et al.*, 2007). There are several physiological factors which affect the expression of oestrus. The underlying cause of poor intensity and reduced duration of oestrus is low oestradiol concentrations which are themselves due to (i) a high metabolic clearance rate of oestradiol (Ireland *et al.*, 2000) (ii) low LH and insulin-like growth factor-I (IGF-I) concentrations brought on by negative energy balance (Diskin *et al.*, 2003), (iii) stressors (such as lameness, mastitis, heat; Mihm *et al.*, 1999) and (iv) genetic selection for high post-partum growth hormone (GH) concentrations (Lucy *et al.*, 2001a).

Luteal phase

During the luteal phase of the oestrous cycle, the CL principally produces progesterone (Hansel and Echternkamp, 1972) in order to prepare the uterus to be able to maintain a pregnancy, by stimulating glandular hyperplasia and secretory activity, and by reducing the activity of the immune system within the uterus, uterine contractility and uterine mucus secretion. If conception occurs, CL has to continue to perform all of these functions, as well as suppressing gonadotrophin secretion to a point at which further ovulations are prevented. During the initial luteal phase, there are increases in progesterone and 20- β -hydroxyprogesterone concentrations in the CL (Hafs and Armstrong, 1968) and in peripheral blood (Hansel *et al.*, 1973) which are maximal between Days 10 and 14 (Donaldson *et al.*, 1970; Schams *et al.*, 1977) after ovulation.

In cattle, LH acts as the main luteotropic hormone (Hansel, 1966), which stimulates luteinisation of the theca and granulosa cells of the preovulatory follicle, producing lutein cells (Alila and Hansel, 1984). The luteal phase is traditionally divided into two periods: metoestrus, the period of the formation of the CL, and dioestrus. During metoestrus, which typically lasts 3–4 days, there is transformation of the collapsed ovulated follicle (corpus haemorrhagicum) into a functioning CL (Forde *et al.*, 2011). Corpus luteum growth following ovulation is very rapid, at a rate that is comparable to that of aggressive tumours (Webb *et al.*, 2002). During dioestrus, the CL continues to increase in size, weight and ability to secrete progesterone until around Day 12 of the cycle. Thereafter, the activity of the organ remains relatively constant until around Day 16–18 of the cycle, when luteal regression (luteolysis) begins (Erb *et al.*, 1971).

The ultrastructural morphology of the bovine CL has been investigated (O'Shea *et al.*, 1989). The three main cell types in the bovine CL which contribute to the function of the CL are: i) small non-steroidogenic cells which comprise mainly of vascular cells (endothelial cells, erythrocytes and leukocytes) and connective tissue cells (e.g. fibrocytes); ii) small steroidogenic cells and iii) large steroidogenic cells (Foley and Greenstein, 1958; Ursely and Leymarie, 1979; Koos and Hansel, 1981). The populations of cells in the CL change during its growth and regression. The large luteal cells occupy 70% of the area of the CL during mid-cycle but account for <10% of the total number of steroidogenic cells (Parry *et al.*, 1980). During the onset of luteolysis, there is an initial decrease in the number of large steroidogenic cells followed by a decrease in the numbers of small steroidogenic cells (Parry *et al.*, 1980).

Several factors are responsible for controlling the growth, development and regression of the CL, including a balance between luteotropic (e.g. LH), luteolytic (e.g. PGF_{2 α}) and metabolic hormones (e.g. IGF-I; McArdle and Holtorf, 1989), and angiogenic and antiangiogenic factors. The regulation of angiogenesis (establishment of blood supply) is a critical factor regulating

luteal function, due to the rapid cyclical changes in luteal growth and regression which demand corresponding rapid changes within its vasculature (Webb *et al.*, 2002). The principal angiogenic factors identified in the ruminant are fibroblast growth factors and vascular endothelial growth factors (Redmer and Reynolds, 1996; Reynolds and Redmer, 1999). Specific receptors for these growth factors, whose concentrations change during the luteal phase, have been identified in luteal tissue (Doraiswamy *et al.*, 1998). Other factors which regulate the function of the CL include immune cells such as lymphocytes and macrophages which are present in the CL in a significant proportion as non-luteal cells (Pate, 1995). For example, secreted products of activated immune cells can be cytotoxic, can inhibit progesterone production and can stimulate luteal cell PGF_{2α} production (Pate and Townson, 1994). The ruminant CL also synthesises and secrets oxytocin (Flint and Sheldrick, 1982; Wathes and Swann, 1982; Wathes *et al.*, 1983). It has been postulated that oxytocin is a key messenger between the large and small luteal cells that augments progesterone secretion in both cell types (Flint and Sheldrick, 1982). However, luteal oxytocin also plays a physiological role in the control of luteolysis (review: Schams, 1987) and, hence, in the duration of the oestrous cycle. Its direct action on luteal cells of all stages is mediated by membrane oxytocin receptors (Okuda *et al.*, 1992).

Ovarian follicular dynamics

Patterns of follicular development

Until the introduction of ultrasonography in the late 1980s, several direct and indirect methods were used in an attempt to study follicle development in cattle e.g. hormone concentrations (gonadotrophic and steroid), the evaluation of ovarian follicles from slaughtered animals and laproscopic observation of growth and development of individual follicles (reviewed by Fortune *et al.*, 1988). Initially, there was disagreement over the numbers and durations of ovarian follicular waves in cattle. For instance, Ireland and Roche (1987) demonstrated a sequential development of three preovulatory-size follicles in cattle, with the first two regressing and the third ovulating, whilst others suggested the existence of a two wave theory (Rajakoski, 1960; Swanson *et al.*, 1972). Conversely, some workers reported that the development of the ovarian follicle was a continuous process (Donaldson and Hansel, 1968; Spicer and Echternkamp, 1986). However, the development of transrectal ultrasonic probes has largely resolved such issues. The use of ultrasound for the study of the cattle ovary was pioneered by Pierson and Ginther (1984). This technique was subsequently used for the analysis of follicle growth patterns during the whole of the bovine oestrous cycle (Fortune *et al.*, 1988; Fortune and Sirois, 1989), which showed that, in cattle, ovarian follicle development occurs in a distinct and consistent pattern.

After analysing several two-wave and three-wave follicle developments in cattle, Ginther *et al.* (1989b) reported that two-wave animals had significantly shorter interovulatory intervals and luteal phases than animals with three waves. Furthermore, Adams (1999) reported that two or three follicular waves were the most common (>95%) forms of follicular waves during bovine oestrous cycles, although there were a few cycles of one or four waves (De Rensis and Peters, 1999). Some studies reported that the majority (>80%) of cycles followed a two-wave pattern (Ginther *et al.*, 1989a; Rajamahendran and Taylor, 1990), others that the majority (>80%) were of three waves (Sirois and Fortune, 1988; Celik *et al.*, 2005), and still others a more uniform spread of two and three waves (Savio *et al.*, 1990a; Sartori *et al.*, 2004). In a two-wave cycle, the second wave starts on Day 9–10 whereas in a three-wave cycle the second and third waves start on Days 8–9 and 15–16, respectively (De Rensis and Peters, 1999; Mapletoft *et al.*, 2002). Table 2.2 shows the average duration of the two- and three-wave patterns of cattle.

Table 2.2 Average duration (days, mean \pm SEM) of two and three- wave patterns of cattle (Noseir, 2003).

Growth wave	Two-wave pattern	Three-wave pattern
1 st	8.7 \pm 0.3	7.2 \pm 0.7
2 nd	10.8 \pm 0.3	7.7 \pm 0.4
3 rd	Not applicable	9.5 \pm 0.4

Although the repeatability of follicular wave patterns has not been studied extensively, some recent findings suggest that there is no breed or age specific predilection for a particular wave pattern, at least in *Bos taurus* cattle (Adams *et al.*, 2008). In *Bos indicus* cattle, parity has shown to influence the wave pattern: Figueiredo *et al.* (1997) reported that in Nellore cattle, the three-wave pattern was more common in the majority (65%) of the heifers, whereas two-wave pattern was more common in the majority of cows (83%). In contrast, in *Bos taurus* (Holstein) cattle, there was no such variation seen between heifers and cows (Sartori *et al.*, 2004). Later, Jaiswal *et al.* (2009) confirmed in *Bos taurus* (Hereford-cross) heifers that the two-wave or three-wave pattern was repeatable within individuals irrespective of the maturity, although the predominance of the two-wave pattern, as well as pattern repeatability, was greater in more mature heifers.

Before about 2004, very little was known about the developmental patterns of antral follicles with cavities <4 mm in diameter because of the poor resolution of the ultrasound probes

available. However, more recently, the advancement of the new ultrasound scanners with high resolution probes which can scan structures as small as 1 mm, has permitted characterisation of the developmental patterns at 1–3 mm. By using such scanners, Souza *et al.* (2011) first identified the future dominant follicle at a diameter of only 1 mm and reported its emergence 6–12 h earlier than the first subordinate follicle, further concluding that follicles as small as 1–3 mm in size develop in a wave-like manner.

Regulation of follicular waves

The regulation of the follicular wave in cattle has not yet been completely elucidated. Follicular waves emerge regularly under the control of a basal level of LH (Sirois and Fortune, 1990). However, the secondary follicle stimulating hormone (FSH) surge also plays a role in regulating follicular waves (Turzillo and Fortune, 1990), as it initiates the regular follicle waves (Figure 1.2) and may play a role in restarting the follicular waves after the LH/FSH surge. However, due to progesterone dominance the dominant follicles which develop during the luteal phase do not ovulate. Negative feedback from progesterone means that, although LH pulse amplitude increases, LH pulse frequency is insufficient to stimulate ovulation of the dominant follicle (Rahe *et al.*, 1980).

Follicular dominance

There are three distinct phases of the follicular wave: recruitment, selection and dominance. During recruitment, a cohort of antral follicles escapes atresia. In the cow, 5–10 follicles generally develop as a cohort (Driancourt, 2001), but the cohort can contain as many as 24 follicles (Mihm and Austin, 2002). Due to regression or ovulation of the previous dominant follicle, the negative feedback effects the follicular hormones (oestradiol and inhibin) is stopped. This in turn leads to a transient increase in FSH secretion from the pituitary gland, which initiates the growth wave of the follicle. Follicle stimulating hormone concentrations peak when the largest follicles attain a diameter of 4–5 mm (Kulick *et al.*, 1999). Follicles of ≥5 mm suppress the FSH-producing capacity of the anterior pituitary (Gibbons *et al.*, 1999).

During the oestrous cycle, there is growth, regression and replacement of the group of follicles (Smeaton and Robertson, 1971; Matton *et al.*, 1981). After approximately three days of growth, when the largest follicle of the wave attains an average diameter of 8.5 mm, there is differentiation (or ‘deviation’: Ginther *et al.* 1997) of the future dominant follicle from its subordinates. Although all growing follicles have the capacity to become the dominant follicle prior to deviation, only the largest follicle further develops into a dominant follicle, whilst the others regress (Ginther *et al.*, 1997).

In each cohort, one follicle will start to dominate oestradiol production as a consequence of its induction by LH via LH receptors present on the granulosa cells (Luo *et al.*, 2010). During the growth period, the FSH-dependence of the dominant follicle reduces while there is an increase in LH dependence (Hansel and Convey, 1983; Mihm *et al.*, 2006). As well as growing faster, the dominant follicle starts producing inhibin. The shift in the dominant follicle from FSH-dependence to LH-dependence allows the dominant follicle to circumvent the effects of the inhibin it produces. Inhibin is an inhibitor of FSH release from the anterior pituitary so retards the growth of the FSH-dependent non-dominant follicles (Hansel and Convey, 1983), which become atretic (Rajakoski, 1960; Ireland and Roche, 1983).

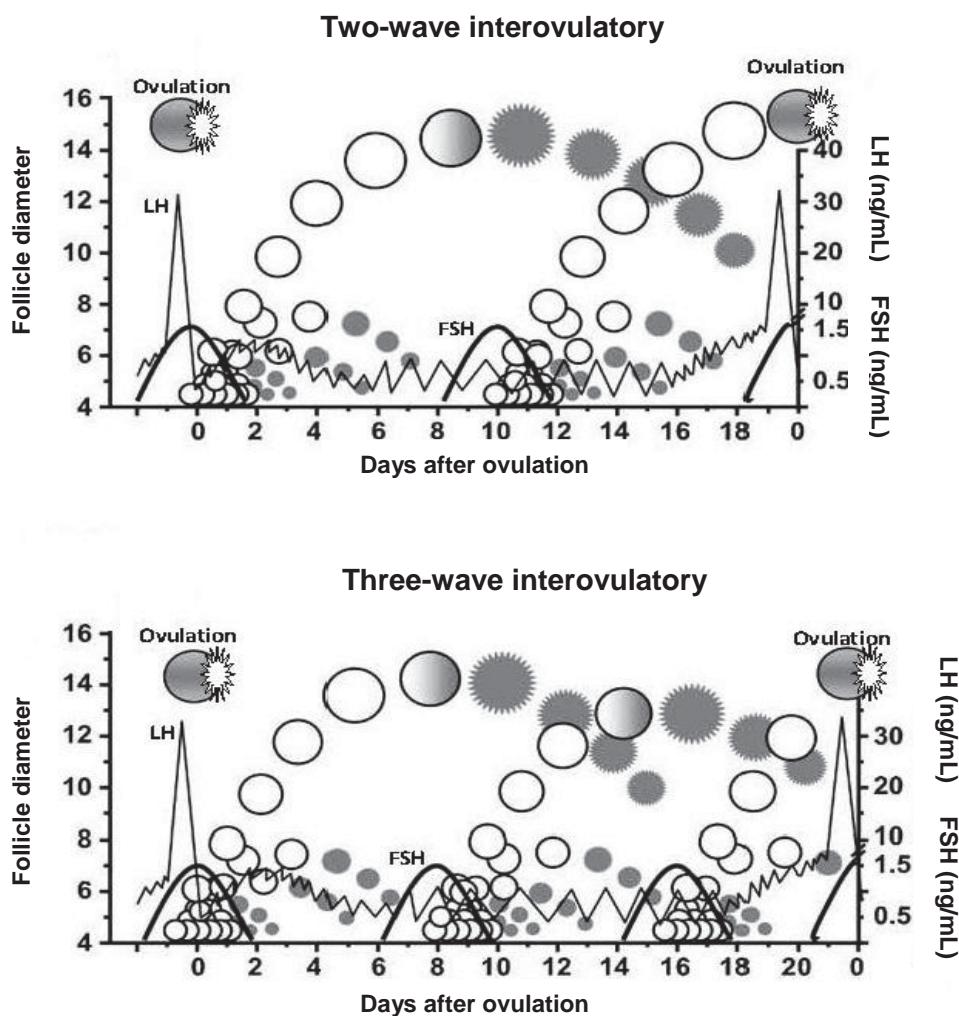


Figure 1.2 Ovarian follicular dynamics and patterns of follicle stimulating hormone (FSH) and luteinising hormone (LH) secretion during two- and three-wave interovulatory interval in cattle. Dominant and subordinate follicles are shown as open (viable) or shaded (atretic) circles. Thick line shows a surge in circulating FSH concentrations preceding emergence of a new wave whereas thin line shows a surge in circulating LH concentrations preceding ovulation. From Adams *et al.* (2008).

Physiology of the post-partum period in dairy cows

Early re-conception after calving is of critical importance in pasture-based dairy farming systems because of the necessity of maintaining a 12-month calving interval to ensure that lactation is synchronised with pasture growth. Because of an average gestation length of 282 days, the processes of uterine involution, resumption of oestrous cycles and conception have to occur within an 83 day period (Macmillan, 2002). Thus, cows that have not ovulated by one week before the planned start of mating have a lesser possibility of being pregnant within the first 21 days of mating (McNaughton *et al.*, 2007). Similarly, cows not displaying oestrus before the planned start of mating have poorer reproductive outcomes than those that have been detected in oestrus (McDougall and Rhodes, 1999; Rhodes *et al.*, 2003).

Normal ovarian physiology of the post-partum period

Calving is followed by a period of anovulation whilst the activity of the reproductive neuroendocrine axis resumes after the prolonged dominance of progesterone during pregnancy. The period between parturition and the re-establishment of ovulations typically lasts for 14–27 days in housed dairy cattle (Fonseca *et al.*, 1983; Stevenson and Call, 1983; Darwash *et al.*, 1997), whereas in pasture-fed dairy cows the mean interval between calving and ovulation is 43 days (McDougall *et al.*, 1995). After parturition, circulating oestradiol and progesterone concentrations return to basal values within 3–5 days, which is followed by recurrent transient increases in FSH concentrations (Crowe *et al.*, 1998) that induce the emergence of the first new follicular wave within 7–10 days post-partum (Murphy *et al.*, 1990; Savio *et al.*, 1990a). However, the first ovulation in both dairy and beef cattle is not generally followed by overt oestrus behaviour (Kyle *et al.*, 1992) and only about 10% of cows stand to be mounted during this first oestrus (Sheldon *et al.*, 2011). The length of the first post-partum oestrous cycle is markedly reduced by the shorter life of the CL formed after the first spontaneous ovulation, due to lack of prior progesterone exposure leading to earlier release of PGF_{2α} from the uterus (review: Garverick *et al.* 1992).

Lactation and suckling generally extend the time to the resumption of oestrous cycles post-partum, although, in dairy cows, there is no clear relationship between prolactin concentrations and the post-partum onset of ovarian cycles (Lamming *et al.*, 1981). In beef cows, the oestrous cycle resumes as the rate of sucking declines, resulting in an anoestrous period of about 6–7 weeks (Fielden *et al.*, 1973). However, in dairy cows, despite regular milking, the inhibitory effect of suckling is largely absent, resulting in first ovulation at 2–4 weeks post-partum, and first oestrus 10–21 days after that (Savio *et al.*, 1990a; Beam and Butler, 1998).

Abnormal ovarian physiology

National animal evaluation data (1990–2004) in New Zealand shows a decline in the six week re-calving rate, with 10% fewer cows (60% *versus* 70%) re-calving within the first 42 days of the seasonal calving period (Harris *et al.*, 2006). This trend of declining fertility largely mirrors that seen in the international dairy herd. Whether the reports of a recent increase in fertility of some New Zealand dairy herds (Brownlie *et al.*, 2013) represent an arrest in the decline in fertility is an intriguing possibility, although whether this is due to heterosis, better feeding management or a genuine change in the genetically-controlled components of fertility remains to be determined.

There are many causes for the global decline in the fertility of dairy cows, to which post-partum anoestrus is an important contributor. Anoestrus is a broad term that has been defined by Peter *et al.* (2009) as “*the lack of typical oestrus expression (or absence of oestrus signs), despite efficient oestrus detection*” and covers all cows not seen in oestrus after a certain period after calving (review: Opsomer *et al.*, 1996). Failure of a cow to be detected in behavioural oestrus by the planned start of mating can be caused by prolonged post-partum anoestrus (McDougall *et al.*, 1993). Prolonged post-partum anoestrus may be due to a failure of oestrus detection, failure of the cow to express behavioural oestrus (even though ovulation has occurred), or failure to recommence ovarian cycles (anovulatory anoestrus) (Peter *et al.*, 2009) . Prolonged anoestrus is a result of interacting management, physiological, pathological and nutritional factors (Macmillan and Clayton, 1980). Cows in true anovulatory anoestrus have no CL, in contrast to cows which have ovulated (missed or silent heat), in which a CL is normally present. Under New Zealand conditions, approximately 20% of cows are not detected in oestrus by the planned start of mating (Rhodes *et al.*, 2003). Generally, a smaller proportion (55%) of cows with anovulatory anoestrous are detected in oestrus during the initial three weeks of mating compared to cycling cows (96%) and such anovulatory anoestrous cows also conceive later than do cycling cows (37 *vs.* 22 days after planned start of mating) (Macmillan, 1997)

Peter *et al.* (2009) classified anoestrous cows into four groups (Figure 2.2). This classification was based on the identification of three functionally critical diameters during follicular growth: ~4 mm (emergence), ~9mm (deviation) and 10 to 20 mm (ovulation). In Type I anoestrus, there is recruitment (emergence) and growth of the follicle, but it fails to progress through the processes of selection and dominance. It is presumed that this condition is due to extreme undernutrition. In Type II anoestrus, there is selection (deviation), growth and/or dominance followed by either atresia or regression. A new follicular wave emerges within 2–3 days after the regression of this follicle. In such cases, successive follicular waves precede the first ovulation (McDougall *et al.*, 1995). McDougall *et al.* (1995) clearly demonstrated the existence

of Type II anoestrus in post-partum New Zealand dairy cows by showing detection of the first dominant follicles within 11 days post-partum but a longer subsequent interval to first ovulation.

In Type III anoestrus, there is recruitment, selection and dominance of the follicle, but it fails to ovulate and develops into a persistent follicle. Type IV anoestrus develops after normal ovulation and formation of the CL, however such animals have an extended luteal phase due to the lack of CL regression.

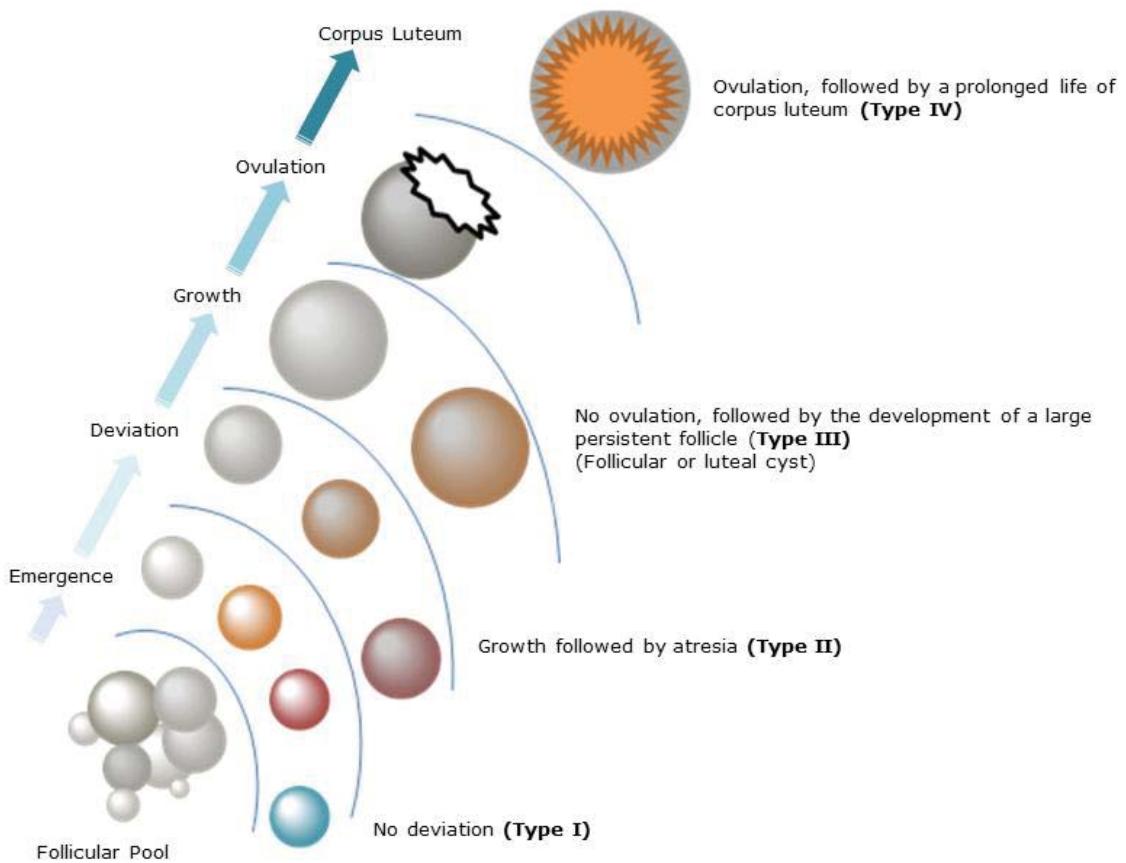


Figure 2.2 Types of post-partum anoestrus conditions based on the ovarian follicular and luteal dynamics. Redrawn from Peter *et al.* (2009).

The first detailed description of anoestrus in pasture-fed dairy cows was that by Fielden *et al.* (1973), who associated the condition with bilateral ovarian hypoplasia (inactive ovaries) at >6 weeks post-partum. However, further studies by McDougall *et al.* (1995) showed that the term inactive ovaries was inappropriate. The absence of large follicles was not the factor limiting the resumption of ovulation after calving, because all cows had large follicles within 11 days of calving. Furthermore, follicles of >14 mm in diameter failed to ovulate (even though

follicles <14 mm in diameter are capable of ovulation). A low LH pulse frequency appeared to be associated with failure of ovulation of the dominant follicle in cows that remained in anovulatory anoestrus (McDougall and Macmillan, 1994), perhaps due to insufficient production of oestradiol by these large follicles (McDougall *et al.*, 1995) and the effects of negative energy balance (Canfield and Butler, 1990).

Negative energy balance and its effects on post-partum reproduction

Across all dairy farming systems, negative energy balance is a consistent cause of anoestrus in the lactating dairy cow because the energy requirements for milk production and maintenance during the early post-partum period exceed energy intake. This is compensated for by increased lipolysis in adipose tissues, increased gluconeogenesis and glycogenolysis in the liver, and mobilisation of protein reserves from the muscles. Eventually, increased gastrointestinal capacity and activity should lead to increased energy intake and a return to positive energy balance (Bauman and Currie, 1980). Cows do not resume cyclic activity until about 10 days after the lowest point of negative energy balance is reached (Butler *et al.*, 1981). Similarly, a nadir (lowest value) of energy balance is related to delayed resumption of luteal activity (de Vries and Veerkamp, 2000). This is as true in New Zealand as elsewhere, despite the dairy system being based on a much lower mean peak milk production (and thus lower energy output) (McDougall *et al.*, 1993). The key impact of negative energy balance in the New Zealand system is to delay the return to ovarian cyclicity and thereby increase the interval from calving to first service and conception (McDougall *et al.*, 1993). Under New Zealand conditions, where a short breeding period is required to maintain the seasonal calving pattern, delayed return to oestrus increases the risk of the cow failing to conceive during the prescribed breeding period and thus increases the risk of culling for infertility (Macmillan and Clayton, 1980; Harris and Kolver, 2001). Additionally, calving date determines length of lactation because cows are dried off on the basis of pasture availability rather than just milk production (Harris and Kolver, 2001), so delayed conception also reduces lactational yield. Thus, delayed onset of ovarian cyclicity can result in significant losses in pasture-based systems.

The somatotrophic system of the body which controls the distribution of nutrients and energy may also affect the reproductive system via effects at several levels of the hypothalamo-pituitary-ovarian axis (Roche, 2006; Chagas *et al.*, 2007). Within the hypothalamus, interactions between the gonadotrophic and somatotrophic systems occur in the pre-optic area (Blache *et al.*, 2007). Releasing hormones produced in this region control the secretion of both gonadotrophins and somatotrophin (Kacsoh, 2000). In addition, the pre-optic area plays a critical role in integrating appetite (Wynne *et al.*, 2005), oestrus behaviour (Pfaff, 2005) and the sensing of

nutritional status (Wade and Jones, 2004). Other metabolic signals involve leptin and NEFA (Liefers *et al.*, 2003; Amstalden *et al.*, 2005).

There are probably carryover effects of the hostile metabolic conditions in the early post-partum during primary follicle growth on the health of the preovulatory follicle two to three months later (Britt, 1992). These follicles may produce inadequate amounts of steroids (oestradiol and progesterone) after ovulation and may contain an oocyte of poorer quality (Britt, 1992; Roth *et al.*, 2001). Conversely, early post-partum positive energy balance influences follicular growth and influences the interval between calving and the first ovulation by influencing the movement of follicles into larger size classes (Lucy *et al.*, 1991).

i. Role of non-esterified fatty acid (NEFA)

Albumin-bound fatty acids in the blood are usually referred to as NEFA to distinguish them from triglyceride fatty acids in chylomicrons and lipoproteins (Cunningham, 2002). During a prolonged period of undernutrition, low glucose availability leads to rapid mobilisation of adipose fatty acids in the form of NEFA. Once the NEFA have been transported into liver hepatocytes, they may follow any of the three potential metabolic paths: complete oxidation for energy production, esterification with triglyceride production or the production of ketone bodies (Cunningham, 2002).

Increased growth hormone and NEFA concentrations antagonise the action of insulin and create a state of insulin resistance in post-partum cows (Lucy, 2007). The direct action of insulin and IGF-I act on the ovary to increase the sensitivity of the ovary to LH and FSH (Webb *et al.*, 2004; Bilby *et al.*, 2006). Therefore, there is increasingly clear evidence that insulin and IGF-I are responsible for mediating the effects of energy balance on the development of follicles and ovulation.

Relatively low blood concentrations of glucose and high NEFA concentrations are present in cows in the initial stages of negative energy balance compared to those of cows in positive energy balance (Murondoti *et al.*, 2004). Consequently, there is mobilisation of stored fat to provide NEFA to generate more adenosine triphosphate (ATP) after oxidation in the liver (Greenfield *et al.*, 2000; Xu *et al.*, 2008). The high circulating concentrations of NEFA further prevent gluconeogenesis by the hepatocytes, thereby increasing the level of negative energy balance (Li *et al.*, 2012).

Only a few studies have examined the possible effects of negative energy balance associated with raised NEFA concentrations upon oocyte quality. Non-esterified fatty acid may directly act

on follicle cells to affect their growth and development. Adding similar concentrations of NEFA, as found during negative energy balance, during *in vitro* maturation of follicle cells in follicular fluid had harmful effects on the viability and functions of follicle cells (Leroy *et al.*, 2005; Vanholder *et al.*, 2005). Such *in vitro* studies suggest that negative energy balance may hamper the fertility of high producing dairy cows through increased NEFA concentrations in follicular fluid affecting oocyte quality (Leroy *et al.*, 2005).

ii. Role of insulin

Insulin is a polypeptide which is synthesised and released from β cells of the pancreatic islets of Langerhans. It acts by lowering blood glucose by facilitating the movement of glucose across cell membranes (Martin and Crump, 2003). Insulin also increases circulating concentrations of IGF-I in lactating dairy cows, either directly or via secondary changes (McGuire *et al.*, 1995). Insulin is the primary modifier of lipid and ketone metabolism (Brockman, 1979) and influences the mobilisation of free fatty acids from adipose tissue and utilisation of ketone bodies by peripheral tissues (Brockman, 1979).

A role of insulin as a regulator of ovarian function was first suggested by Channing *et al.* (1976). Regardless of the species, the general effects of insulin on ovarian cells are positive, including stimulation of the proliferation of granulosa cells, production of progesterone and enhancing luteal cell steroidogenesis (review: Poretsky and Kalin, 1987). Several *in vitro* and *in vivo* studies in cattle suggest that insulin is a better stimulator of granulosa cell oestradiol production than IGF-I (review: Spicer and Echternkamp, 1995). Insulin infusion during the early post-partum period has been shown to increase oestradiol secretion by the dominant follicle of the first follicular wave in dairy cows (Butler *et al.*, 2004).

Low post-partum insulin and glucose concentrations can suppress GnRH secretion from the hypothalamus and subsequent pituitary LH release (Diskin *et al.*, 2003). Since insulin has been shown to stimulate follicular growth, maturation and steroidogenesis, reduced post-partum concentrations are associated with ovarian dysfunction (Armstrong *et al.*, 2002; Butler *et al.*, 2004; Kawashima *et al.*, 2007). When cows were fed with experimental diets for the first 50 days of lactation, the diet used to induce high insulin secretion advanced the first ovulation post-partum in both high and low genetic merit cows (Gong *et al.*, 2002).

iii. Role of IGF-I

Insulin-like growth factor-I is related structurally and functionally to insulin. It was first purified and sequenced in 1978 by Rinderknecht and Humbel (1978). It is a single-chain peptide whose α and β domains are approximately 50% identical with the α and β chains of insulin (Martin and

Crump, 2003), and which has potent insulin-like effects. Administration of IGF-I stimulates the uptake and utilisation of glucose by muscle and adipose tissue, which results in a marked decrease in blood glucose (Martin and Crump, 2003). It also decreases plasma glucose concentrations by stimulating peripheral glucose uptake and reducing hepatic glucose production (Boulware *et al.*, 1992; Boulware *et al.*, 1994).

Though structurally related to insulin, most of the circulating IGF-I originates from the liver rather than the pancreas. It is released from the liver in response to growth hormone and is believed to control growth and lactation (Renaville *et al.*, 2002). Insulin-like growth factor-I is also produced by ovarian cells and thus contributes to an autocrine or paracrine system which regulates follicular growth and gonadotrophin-induced differentiation (Spicer and Echternkamp, 1995; Martin and Crump, 2003).

Positive correlations between energy balance and serum progesterone, and between energy balance and serum IGF-I, have been demonstrated for post-partum dairy cows (Spicer *et al.*, 1990). Dairy cows with positive energy balance (+14 megajoules/day) during the first 12 weeks post-partum had greater serum concentrations of IGF-I and greater luteal phase progesterone concentrations than did cows with negative (-7 megajoules/day) energy balance. Likewise, reduced luteal activity during negative energy balance has been associated with reduced serum concentrations of IGF-I (Spicer *et al.*, 1990).

Insulin-like growth factor-I has also been described as a hormonal mediator of ovarian function because some *in vitro* studies have shown it to be a potent stimulator of bovine granulosa and luteal cell steroidogenesis (Schams *et al.*, 1988; McArdle and Holtorf, 1989). Insulin-like growth factor-I plays an important role in gonadotrophin-induced folliculogenesis, ovarian steroidogenesis and CL function, and is considered as a key factor that signals nutritional status to the reproductive axis (review: Zulu *et al.*, 2002). Insulin and IGF-I act directly on ovarian cells and therefore are important regulators of follicle growth and development (Spicer and Echternkamp, 1995; Gong, 2002). Consequently, a negative influence of low peripheral IGF-I concentrations was established on the commencement of post-partum ovarian activity (Beam and Butler, 1997; Ortega *et al.*, 2008).

Genetic selection for high milk yield *versus* fertility

An antagonistic relationship between milk production and reproduction in lactating dairy cattle has been widely reported overseas (Butler and Smith, 1989; Lean *et al.*, 1989; Ray *et al.*, 1992) however, the average New Zealand dairy cow has not reached this apex in this relationship. Lactating dairy cows that ovulated early during the post-partum period (15 to 21 days after

calving) consumed more food and tended to produce more fat-corrected milk than cows ovulating later (22 to 42 days *versus* >42 days; Lucy *et al.*, 1992). Early lactation fat percentage might serve as an indicator of energy balance in dairy cattle, as the highest correlation was seen between nadir of energy balance and a decline in milk fat percentage (de Vries and Veerkamp, 2000).

Although the most serious fertility problems are attributed to the high-producing dairy cow, cows with moderate milk production (such as most dairy cows in New Zealand) also have reproductive problems, which, taken together, indicate that in dairy cows the general effects of lactation on reproduction cannot be ignored (Lucy, 2001). The energy status of dairy cows during the periparturient period can be evaluated on the basis of energy intake and energy requirements (energy status = energy intake - energy requirements; review: Adewuyi *et al.*, 2005). In dairy cattle in New Zealand, the period of negative energy balance lasts for 4–14 weeks post-partum, with significant variation between cows in both the length and the depth of negative energy balance (McDougall *et al.*, 1993). The loss of body condition is correlated with the mobilisation of body fat (Wright and Russel, 1984; Komaragiri *et al.*, 1998) and, therefore, body condition score (BCS) is also used as indicator of energy balance during early lactation. Calving condition score has a great deal of influence on post-partum ovarian activity, mainly because cows with adequate energy reserves can attempt to minimise the negative effects of energy shortage and can commence an ovarian cycle and ovulation during the early post-partum period (Fielden *et al.*, 1973; Macmillan, 1997).

Based on many studies of nutrient partitioning during early lactation in cows, Lucy *et al.* (2001a) proposed that the decrease in feed intake and hormonal events around parturition (such as decreasing progesterone, increasing oestradiol and increasing glucocorticoids), together with intense selection for high post-partum GH concentrations, may collectively cause a decrease in the ability of GH to elicit IGF-I secretion by the liver. The decrease in liver IGF-I secretion results in low blood IGF-I concentrations and, because circulating IGF-I acts as the main negative feedback hormone for GH, an increase in blood GH concentrations. The effects of these hormonal changes on reproductive functions of the early post-partum dairy cows are summarised in Figure 3.2.

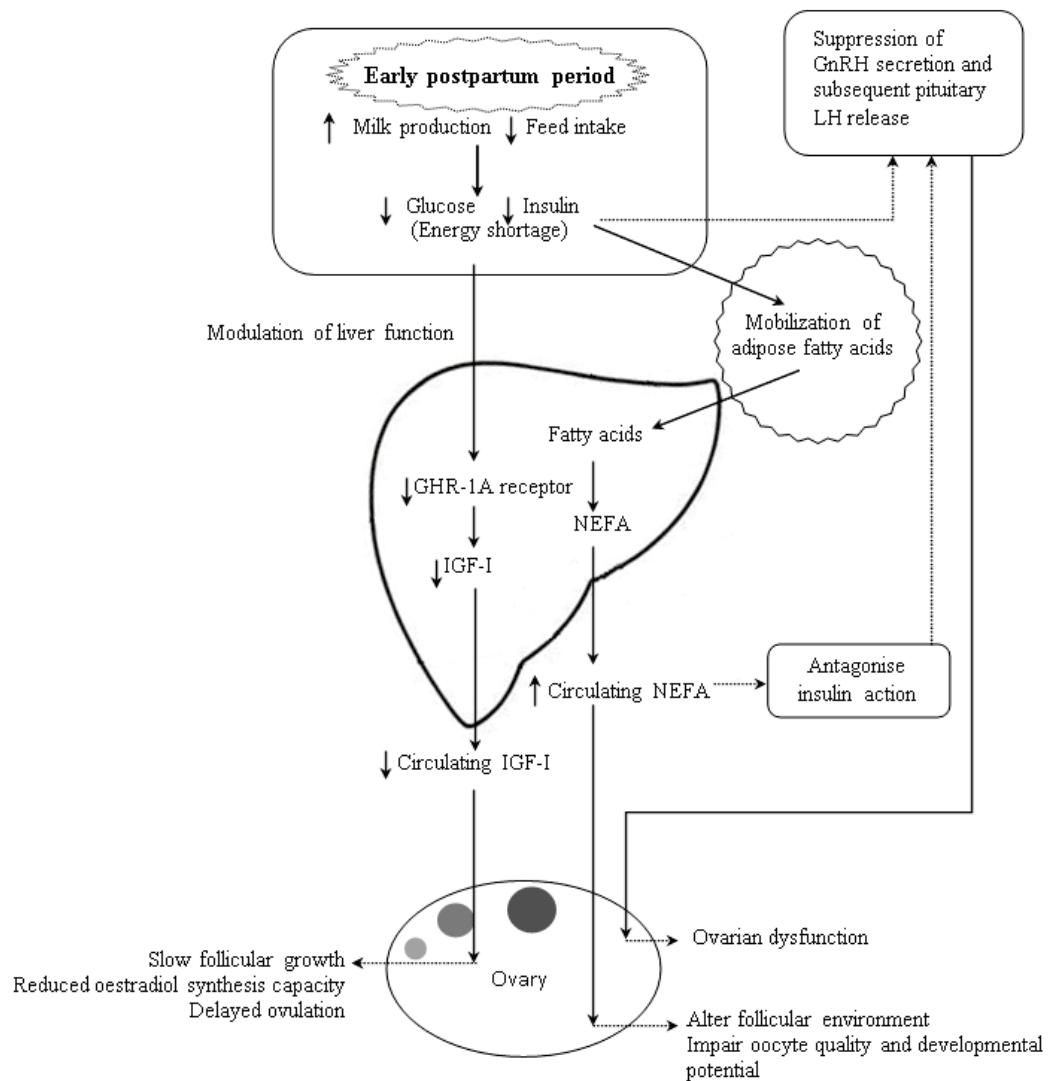


Figure 3.2 Effect of negative energy balance and concentrations of insulin-like growth factor-I (IGF-I), insulin and nonesterified fatty acid (NEFA) on post-partum reproductive function in lactating cow (modified from Wathes *et al.* 2007). GHR-1A= growth hormone receptor 1A.

Physiological basis of oestrus synchronisation

Exogenous hormonal treatments can be used to modify the activity of the hypothalamo-pituitary-ovarian axis and, thus, modify ovarian activity. Receptors for GnRH are not present in the bovine ovary (Brown and Reeves, 1983; Ireland *et al.*, 1990); it induces its effects by inducing the release of LH and FSH (Chenault *et al.*, 1990) from the anterior pituitary. These in turn act upon gonadotrophin responsive tissues in the ovary by binding to their corresponding receptors on these cells. During pro-oestrus, there is a sudden decrease in circulating progesterone concentration (to <1 ng/mL) and an increase in production of oestradiol 17β by the preovulatory follicle. High concentrations of circulating oestradiol are

responsible for oestrus behaviour and cause positive feedback effects on the hypothalamus (Ireland, 1987; Fortune *et al.*, 1988), resulting in ovulation of the dominant follicle (Nett, 1987). However, the ovulation response to exogenous GnRH depends on several further factors: for example, GnRH is unable to cause the ovulation of an already regressing follicle (Silcox *et al.*, 1993), which may be due to the decrease in the LH receptors in such follicles (Guilbault *et al.*, 1993). Similarly, ovulation cannot occur during the luteal phase of the cycle, due to the negative feedback effects of progesterone upon LH pulsatility (Roberson *et al.*, 1989).

The concept of oestrus synchronisation dates back to the 1960s, when attempts to synchronise behavioural oestrus were made using a variety of progestagens to produce an artificial luteal phase. Exogenous progesterone can be administered to artificially lengthen the luteal phase, consequently increasing the inter-oestrus interval. This is the most basic means of using progesterone to manipulate the length of the oestrous cycle and is a common element of oestrus synchrony (Macmillan, 1990). Melengestrol acetate (MGA) was the first commercially available progestagen used for oestrus synchronisation. This orally active progestin could synchronise behavioural oestrus over a four day period from two days after its removal. The first bovine oestrus synchronisation programme introduced in New Zealand used progesterone alone (Lammond, 1967a). This was used in an attempt to stimulate the resumption of oestrous cycles during the early post-partum period in both beef and dairy cows, however results were variable (Brown *et al.*, 1972; Miksch *et al.*, 1978; Kyle *et al.*, 1992; Fike *et al.*, 1997). Further research focussed on progesterone administration led to the introduction of the intravaginal progesterone releasing device, which significantly lessened the inconvenience of repeated animal handling required for oral or injectable forms of progesterone. The period of time over which the progesterone inserts were kept in the vagina had a key impact on the subsequent oestrus synchronisation rate and fertility. In general, the longer the length of progesterone administration, the higher the rate of oestrus synchronisation, but the lower the fertility of synchronised animals (Cooper, 1974). Long-term progesterone administration also causes development of persistent, oestradiol-secreting follicles (Lammond, 1967b; Sirois and Fortune, 1990). Such persistent follicles develop due to increased gonadotrophin secretion from the anterior pituitary after the regression of the CL in the ovary (Lammond, 1967a; Savio *et al.*, 1993a). The poor conception rates after its use for 14–18 days led to the modification of progesterone-based programmes by the addition of other hormones, including oestradiol, oestradiol esters (benzoate, cypionate) and gonadotrophins [(GnRH, human chorionic gonadotrophin (hCG) and equine chorionic gonadotrophin (eCG)].

Oestradiol was added to progesterone-based programmes to stimulate ovulation and behavioural oestrus after the treatment with progesterone. Some of the earlier studies, involving long-term

progesterone injections (9 to 14 days), followed by a single oestradiol injection, resulted in successful induction of oestrus and ovulation in beef cattle (Ulberg and Lindley, 1960; Saiduddin *et al.*, 1968). Similarly, an injection of 5 mg of oestradiol valerate at the end of a long-term oral progesterone treatment significantly reduced the interval to first oestrus and ovulation (Brown *et al.*, 1972), although conception rates within three services were less than those of untreated cows (88% *versus* 96%). Before the European Union prohibited the use of oestradiol esters in food producing animals (European Union, 2003), oestradiol benzoate/progesterone programmes were the main protocols for the induction of oestrus in anoestrous cows in New Zealand (Rhodes *et al.*, 2003; Lucy *et al.*, 2004). Oestradiol has two actions: (i) if progesterone concentrations are high, or if the follicle has reached preovulatory maturity, it causes atresia and regression of large antral follicles, and the emergence of a new follicular wave; or (ii) where progesterone concentrations are low, it induces a positive feedback (GnRH and LH surge) leading to ovulation and luteinisation (Lucy *et al.*, 2004).

After the demonstration of the luteolytic effects of PGF_{2α} in cattle (Rowson *et al.*, 1972), its use gained in popularity, with oestrus synchronisation being achieved by using single or double injection (10–12 days apart) of a luteolytic dose of PGF_{2α}. This was once the most frequently used programme in cattle (Odde, 1990). Prostaglandin F_{2α} is only effective if there is functional luteal tissue (CL or luteinised follicle; Macmillan, 1990). However, PGF_{2α} administration is ineffective between the onset of spontaneous luteolysis and Days 6–7 after ovulation (Macmillan, 1990), due to refractoriness of the metoestrus CL to its effects. A PGF_{2α} responsive CL is usually present between Days 5 to 17 (heifers) and 7 to 17 (cows) of the oestrous cycle, but responses are usually greatest, intermediate and least for cows in the late (Days 14–19), middle (Days 10–13) or early (Days 5–9) stages of the oestrous cycle, respectively (Xu *et al.*, 1997). Moreover, in cows which responded to the treatment, the accompanying oestrus was spread over a 6-day period (Macmillan and Henderson, 1984). Prostaglandin F_{2α} treatment based on *per rectal* confirmation of the presence of a CL in the ovary, led to an average of 52% (range 36 to 68%) of cows showing an oestrus response within six days of treatment (Dailey *et al.*, 1983; Plunkett *et al.*, 1984; Whittier *et al.*, 1989), while treatment of cows that had already resumed oestrous cycles resulted in 63 to 88% being detected in heat within seven days of treatment (Xu *et al.*, 1997).

The two injection protocol of PGF_{2α} gained popularity as it also allowed for single (80 h after treatment) or double (72 and 96 h after the treatment) fixed-time artificial insemination (FTAI) without the need for oestrus detection. Two-dose PGF_{2α} treatment protocols at an 11 day interval were developed to maximise the chance of a PGF_{2α}-responsive CL being present when the second dose of PGF_{2α} was given. Smith (1976) demonstrated that two injections of

cloprostenol given to Angus x Jersey heifers at an 11-day interval resulted in 80% of the heifers exhibiting oestrus within a period of 36 to 168 h (mean, 68.3 ± 27.4 h) after the second injection. Hafs *et al.* (1975) treated maiden Friesian heifers with two injections of PGF_{2α} at an 8-day interval, the second injection being followed by two inseminations 70 h and 88 h later. Seventy percent of the treated heifers were observed in oestrus over a two-day period and 54% conceived to the double insemination. Cooper (1974) obtained an exceptional degree of synchronisation with cloprostenol given to maiden dairy heifers at an 11 day interval (98% were in oestrus over a 24 h period). Macmillan *et al.* (1978) gave cloprostenol to maiden heifers (105 dairy and 119 beef) at an 11-day interval, after which they were inseminated at 72 h and 96 h after the second injection. However, the pregnancy rates following the use of this programme were not always consistent (23%–57.8%) (Macmillan *et al.*, 1978; Stevenson *et al.*, 1987; Kaim *et al.*, 1990).

Although PGF_{2α} was used extensively for oestrus synchronisation, its effect in synchronising oestrus was not very consistent. This was due to the inability of PGF_{2α} to affect the follicular dynamics. Ovarian ultrasonography has shown that the timing of the onset of oestrus is mainly dependent upon the follicular rather than the luteal status at the time PGF_{2α} injection (Kastelic and Ginther, 1991). If administered at the time of dominance of a follicular wave, the interval to the onset of oestrus will be short (2–3 days: Savio *et al.*, 1990c). Likewise, injection during the late growing (when there is increase in dominant follicle size in relation to time) or early static (when there is no noticeable increase in dominant follicle size in relation to time) phase causes ovulation within three to four days. On the other hand, injection during the mid to late static phase will cause the ovulation of the dominant follicle from the succeeding follicular wave within 5–7 days (Kastelic and Ginther, 1991).

Prostaglandin F_{2α} was combined with progesterone/progestins to decrease the length of progesterone administration. The combination treatment in which PGF_{2α} was given at the end of a 5–7 day progesterone treatment resulted in an enhanced oestrous response in Friesian heifers (Wishart, 1974) and a conception rate of 63.8% in beef heifers (Heersche *et al.*, 1974). Further modification to the protocol, by which PGF_{2α} injection was given 24 h before progesterone device removal (Hansel and Beal, 1979), resulted in excellent synchrony of oestrus allowing single FTAI. Inclusion of progesterone for 5 days in a double PGF_{2α} programme was also effective as it increased both submission and conception rates (Xu *et al.*, 1997). Use of a progesterone device for 7 days and PGF_{2α} on Day 6 in beef cows effectively reduced the interval to first oestrus compared with untreated controls (8 *versus* 11 days) (Lucy *et al.*, 2001b).

In the US, during the period of time when progesterone devices were not commercially available, the inconsistency associated with PGF_{2 α} -based synchronisation led to the development of the GPG (Ovsynch) programme. The GPG programme not only synchronised CL development and luteolysis but also synchronised follicular development, resulting in an improvement in pregnancy rates to FTAI. A GPG programme which involves consecutive administration of GnRH, PGF_{2 α} and GnRH at intervals of 7 and 2 days (Figure 4.2), followed by either FTAI 16 to 24 h after the Day 9 GnRH injection or insemination to observed oestrus, was first described by Pursley *et al.* (1995). The purpose of the GnRH on Day 0 is to cause atresia/ovulation of the dominant follicle, leading to the emergence of a new follicular wave and development of a CL (or luteinised/progesterone-secreting tissue) by Day 7. Prostaglandin F_{2 α} treatment on Day 7 causes luteolysis and the second administration of GnRH on Day 9 causes ovulations that are synchronised within a period of about 8 h. Generally, cows treated with this programme do not exhibit oestrus behaviour at the time of FTAI due to ovulation of the follicle before peak oestradiol concentrations have been attained.

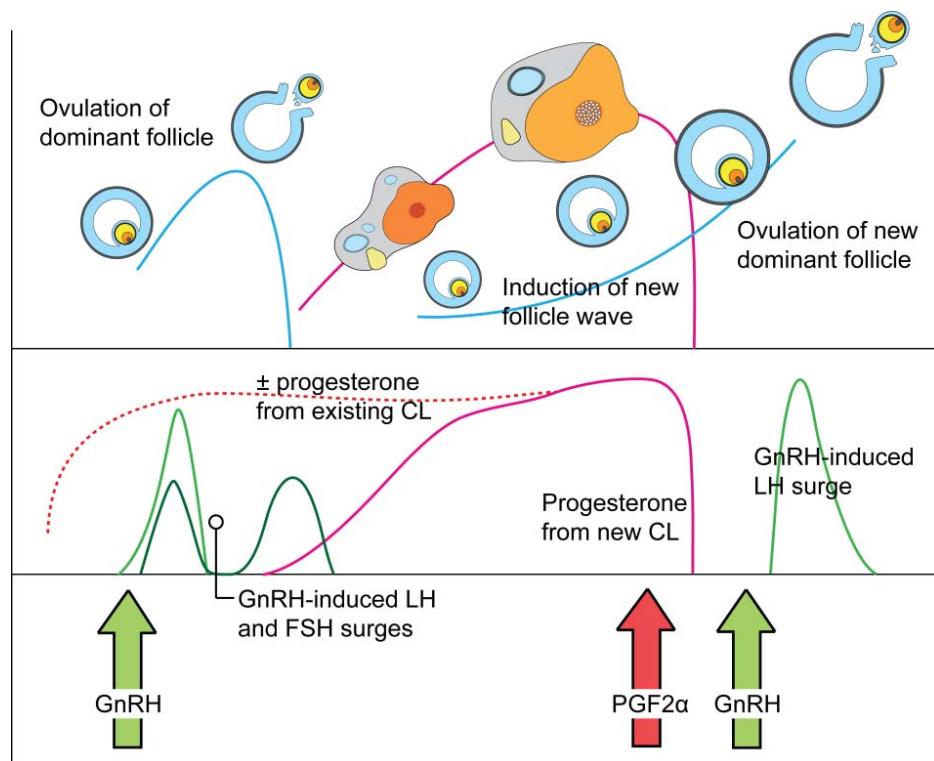


Figure 4.2 Physiological actions of gonadotrophin releasing hormone (GnRH) given on Day 0 and Day 9 and prostaglandin F₂ alpha (PGF_{2 α}) given on Day 7 for the synchronisation of luteolysis and ovulation in an GPG (Ovsynch) programme. Based on Pursley and Martins (2011) and Wiltbank *et al.* (2011).

After the initial development of the GPG programme for cattle, several drawbacks of the programme became apparent. For example, failure to respond to the first GnRH treatment leads to poor conception rates because of the inconsistent response of the ovary to the PGF_{2α} treatment (Vasconcelos *et al.*, 1999). This initial poor response can further lead to premature oestrus before the second GnRH injection of the programme (Roy and Twagiramungu, 1999; DeJarnette *et al.*, 2001). Moreover, incomplete luteal regression after PGF_{2α} injection on Day 7 has also been shown to affect the conception rate (Burke *et al.*, 1996). These two shortcomings of GPG can be corrected in lactating dairy cows by the inclusion of a progesterone releasing device between Days 0 and 7 (Kim *et al.*, 2003). The addition of this intravaginal progesterone device inhibits the premature oestrus (Roy and Twagiramungu, 1999; DeJarnette *et al.*, 2001).

Effectiveness of oestrus synchronisation in dairy cattle

Anoestrous and cycling cows

The effectiveness of double injections of PGF_{2α} in lactating cows to synchronise oestrus has been the subject of debate. Firstly, the response depends upon stage of the follicular wave at the onset of treatment. Macmillan (1978) showed that the interval to oestrus was influenced by the stage of the cycle: 89% of the oestrous responses in cows treated on Days 6 to 9 occurred within 72 h of injection, whereas for cows treated on Days 10 to 13 and Days 14 to 17 the figures were 48% and 70%, respectively. Macmillan (1978) concluded that the most effective use of PGF_{2α} for oestrus synchronisation in lactating dairy cows should involve treatment of animal groups at similar stages of their oestrous cycles and that insemination should be done at observed oestrus. A poor response to two injections of PGF_{2α} 14 days apart in lactating dairy cows was reported by Stevenson *et al.* (1999), with only 55% of cows detected in oestrus, although the conception rate in the cows that were inseminated was adequate (52%; Stevenson *et al.* 1999). Use of a GPG protocol in anoestrous pasture-based dairy cows resulted in similar conception rates to those of cows treated with progesterone and oestradiol benzoate and inseminated to detected oestrus (McDougall *et al.*, 2001). Cartmill *et al.* (2001) suggested that the GPG protocol may be of benefit in treating anoestrous cows in locations where detection of oestrus is a problem.

Several later field studies have shown that the addition of progesterone to a GPG-based synchronisation programme in both cycling and anoestrous dairy cows significantly improved conception rates. For example, an increase in conception rate of 10 to 20% was shown in several North American studies (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b). However, not all studies have shown such an effect: Stevenson *et al.* (2006) reported no improvement in 56 day conception rates in either cycling (GPG = 39%, GPG+ P4 = 43%) or noncycling (GPG = 39%, GPG + P4 = 34%) lactating dairy cows.

Under New Zealand conditions, the addition of progesterone to a GPG programme does seem to improve conception rates. McDougall (2010a) reported that a GPG+P4 programme resulted in a higher conception rate in anoestrous dairy cows compared with GPG alone (45.7 *versus* 33.9%). This increase in conception rate may be due to the prevention of premature oestrus (5–11.8%) between the first GnRH and PGF_{2a} injections (Roy and Twagiramungu, 1999; DeJarnette *et al.*, 2001), leading to a more synchronised ovulation.

Maiden heifers

Although the conception rates that follow GPG programmes in lactating cycling dairy cows are acceptable (35–40%) for such animals (Pursley *et al.*, 1997; Moreira *et al.*, 2001; Cerri *et al.*, 2004), conception rates of <40% are not acceptable for nulliparous dairy heifers; particularly since conception rates after insemination to detected oestrus are >60% in such heifers (Schmitt *et al.*, 1996b; Pursley *et al.*, 1997; Tenhagen *et al.*, 2005).

It has been suggested that this poor response occurs because heifers' follicles are responsive to GnRH for a relatively short period of time (Haughian and Wiltbank, 2002; Colazo and Ambrose, 2011). This means that the first GnRH injection fails to stimulate follicle growth in many heifers and that, as a result, too few heifers ovulate and start a new follicular wave (Haughian and Wiltbank, 2002). As a consequence, a significant proportion of heifers have no CL present on the day of PGF_{2a} injection. These heifers can undergo premature oestrus around the time of PGF_{2a} administration rather than after the second GnRH injection (DeJarnette *et al.*, 2001); this can be prevented by progesterone administration between the Day 0 GnRH and the PGF_{2a} injection (seven days) (Peeler *et al.*, 2004; Ambrose *et al.*, 2005; Cavalieri *et al.*, 2007; McDougall *et al.*, 2013).

McDougall *et al.* (2013) compared the efficacy of three programmes in heifers: (i) double PGF_{2a} (two injections of PGF_{2a} 11 days apart, with AI following detection of oestrus within four days of the second PGF_{2a}; n = 380); (ii) GPG+P4 (two treatments of GnRH (given nine days apart) with a PGF_{2a} injection on Day 7, combined with intravaginal progesterone from Days 0 to 7, with FTAI 16–20 h after the Day 9 GnRH injection; n = 383); and (iii) Cosynch (the same sequence of treatments as for GPG+P4 but with the FTAI coincident with the final GnRH; n = 374). They found that Cosynch was the best of the three programmes as it was associated with the highest first service conception rate (GPG+P4-Cosynch: 57%, double PGF_{2a}: 48%, GPG+P4: 47%) and highest 21 day pregnancy rate (GPG+P4-Cosynch: 76%, double PGF_{2a}: 63%, GPG+P4: 72%). However, this study did not show whether the incorporation of a progesterone device into a GPG+P4 programme could have improved the conception rate.

Summary of meta-analyses of the response to oestrus synchronisation in post-partum cows

Rabiee (2004) used a Bayesian meta-analysis to examine 71 GPG treatment (and variants thereof) and control comparisons from 53 field studies. They concluded that the GPG programme was beneficial in lactating cows principally because it allowed for FTAI and thereby obviated the need for oestrus detection: i.e. it was most useful in herds with poor oestrus detection. Rabiee (2004) also concluded that in lactating cows there was no difference in conception and pregnancy outcomes of GPG programmes compared to several other programmes such as prostaglandin, Selectsynch, and modified GPG (including Presynchronisation and Cosynch). This suggests that GPG programmes are not necessarily optimal on a cost-benefit basis.

Rabiee *et al.* (2004) used a similar approach for the meta-analysis of 25 synchrony trials to study the effects of adding a progesterone device to synchronisation programmes on the reproductive performance of cycling and anoestrous cows. Although, in cycling dairy cows, there was an improvement in submission rates compared to untreated controls, there was no effect on the risk of conception or on the subsequent risk of pregnancy. However, addition of progesterone advanced the date of first oestrus and, thereby, shortened the calving to conception interval. In anoestrous cows, there were insufficient trials to make definitive conclusions, but Rabiee *et al.* (2004) found that the use of progesterone-based programmes did not reduce the risk of conception after first service, although it also did increase the risk of pregnancy during the breeding season. This finding is consistent with the work of McDougall (2010) who reported that although treatment of anoestrous cattle with a GPG+P4 programme reduced calving to conception interval it did not improve six-week in-calf rate or reduce the not-in-calf rate at the end of the breeding season.

Variants of GPG programme

After the introduction of the basic GPG protocol, several methods such as Cosynch (Day 0: GnRH, Day 7: PGF_{2α}, Day 9: GnRH and FTAI; Geary and Whittier 1998), Heatsynch (Day 0: GnRH, Day 7: PGF_{2α}, Day 8: oestradiol cypionate, Day 10: FTAI; Pancarci *et al.* 2002), Selectsynch (Day 0: GnRH, Day 7:PGF_{2α}, heat detection and AI, and Day 10: GnRH and FTAI for nonresponders; Geary *et al.* 2000; DeJarnette, ND) and their modifications also have been developed (Figure 5.2). These FTAI protocols have been widely used all over the world, mainly in dairy and beef cows, due to the management advantages such as elimination of the need for oestrus detection.

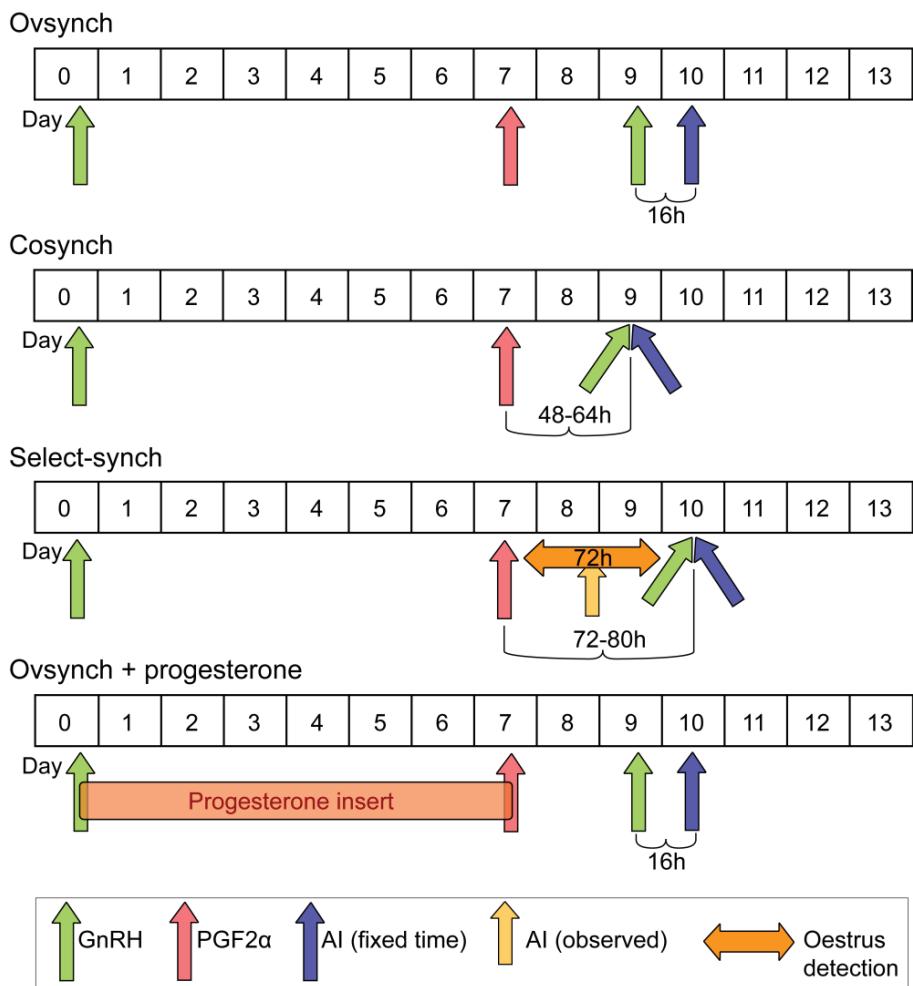


Figure 5.2 Variants of the GPG (Ovsynch) programme without presynchronisation. Based on DeJarnette (ND) and McDougall (2010).

Further modifications of these programmes include the use of presynchronisation of the oestrous cycle before the commencement of the GPG treatment (Figure 6.2) (Moreira *et al.*, 2001). Such oestrus presynchronisation was mainly based on reports of Vasconcelos *et al.* (1999) that starting a GPG programme during early to mid dioestrus (between Days 5 and 12 of the oestrous cycle) improves pregnancy rates. In cyclic cows this goal can be achieved by two injections of PGF_{2α}, 14 days apart and 11–14 days before starting the GPG treatment (El-Zarkouny *et al.*, 2004).

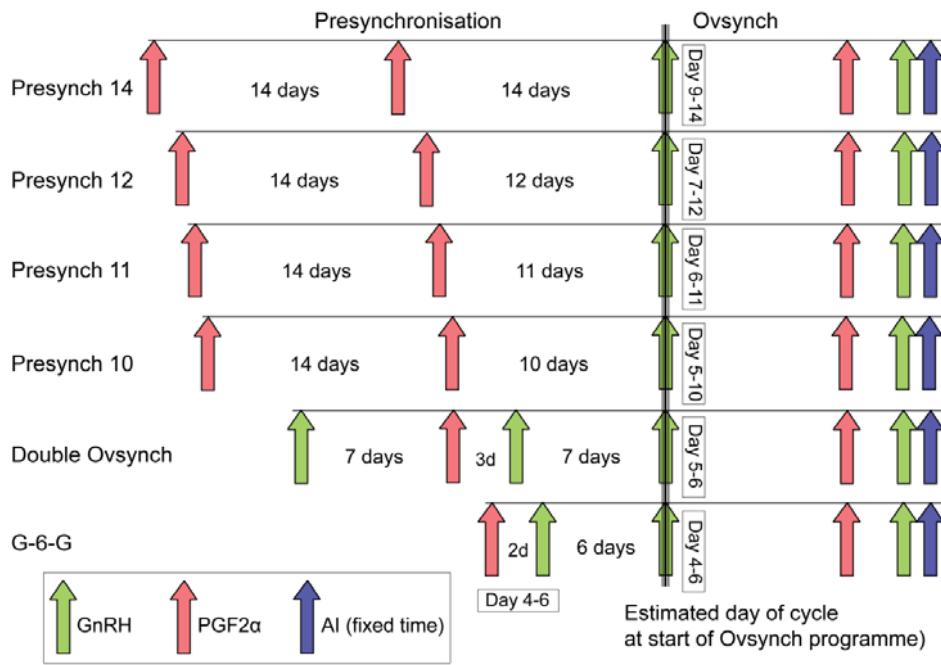


Figure 6.2 Variants of the GPG (Ovsynch) programme which were aimed at synchronising the follicular wave before commencing the GPG treatment. Based on Stevenson (2011; 2014).

Conclusions

Even though it is a long time since oestrus synchronisation was first attempted in cattle, there is still no definitive programme by which oestrus and ovulation can be reliably and effectively synchronised under all circumstances to a sufficient degree to allow for insemination to detected oestrus, FTAI. There are several issues and hurdles in developing programmes which are equally effective under different dairy production systems for cattle of different parities and with varying levels of milk production. Oestrus synchronisation is a management tool for reducing the costs associated with subfertility, so the cost of treatment is one of the key factors for choosing a suitable programme. New Zealand dairy cows are different from North American dairy cows, particularly with regard to their reproductive function and milk production; that is New Zealand cows generally have better reproductive function, although this is achieved in the face of markedly lower milk production. Therefore, there needs to be thoughtful selection of proper synchronisation programme for use in New Zealand cattle, in which the costs of treatment is balanced against the relatively high fertility of the cows and the relatively low price for milk. The most important biological effect of any oestrus synchronisation programme is its ability to synchronise the ovulation and/or luteolysis, followed by favourable postovulatory hormonal changes in the circulation. Although the efficacy of a GPG programme has been demonstrated through many studies, meta-analyses have shown its overall efficacy to be no

better than the simple and very cheap double PGF_{2α} programme (Rabiee *et al.*, 2004). Similarly, the way in which the expensive progesterone-releasing device in a GPG programme improves the response of anoestrus dairy cows is not very clear. The role of the first GnRH injection in such programmes is particularly unclear in nulliparous heifers and post-partum anoestrous cattle as such cattle are less likely to respond to that injection.

Overall conclusions

The dairy industry plays a pivotal role in the economy of New Zealand. Therefore, proper reproductive management of the dairy cow is essential for optimising the profit produced by this industry. Although dairying in New Zealand is generally based on low cost production systems, it has several constraints, which include the pressure to maintain a 365 day inter-calving interval and to maintain an industry target of a maximal six-week in-calf rate. One of the key problems preventing farms from meeting their reproductive targets is the relatively high percentage of cows that are not diagnosed in oestrus by the planned start of the breeding season. Such cows need to be treated as soon as possible with an effective programme if the profitability of the farm is to be maintained. Similarly, dairy heifers can benefit from oestrus synchronisation, as this will allow for the most efficient use of AI and optimal genetic gain.

The main consideration in selecting an oestrus synchronisation/anoestrus treatment programme is the balance between the benefits of earlier conception and the cost of the programme. In other words, whether the improvement in reproductive outcome justifies the difference between a costly or a cheaper treatment programme. Moreover, nulliparous dairy heifers have relatively better reproductive health than the pluriparous cows. Therefore, a more simple hormonal treatment may be equally effective in heifers to a GPG+P4 programme. In contrast, it is likely that the currently-recommended treatment option for the anoestrous cow, i.e. GPG+P4, is at the moment the optimal treatment programme, even though it is costly. Although a GPG+P4 programme has been evaluated only with regard to conception and pregnancy rates, the actual pharmacological action of the programme has not been evaluated by examining its effects on ovarian follicular and luteal dynamics, nor has it been evaluated in terms of the overall synchronisation of the induced ovulation. A better understanding of such dynamics could aid the design of equally effective but cheaper programmes. Additionally, the economics of synchronisation and the optimal programme could be affected by the energy status of the treated cows, as several indicators of negative energy balance including (but not limited to) NEFA, insulin, IGF-I and milk production can adversely affect the growth and development of follicle and luteal tissues. However, whether, or to what extent these measures of negative energy balance affect the responses to oestrus synchronisation in New Zealand dairy cows has not been studied.

CHAPTER 3

Response to the removal of either Day 0 GnRH or the progesterone device from a GPG+Progesterone programme and the effect of post-partum energy balance on development of the Day 7 dominant follicle and corpus luteum in dairy cows that have not been observed in oestrus

Abstract

The effect of energy status (as measured using non-esterified fatty acid [NEFA], insulin and insulin-like growth factor-I [IGF-I] concentrations) on the development of the Day 7 dominant follicle and corpus luteum in response to gonadotrophin and prostaglandin-based treatments was studied in postpartum dairy cows. Friesian and Friesian x Jersey cows ($n = 81$), which had not been observed in oestrus were first blocked on corpus luteum (CL) status and then randomly allocated to one of three oestrus synchronisation programmes: GPG+P4 ($n = 29$; 100 µg GnRH on Day 0, a progesterone releasing intravaginal device from Day 0 to Day 7, 500 µg PGF_{2α} on Day 7 and 100 µg GnRH on Day 9, and fixed-time AI 16–20 h later); GPG ($n = 28$; as Group 1 with the exclusion of the progesterone device); and P+G+P4 ($n = 24$; as Group 1, but excluding the GnRH treatment on Day 0). Transrectal ultrasonography was performed on Days 0 and 7 to measure dominant follicle and CL size. Plasma concentrations of insulin, IGF-I and NEFA were measured on Days 0 and 7.

Mean dominant follicle diameter on Day 0 did not differ between treatment groups irrespective of CL status on Day 0. Mean diameters of dominant follicles on Day 7 were not affected by Day 0 CL status or treatment ($P > 0.05$). When data for CL positive and negative cows were combined, the proportion of cows with a CL on Day 7 was significantly different between treatment groups (GPG: 78% [22/28], GPG+P4: 69% [20/29], P+G+P4: 42% [10/24]; $P = 0.02$). There was no effect of treatment on the volume of the CL on Day 7 (CL negative: [GPG: $3366 \pm 550 \text{ mm}^3$, GPG+P4: $2927 \pm 388 \text{ mm}^3$ and P+G+P4: $1674 \pm 486 \text{ mm}^3$; $P = 0.23$]; CL positive: [GPG: $2400 \pm 370 \text{ mm}^3$, GPG+P4: $4325 \pm 833 \text{ mm}^3$ and P+G+P4: $3464 \pm 1003 \text{ mm}^3$; $P = 0.11$]). When energy status data were included alongside other measures the volume of the CL on Day 7 was significantly associated with treatment, Day 0 dominant follicle diameter, treatment by Day 0 dominant follicle diameter, treatment by time post-partum, Day 0 insulin concentrations, and Day 7 NEFA concentrations.

In cows without a CL present on Day 0 of the oestrus synchronisation programme, removal of the Day 0 GnRH treatment led to reduced CL development; however no effect of adding progesterone was found. In contrast, in cows with a CL present on Day 0 of the oestrus synchronisation programme inclusion of a progesterone device led to a higher CL volume, but removal of the first GnRH injection had no effect.

(Key words: postpartum anoestrus, NEFA, insulin, IGF-I, GPG).

Introduction

In the pasture-based, seasonal calving dairy systems of New Zealand, failure of cows to return to oestrus by the start of the breeding season is a significant limiter of herd fertility (Rhodes *et al.*, 2003), especially as this problem typically affects around 20% of the cattle in a herd. Such animals may be in anovulatory anoestrus, or may have ovulated without being detected in oestrus (McDougall, 2010b). The main financial impact is via increased intervals between calving and conception and lower pregnancy rates, resulting in shorter lactations and increasing the risk of culling (Xu and Burton, 2000; McDougall, 2001).

Hormonal intervention (oestrus synchronisation) is the main treatment option for such cows. The aim of this treatment is to induce ovulation by stimulating maturation and ovulation of ovarian follicles, including by directly or indirectly causing preovulatory LH surges (Rhodes *et al.*, 2003). Before 2007, this was most commonly achieved using programmes based upon oestradiol benzoate/progesterone (Lucy *et al.*, 2004; McDougall, 2010a). However, in 2007 oestradiol treatments for dairy cows were banned for use in animals providing food products to the European Union (European Union, 2003), with a consequent prohibition of their use in New Zealand. Treatments were revised, with gonadotrophin releasing hormone (GnRH) being substituted for oestradiol, and fixed time AI (FTAI) replacing insemination at observed oestrus (McDougall, 2010a). The current recommended protocol for treatment of anoestrous dairy cows is two administrations of GnRH 9 days apart, with a progesterone releasing intravaginal device from Day 0 to Day 7, and a PGF_{2α} injection on Day 7, followed by FTAI, 16-20 h after the second GnRH injection (McDougall, 2010a).

Field studies have generally shown that the addition of progesterone to a GnRH-based synchronisation programme significantly improves conception rates in both cycling and anoestrous dairy cows. For example, increases of pregnancy rate by 10 to 20% have been shown in several North American studies (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b). However, not all studies have shown such an effect: For example, Stevenson et

al (2006) showed no improvement in 56 day conception rate in either cycling (GPG = 39%, GPG+P4 = 43%) or noncycling (GPG = 39%, GPG+P4 = 34%) lactating dairy cows. There has been only one such peer-reviewed study undertaken in New Zealand (McDougall, 2010a). That study also reported increased conception rates in the GPG+P4 programme as compared to GPG alone (45.7 ± 2.6 days *versus* 33.9 ± 2.4 days).

The mechanism by which the addition of progesterone improves the conception rate is not clear. It may be due to inhibition of the premature oestrus that occurs in 5–12% of cows between the first GnRH and the PGF_{2α} injections (Roy and Twagiramungu, 1999; DeJarnette *et al.*, 2001), or to modification of the negative feedback control of FSH and LH release which may lead to more synchronous follicular development and synchronised ovulation after the second GnRH injection (Nett, 1987; Sirois and Fortune, 1988). It may also be due to the maintenance of a higher progesterone concentration during the late luteal phase after FTAI since higher progesterone concentrations during this time are associated with improved conception rates (Folman *et al.*, 1973; Erb *et al.*, 1976; Rosenberg *et al.*, 1990) largely due to progesterone-induced favourable changes in the uterine environment (Clemente *et al.*, 2009) and, hence, increased embryonic growth rate (Stronge *et al.*, 2005; McNeill *et al.*, 2006). Moreover, cows with incomplete luteal regression 48 h after PGF_{2α} injection are also at risk of conception failure, so such cows will have a decreased conception rate to FTAI after GPG programmes (Burke *et al.*, 1996; Peters *et al.*, 1999b; Moreira *et al.*, 2000b; Kim *et al.*, 2003). Therefore, improvements in conception rates due to the addition of a progesterone device may be due in part to the development of a CL that is susceptible to complete luteolysis by the PGF_{2α} injection given on Day 7. Consequently complete luteolysis of a responsive CL may later lead to a more synchronised ovulation and elevated circulating progesterone concentrations during the post-insemination period, resulting in an increased pregnancy rate. A positive correlation between serum progesterone concentration before AI and conception rate (Fonseca *et al.*, 1983) and decreased conception rates in dairy cows without a CL at the end of progesterone treatment as compared to those having a CL (Smith and Stevenson, 1995) shows that the effectiveness of a programme to synchronise ovulation might be determined by the activity of the CL on Day 7 of the treatment. Moreover, the dominant follicle size on Day 7 is likely to be an important factor affecting the ovulation synchronisation after Day 9 GnRH treatment. Therefore, Day 7 follicular and luteal size could potentially be the important estimates of the efficacy of the synchronisation of ovulation.

Even though the effects of progesterone devices upon follicular and luteal development during oestrus synchronisation are not clear, their use seems to improve the reproductive outcomes of anoestrous cows, albeit also significantly adding to the cost of treatment. On the other hand, the

benefits of using GnRH at the start of such programmes in anoestrous cattle has not been unequivocally demonstrated. The aim of GnRH treatment on Day 0 is to stimulate the ovulation of large dominant follicles. However, in cyclic dairy cattle, a lower response rate (64%) to the first GnRH may result in reduced pregnancy rates following FTAI due to asynchronous ovulation (Vasconcelos *et al.*, 1999). In anoestrous cattle, a high proportion of treated cattle may have dominant follicles that are non-responsive to GnRH. If this is the case then removal of the first GnRH could save cost without affecting follicular dynamics and conception rates.

Nonetheless, many of the foregoing studies of both GPG and GPG+P4 programmes for oestrus induction and synchronisation highlight Day 7 of the programme as a critical stage of this treatment procedure. At that time, the ovary should not only have a dominant follicle from the induced new follicular wave but should also have a well-developed CL which is responsive to PGF_{2a} injection. Thus, the early stages of the treatment protocol (i.e. either the placement and removal of the progesterone device or the first GnRH treatment) may exert their effects upon reproductive outcome depending on the extent to which they affect Day 7 follicular and luteal development. However, there is no information in the literature directly comparing Day 7 follicle and luteal development in response to oestrus synchronisation treatment in anovulatory anoestrous cows.

Whether the response to oestrus induction and synchronisation regimens is moderated by the nutritional status of the cows is also not well understood – particularly for pasture-fed cows. It has been established that high postpartum concentrations of non-esterified fatty acid (NEFA), low insulin and low insulin-like growth factor-I (IGF-I) are indicative of negative energy balance and negatively affect the ovarian activity of lactating dairy cows (Beam and Butler, 1997; Butler *et al.*, 2004; Leroy *et al.*, 2005; Vanholder *et al.*, 2006; Kawashima *et al.*, 2007; Ortega *et al.*, 2008). In the seasonal calving system of New Zealand, the planned start of mating coincides with peak lactation, when many cows are still in negative energy balance (Kay *et al.*, 2009). Therefore, it is likely that negative energy balance, which is demonstrated by the time after parturition and varying concentration of insulin, IGF-I, NEFA, may affect the dominant follicle and CL development and thereby affect the synchronisation of ovulation.

This study was therefore undertaken to assess, firstly, the effects of a GPG+P4 programme in comparison to programmes with either (i) no administration of GnRH on Day 0 or (ii) no progesterone device, on Day 7 CL and dominant follicle size. Secondly, the study assessed the effects of energy balance, as determined by concentrations of insulin, IGF-I and NEFA, upon the ovarian responses to each of these programmes.

Materials and methods

All animal use was approved by Massey University Animal Ethics Committee, Palmerston North.

Animals

This study was conducted at No. 4 Dairy Farm, a spring-calving, pasture-based, 460-cow dairy farm in Palmerston North (latitude 40°2'S, longitude 175°4'E), New Zealand, during September to November (spring) 2010. Oestrus detection had been undertaken by farm staff using a combination of tail paint and observation from 40 days prior to the planned start of mating. Nine days before the planned start of the breeding season, all lactating cows ($n = 81$) which had been calved for >30 days, had not had an assisted calving or a diagnosis of endometritis and which had not been recorded as being observed in oestrus were enrolled in the study. The reproductive tracts of all animals were examined by transrectal ultrasonography to confirm the absence of utero-ovarian pathology and to determine the presence or absence of CL. Body condition score (BCS, Scale 1 to 10; Macdonald and Roche, 2004) of each animal was also recorded at the time of enrolment.

Cows were first separated into two groups based on the CL status at the time of enrolment (CL negative or positive). Thereafter, using a random allocation sheet (created using Excel 2007 [Microsoft, USA]), the cows in each group were allocated to one of the three treatment regimens (Figure 1.3) based on their order through the race on Day 0. Treatments were: (1) 100 µg i/m of GnRH (Ovurelin, Bomac Laboratories Ltd, Auckland, New Zealand) on Day 0 (morning), a progesterone-releasing intravaginal device (1.56 g progesterone, Cue-Mate, Bomac Laboratories Ltd) from Day 0 to Day 7, 500 µg cloprostenol i/m, (Ovuprost, Bomac Laboratories Ltd) on Day 7 (late afternoon) and a second dose of GnRH on Day 9 (late afternoon), followed by FTAI on Day 10 (16–20 h after second GnRH treatment) (GPG+P4; $n = 29$); (2) As for GPG+P4, with the exclusion of a progesterone-releasing intravaginal device (GPG; $n = 28$); and (3) as for GPG+P4, but omitting the GnRH treatment on Day 0 (P+G+P4; $n = 24$).

Ultrasonography

Ovarian structures of all cows were monitored and studied as previously described (Pierson and Ginther, 1984) using a real time B-mode ultrasound scanner (DP-6600 Vet, Mindray, Szechuan, China), equipped with a variable linear transducer set at 7.5 MHz. On Day 0, dominant follicle size, and CL status and size was established in all cows. In both CL negative and CL positive groups, ultrasonography was performed on Day 0 and 7 to measure dominant follicle and CL

size. Digital ultrasound images of both ovaries were recorded on each occasion and a corresponding ovarian map was also drawn manually on the recording sheet to locate and identify the dominant follicle and CL.

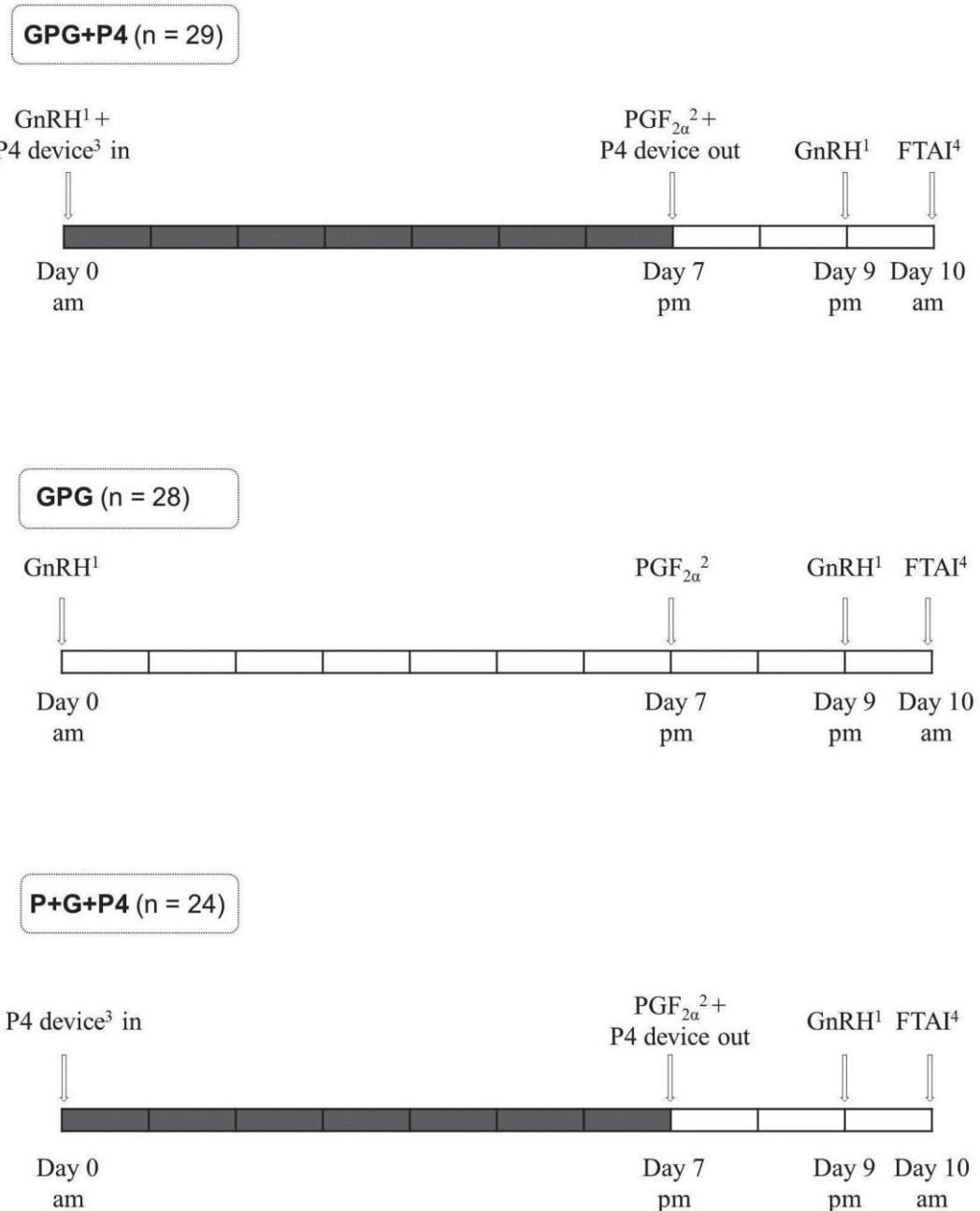


Figure 1.3 Synchronisation protocol for three groups (grey shading indicates progesterone releasing intravaginal device).

¹ 100 µg GnRH, i/m.

² 500 µg PGF_{2α}, i/m.

³ Progesterone releasing intravaginal device containing 1.56 g of progesterone.

⁴ Fixed-time artificial insemination.

The impact of treatment on follicle and CL size was evaluated by measuring the size of the largest dominant follicle and CL using ImageJ (v1.46d, National Institutes of Health, USA). The diameter of the dominant follicle was estimated by taking the average of two measurements: size at the widest point and size at right angles to the first measurement. The volume of the CL was calculated by using the formula $\frac{4}{3}\pi r^3$, in which r was the radius of the CL. If the CL had a fluid filled cavity, the volume of cavity was calculated in a similar way and was subtracted from the total volume of the CL (Figure 2.3). Response to first GnRH injection, as shown by the development of a CL (induction rate), was defined as the proportion of animals with a CL on Day 7 regardless of their initial CL status.

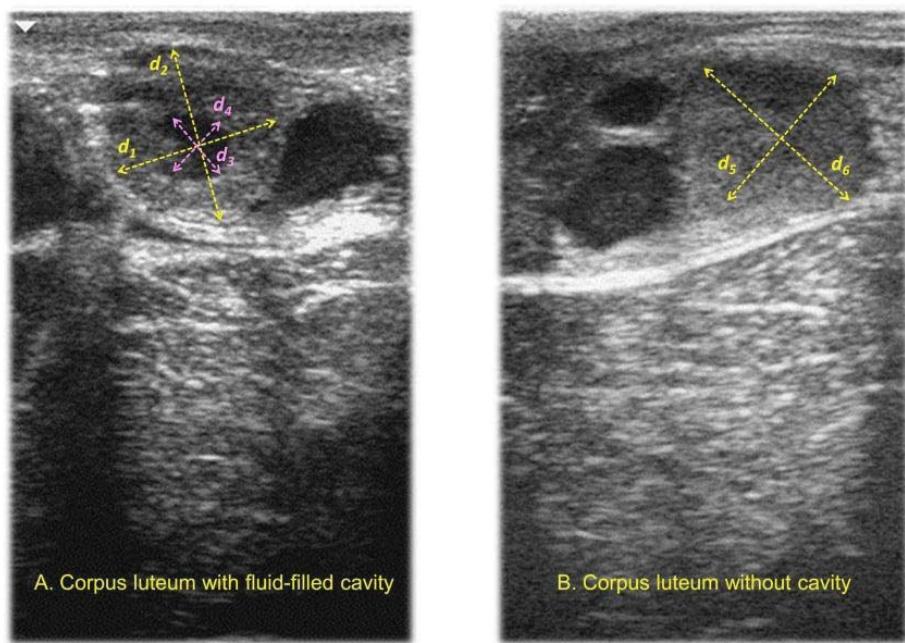


Figure 2.3 Measurement of volume of corpus luteum (CL) with fluid filled cavity (A) $CL\ volume = \left[\frac{4}{3}\pi \left(\frac{d_1+d_2}{2} \right)^3 \right] - \left[\frac{4}{3}\pi \left(\frac{d_3+d_4}{2} \right)^3 \right]$ and without cavity (B) $CL\ volume = \frac{4}{3}\pi \left(\frac{d_5+d_6}{2} \right)^3$; where d_1 to d_6 are the diameters of CL measured in millimetres.

Blood samples and hormone assay

Blood samples (10 mL) were collected via coccygeal venepuncture into heparinised vacutainers (Becton Dickinson New Jersey, USA) on Days 0 and 7 for the estimation of plasma insulin, IGF-I and NEFA concentrations. Plasma was separated by centrifugation at 1500 g for 20 min at 40°C within 2 h of collection. The plasma duplicates were taken and then stored at -20°C until assay.

Insulin assay

Plasma insulin concentrations were measured in duplicate 20 µL aliquots by radioimmunoassay, using an INSIK-5 kit (DiaSorin Inc., USA). The sensitivity of the assay was 4.4 µU insulin /mL. The intra-assay coefficients of variation at 80 and 50% binding on the standard curve were 11.2% and 15.5%, respectively; the inter-assay coefficients of variation were 12.9 % and 11.3% for low and high solutions, respectively. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer (see Appendix 3 for a description of the method).

Analysis of IGF-I

Concentrations of IGF-I were measured in duplicate 20 µL aliquots by ELISA, using the DSL-10-2800 ACTIVE IGF-I ELISA (Diagnostic Systems Laboratories, USA). The limit of sensitivity of the assay was 1.5 ng/mL. Control solutions of IGF-I were used as low and high quality controls in every plate. The mean concentrations of IGF-I in these solutions were 188.9 ± 8.7 and 353.9 ± 10.8 ng/mL, respectively. The intra-assay coefficients of variation for low (~200 ng/mL) and high (~350 ng/mL) control solutions were 8.1% and 2.5%, respectively whilst the inter-assay coefficients of variation were 6.5% and 4.3%. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer (see Appendix 4).

Non-esterified fatty acid (NEFA)

Plasma samples were sent immediately after collection to New Zealand Veterinary Pathology (Palmerston North) for the analysis of NEFA concentrations. These were measured in duplicate 50 µL aliquots using the commercial Wako NEFA C test kit (Wako Chemicals GmbH, Neuss, Germany). Optical density was read using the Hitachi Modular P800 analyser (Roche Diagnostics, Mannheim, Germany). The sensitivity of the method expressed as absorptivity (using Hitachi 556 spectrometer at 550 nm) was $52 \text{ I/mEq}^* \text{cm}$. Within-run coefficient of variation (CV) of three repeated assays ($n = 20$) was 2.7% or less.

Statistical analyses

There were two key outcome variables: Day 7 dominant follicle size and CL volume. Corpus luteum volume was included as the outcome variable instead of CL diameter due to the assumption that some corpora lutea contain a fluid filled cavity and therefore volume calculation might give a more accurate estimate of the CL size as the inclusion of the vacuole is excluded in this calculation (Figure 2.3). Normality test for Day 7 CL volume and dominant follicle size was done by D'Agostino-Pearson omnibus test ($\alpha = 0.05$). The predictor variables

evaluated were treatment group, days postpartum, diameter of Day 0 dominant follicle, presence or absence of CL on Day 0 and indicators of energy balance (NEFA, insulin, IGF-I concentrations on Day 0 and 7). Significant outliers were identified using Grubb's test ($P \leq 0.05$) and removed if not biologically plausible.

The balance of the treatment group for age and BCS was compared by one-way ANOVA to ensure that the groups were balanced for these factors which are known to have a key influence on fertility (McDougall, 2001; Roche *et al.*, 2007).

Differences in the dominant follicle and CL diameter and CL volume from Day 0 to 7 in both CL groups were first subjected to separate one-way ANOVA with respect to treatment. Thereafter, data from both groups were combined and subjected to two-way ANOVA with respect to CL status and treatment using the Prism software (v 5.03, GraphPad Software Inc., La Jolla, USA).

The effects of treatment, Day 0 dominant follicle diameter, presence or absence of CL on Day 0, time postpartum, and NEFA, insulin and IGF-I concentrations on Day 0 and Day 7, on Day 7 dominant follicle size, and the CL volume were predicted by general regression models. The independent variables were first identified based on univariable models. Those variables with a P value of <0.2 were kept in the model, unless there was a significant correlation ($r > 0.7$) between the predictor variables when the variable with the smallest P value was kept in the model. All two-factor interactions were also explored and kept in the model if they were significant ($P \leq 0.05$). The model was checked by plotting the residuals of deviance against fitted value and if there was a random pattern of the residuals indicating no any concern with any of the observation, the model was finalised. Furthermore, the presence or absence of CL on Day 7 (binary outcome) was predicted by the binary logistic regression. Minitab (v16, Minitab Inc., State College, PA, USA) was used for fitting general and binary logistic regression model.

Results

The distributions of age, BCS and postpartum interval of the cows in the different groups are shown in Table 1.3. There was no difference between the groups in age ($P = 0.13$), BCS ($P = 0.24$) and interval from calving to study commencement ($P = 0.43$) which shows that the treatment groups were balanced for these factors. The mean BCS of cows used in this study was significantly less (3.4–3.7) than the industry target (Dairy NZ, 2007) of ≥ 4 at the time of mating.

Concentrations of NEFA, IGF-I and insulin are shown in Table 2.3. Two significant outliers from Day 0 NEFA concentrations, one from each the GPG+P4 (CL negative) and GPG (CL positive) groups (both 1.52 mMol/L), were removed from the data analysis as they were not biologically plausible. Concentrations of NEFA were higher on Day 0 in cows with a CL in the P+G+P4 group than in similar animals in the GPG + P4 group ($P = 0.04$). However, there were no such differences in NEFA concentrations on Day 7 between the groups ($P = 0.22$). No other significant differences of NEFA concentrations were present between groups. There was no difference in overall mean IGF-I concentrations between the treatment groups, either in animals that had a CL on Day 0 (Day 0: $P = 0.47$; Day 7: $P = 0.26$) or animals that did not (Day 0: $P = 0.68$; Day 7: $P = 0.83$). There were no differences in insulin concentrations between treatment groups, or between CL groups or Day 0 or 7 of the study ($P > 0.57$).

Table 1.3 Descriptive data of the cows enrolled in GPG, GPG+P4 and P+G+P4 treatment groups. Data are presented as mean (range).

Parameter	Treatment			P value
	GPG	GPG+P4	P+G+P4	
N (CL negative)	14	19	12	
N (CL positive)	14	10	12	
Age (months)	52.5 (25.7–111.4)	45.1 (25.2–98.9)	59.2 (26.5–159.2)	0.13
BCS (1-10)	3.4 (2.5–5.0)	3.5 (2.5–4.5)	3.7 (2.5–5.0)	0.24
Days postpartum	60.4 (37–97)	60.6 (37–101)	54.3 (30–98)	0.43

BCS: Body condition score (Scale 1–10, Macdonald and Roche, 2004).

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 2.3 Comparison of concentrations of NEFA, insulin and IGF-I concentrations in GPG, GPG+P4 and P+G+P4 groups on Day 0 and 7 separated by corpus luteum status (mean \pm SEM).

	Treatment									
	GPG					GPG+P4			P+G+P4	
	Days	NEFA	Insulin	IGF-I	NEFA	Insulin	IGF-I	NEFA	Insulin	IGF-I
<i>CL negative</i>	0	0.4 \pm 0.1	11.9 \pm 0.9	40.3 \pm 5.1	0.4 \pm 0.04	12.9 \pm 1.2	41.9 \pm 3.9	0.4 \pm 0.1	13.3 \pm 1.5	36.4 \pm 4.6
	7	0.4 \pm 0.04	13.0 \pm 1.2	47.2 \pm 4.5	0.4 \pm 0.1	12.8 \pm 1.2	50.4 \pm 4.0	0.3 \pm 0.03	11.7 \pm 0.7	21.2 \pm 5.3
<i>CL positive</i>	0	0.4 \pm 0.1	13.4 \pm 1.6	40.0 \pm 6.7	0.4 \pm 0.1	11.4 \pm 0.9	42.0 \pm 5.6	0.6 \pm 0.1	12.1 \pm 1.4	32.5 \pm 4.0
	7	0.5 \pm 0.1	11.0 \pm 0.7	52.3 \pm 7.4	0.4 \pm 0.1	10.4 \pm 0.4	51.0 \pm 4.3	0.6 \pm 0.1	11.5 \pm 1.1	40.0 \pm 4.3

Abbreviations: G, GnRH; P, PGF_{2*a*}; P4, progesterone.

NEFA: non-esterified fatty acids (mMol/L); insulin (μ U/mL); IGF-I: insulin-like growth factor-I (ng/mL).

CL negative cows

Day 0 dominant follicle size

The diameter of the dominant follicle on Day 0 did not differ between treatment groups (GPG: 13.2 ± 0.5 mm, GPG+P4: 13.6 ± 0.6 mm, P+G+P4: 13.6 ± 0.7 mm; $P = 0.87$).

Proportion of cows with CL on Day 7

The proportion of cows which had single or multiple luteal structures present on Day 7 were 64% (9/14), 63% (12/19) and 25% (3/12) for GPG, GPG+P4 and P+G+P4 groups respectively. The average number of CL on ovary on Day 7 was 0.9, 0.8 and 0.3 in GPG, GPG+P4 and P+G+P4 treated cows respectively. There were three and two cows in the GPG and GPG+P4 groups, respectively, with multiple CL on Day 7, whereas no cows in the P+G+P4 group had multiple CL.

Day 7 dominant follicle and CL size

Figure 3.3 shows the diameters of the dominant follicles in GPG, GPG+P4 and P+G+P4 groups on Day 7. The diameters of these dominant follicles did not differ between treatment groups (GPG: 13.7 ± 1.0 mm, GPG+P4: 14.1 ± 0.6 mm, P+G+P4: 15.4 ± 1.1 mm; $P = 0.47$).

There were no differences in CL diameter between groups of animals (GPG: 18.0 ± 1.2 mm, GPG+P4: 17.2 ± 0.9 mm, P+G+P4: 14.4 ± 1.6 mm; $P = 0.20$). Likewise, there were no differences in luteal volume (GPG: 3357 ± 549 mm³, GPG+P4: 2919 ± 387 mm³, P+G+P4: 1670 ± 485 mm³; $P = 0.23$) on Day 7 (Figure 4.3).

CL positive cows

Day 0 dominant follicle and CL size

The diameter of the dominant follicle on Day 0 did not differ between treatment groups (GPG: 13.6 ± 0.9 mm, GPG+P4: 13.8 ± 1.3 mm, P+G+P4: 13.9 ± 1.1 mm; $P = 0.97$). Similarly, there were no differences between treatment groups in either the diameter (GPG: 19.1 ± 1.5 mm, GPG+P4: 18.9 ± 1.5 mm, P+G+P4: 20.1 ± 1.7 mm; $P = 0.84$) or the volume (GPG: 4597 ± 1092 mm³, GPG+P4: 4130 ± 1013 mm³ and P+G+P4: 5397 ± 1201 mm³; $P = 0.78$) of the CL that were present on Day 0.

Proportion of cows with CL on Day 7

The proportion of cows with single or multiple luteal structures on Day 7 were 94% (13/14), 80% (8/10), and 58% (7/12) for GPG, GPG+P4 and P+G+P4 groups respectively. The average number of CL on ovary on Day 7 was 1.6, 1.2 and 0.6 in GPG, GPG+P4 and P+G+P4 treated

cows respectively. There were six and four cows in the GPG and GPG+P4 groups, respectively with multiple CL present on Day 7, whereas no cows in the P+G+P4 group had multiple CL.

Day 7 dominant follicle and CL size

Figure 3.3 shows the diameters of the dominant follicles in GPG, GPG+P4 and P+G+P4 groups on Day 7. The diameters of the dominant follicles did not differ between treatment groups (GPG: 13.4 ± 0.7 mm, GPG+P4: 13.4 ± 1.1 mm and P+G+P4: 15.7 ± 0.6 mm; $P = 0.08$).

There were no differences in CL diameter between groups (GPG: 16.5 ± 0.8 mm, GPG+P4: 17.8 ± 1.7 mm, P+G+P4: 19.4 ± 1.5 mm; $P = 0.26$). Likewise, there were no differences in luteal volume between animals (GPG: 2577 ± 345 mm³, GPG+P4: 4314 ± 830 mm³, P+G+P4: 3454 ± 1003 mm³; $P = 0.17$; Figure 4.3).

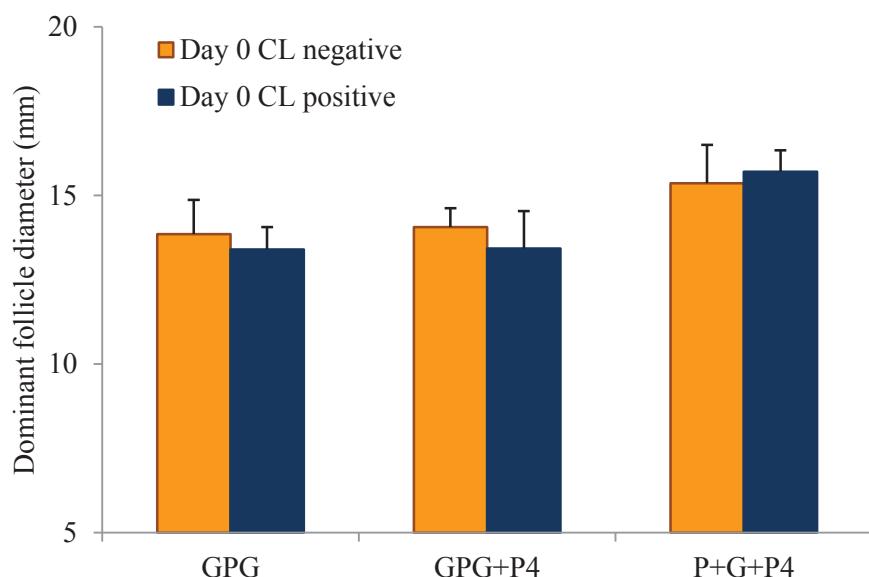


Figure 3.3 Mean (\pm SEM) diameters of dominant follicles present on Day 7 in GPG, GPG+P4 and P+G+P4 groups, separated by CL status on Day 0. There was no effect of treatment or Day 0 CL status on Day 7 dominant follicle diameter ($P > 0.05$). Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

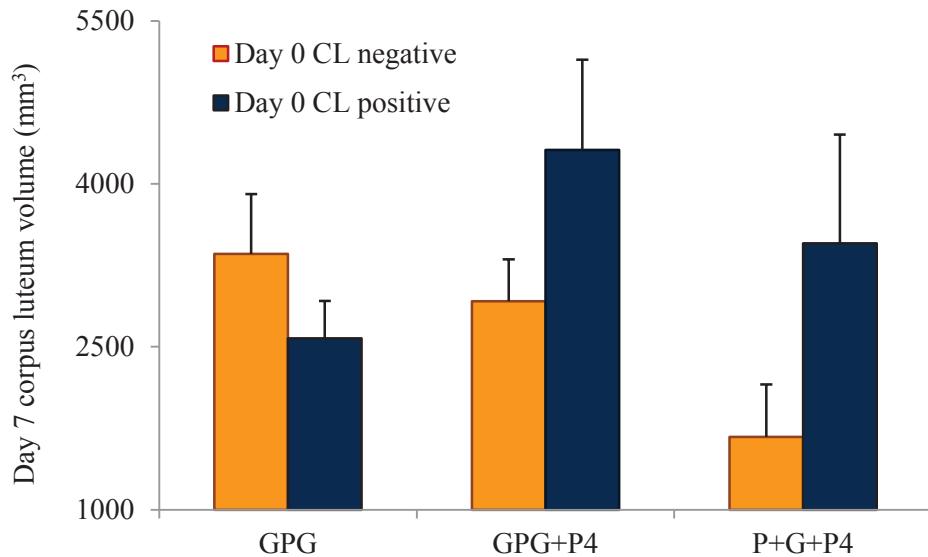


Figure 4.3 Mean (±SEM) volume of corpora lutea present on Day 7 in cows treated with GPG, GPG+P4 or P+G+P4 groups, separated by CL status on Day 0. There was no effect of treatment or Day 0 CL status on Day 7 CL volume ($P>0.05$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Combined data

Proportion of cows with CL on Day 7

The proportion of cows with single or multiple luteal structures in the combined CL positive and negative groups on Day 7 was 78% (22/28), 69%(20/29) and 42% (10/24) for GPG, GPG+P4 and P+G+P4 groups respectively ($P = 0.02$, Figure 5.3).

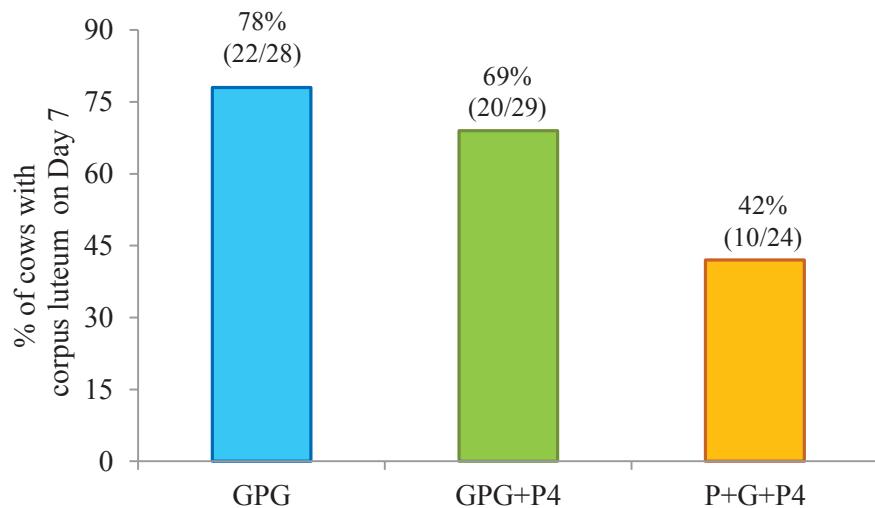


Figure 5.3 Comparison of percentage of cows in three treatments (CL negative and positive groups together) with observable corpus luteum on Day 7 ($P = 0.02$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Day 7 dominant follicle and CL size

There were no effects of treatment, Day 0 CL status or interaction between treatment and CL status, on Day 7 dominant follicle diameter ($P>0.05$ for all). Likewise, there were no effects of treatment, Day 0 CL status or interaction between treatment and CL status, on Day 7 CL diameter or volume ($P>0.05$ for all).

Prediction of Day 7 follicular and luteal status

Model 1

In Model 1, the effects of treatment, days postpartum, Day 0 dominant follicle diameter, presence or absence of CL on Day 0 and their interactions were used to predict CL volume, dominant follicle diameter and the presence or absence of a CL on Day 7. The predictors for the best-fitted model ($R^2 = 46.2\%$) for predicting Day 7 CL volume were treatment ($P = 0.06$), Day 0 dominant follicle diameter ($P = 0.05$), treatment by days postpartum ($P = 0.003$), treatment by Day 0 dominant follicle diameter ($P = 0.03$), treatment by Day 0 presence or absence of CL ($P = 0.03$) and days postpartum by Day 0 presence or absence of CL ($P = 0.04$). In contrast, no significant predictive relationship could be established for either Day 7 dominant follicle diameter ($P>0.05$) or the presence or absence of a CL on Day 7 ($P>0.05$).

Model 2

In Model 2, the effects of treatment, days postpartum and indicators of energy balance (NEFA, insulin and IGF-I concentrations on Day 0 and Day 7) and their interactions were used to predict CL volume, dominant follicle diameter and the presence or absence of a CL on Day 7. A significant correlation was seen between the predictors (Table 3.3). There was a significant negative correlation between days postpartum and NEFA concentrations on Days 0 and 7, whereas Day 0 and 7 IGF-I concentrations had significant positive correlations with days postpartum. Conversely, there were significant negative correlations between IGF-I (Days 0 and 7) and NEFA (Days 0 and 7) concentrations. In contrast, there were significant positive correlations between Day 0 and Day 7 NEFA and Day 0 and Day 7 IGF-I concentrations.

The significant predictors for the best-fitted model ($R^2 = 49.1\%$) for Day 7 CL volume were Day 0 insulin concentration ($P = 0.01$), treatment by days postpartum ($P = 0.01$) and treatment by Day 7 NEFA concentration ($P = 0.003$). However, treatment by Day 0 IGF-I concentration was not significant in the model ($P = 0.18$). Additionally, Day 7 dominant follicle diameter was not related to any of the predictors in the model ($P>0.05$). Similarly, none of the predictors were significantly related to the presence or absence of a CL on Day 7 ($P>0.05$).

Table 3.3 Pearson correlations (P values in parentheses) between the predictors of Model 2 for Day 7 corpus luteum volume.

	<i>Days postpartum</i>	<i>Day 0 NEFA</i>	<i>Day 7 NEFA</i>	<i>Day 0 insulin</i>	<i>Day 7 insulin</i>	<i>Day 0 IGF-I</i>
<i>Day 0 NEFA</i>		-0.58* (<0.001)				
<i>Day 7 NEFA</i>		-0.50* (<0.001)	0.56* (<0.001)			
<i>Day 0 insulin</i>		0.16 (0.19)	-0.21 (0.07)	0.06 (0.61)		
<i>Day 7 insulin</i>		0.05 (0.68)	-0.08 (0.53)	-0.01 (0.92)	0.19 (0.11)	
<i>Day 0 IGF-I</i>		0.47* (<0.001)	-0.36* (0.002)	-0.23* (0.05)	-0.06 (0.63)	-0.13 (0.28)
<i>Day 7 IGF-I</i>		0.43* (<0.001)	-0.29* (0.01)	-0.25* (0.03)	-0.08 (0.52)	0.85* (<0.001)

*Significant.

NEFA: Non-esterified fatty acids mMol/L.

Insulin: μ U/mL.

IGF-I: insulin-like growth factors-I in ng/mL.

Based on Model 2, a higher insulin concentration on Day 0 led to smaller Day 7 CL volume in all the treatment groups. More days postpartum was associated with larger CL volume in both the GPG+P4 and P+G+P4 groups whereas in the GPG group it was associated with smaller CL volume. Higher Day 7 NEFA concentration was associated with larger Day 7 CL volume in both the GPG and P+G+P4 groups whereas it was associated with a lower CL volume in the GPG+P4 group. In contrast, higher Day 0 IGF-I concentration was associated with larger CL volume in both the GPG and GPG+P4 groups whereas in P+G+P4 group it decreased the Day 7 CL volume.

PGP:

$$\text{Day 7 CL volume} = 4991.1 - 158.9 \text{ Day 0 insulin} - 42.1 \text{ Days postpartum} + 2457.8$$

$$\text{Day 7 NEFA} + 27.8 \text{ Day 0 IGF-I}$$

GPG+P4:

$$\text{Day 7 CL volume} = 4991.1 - 158.9 \text{ Day 0 insulin} + 19.7 \text{ Days postpartum} - 2500.3$$

$$\text{Day 7 NEFA} + 13.5 \text{ Day 0 IGF-I}$$

P+G+P4:

$$\text{Day 7 CL volume} = 4991.1 - 158.9 \text{ Day 0 insulin} + 22.4 \text{ Days postpartum} + 42.5$$

$$\text{Day 7 NEFA} - 41.3 \text{ Day 0 IGF-I}$$

Discussion

This study compared the ovarian responses of lactating dairy cows which were observed in oestrus to two variations on the core GPG+P4 oestrus synchronisation protocol, based upon the hypotheses that the exclusion of progesterone from the GPG+P4 programme would lower the synchronisation of Day 7 CL and dominant follicle development, whereas no any effects would be evident if the first GnRH injection is omitted. By comparing the responses to these three treatment regimens in cows that either had ovulated before the start of the study and had a CL present, or those in which no CL was present and which were, therefore, presumed not to have ovulated, the overall usefulness of these protocols for treatment of apparently anoestrous cows of unknown ovarian status could be inferred. Moreover, the study evaluated the interrelationship between treatment regimen and time postpartum, NEFA, insulin and IGF-I concentrations on ovarian activity on Day 7 of the protocol, the time which is arguably the key stage of the process in terms of follicular and luteal development. The study showed that Day 7 luteal volume was significantly affected by treatment, Day 0 dominant follicle diameter, Day 0 insulin concentration, treatment by days postpartum, treatment by Day 0 dominant follicle diameter and interaction of presence or absence of CL by the treatment and by days postpartum.

Effect of CL status at the start of treatment

The mean size of the dominant follicle on Day 0 did not differ between cows that had, or lacked, a CL at the start of treatment (range 13.2 ± 0.5 mm to 13.9 ± 1.1 mm). As this size range is in agreement with that previously reported (Herlihy *et al.*, 2012) in cyclic dairy cows (range of 12.5 to 14.7 mm), it can be inferred that all of the cows across the different treatment groups had follicles that were potentially responsive to GnRH injection (i.e. as follicles of ≥ 8.5 mm in diameter have the ability to ovulate after GnRH treatment). This finding is important in considering the response to the first GnRH of the treatment in the GPG and GPG+P4 programmes, inasmuch as, if the first GnRH were not effective, then the response to the GPG and GPG+P4 programme should not be different from the group that did not receive GnRH on Day 0 (P+G+P4). In fact, there were no differences between the GPG and GPG+P4 treatments in terms of dominant follicle and CL size, luteal volume and the proportion of cows with a CL on Day 7 irrespective of CL status on Day 0.

The overall range of diameters of dominant follicles on Day 7 (13.4 ± 1.1 mm to 15.7 ± 0.6 mm) was slightly greater than the 12.2 mm previously reported by Peters *et al.* (1999a) seven days after giving GnRH alone in cyclic cows. The size and maturity of the dominant follicle on Day 7 (i.e. at the time of PGF_{2α} treatment) has been shown to be an influencing factor in the synchronisation of oestrus and ovulation (Savio *et al.*, 1993b; Diskin *et al.*, 2002). On Day 7 of the treatment, dominant follicles were similarly sized between cows that had been CL negative or positive at the start of the trial (Table 2.3).

The diameters of CL present on Day 0 (GPG: 19.1 ± 1.5 mm, GPG+P4: 18.9 ± 1.5 mm, P+G+P4: 20.1 ± 1.7 mm) in the present study was in agreement with the previously reported range of 19.5 mm to 22.3 mm in cycling pasture-fed lactating dairy cows in Ireland (Herlihy *et al.*, 2012). The variation in the average numbers of CL on Day 7 in cows that had (GPG: 1.6, GPG+P4: 1.2, P+G+P4: 0.6) or lacked (GPG, 0.9; GPG+P4, 0.8; P+G+P4, 0.3) a CL on Day 0 indicates some degree of variation in response to treatment between CL positive and CL negative animals. The response in the CL positive cows is in agreement with the data of Peters *et al.* (1999b) who also reported an increase in the mean number of CL from 1.0 to 1.3 between Days 0 and Day 7 after GnRH administration to cyclic cows. Indeed, administration of GnRH on Day 5 of the oestrous cycle can lead to ovulation and formation of an accessory CL, which is indicative of an optimum response to the GnRH treatment. In cycling cows this induction rate has been varied from 50% (Wolfenson *et al.*, 1994) to $\geq 90\%$ (Pursley *et al.*, 1995; Schmitt *et al.*, 1996a). In the present study, relatively fewer cows that lacked a CL on Day 0 had a CL on Day 7 (GPG: 64%, 9/14; GPG+P4: 63%, 12/19; P+G+P4: 25%, 3/12), whereas the majority of CL positive cows did so (GPG: 94%, 13/14; GPG+P4: 80%, 8/10 and P+G+P4: 58%, 7/12). It

seems that the lower response rate in CL negative animals could be due to the fact that they were truly acyclic, or had asynchronous follicular growth and improper development of the gonadotrophin receptors in their follicles (Bao and Garverick, 1998; Webb *et al.*, 1999).

Thus the major conclusion from the comparison of response of cows with or without a CL at the start of treatment was that all responded equally well to the three different types of synchronisation programme. Moreover, luteal status did not affect CL size on Day 7 via differences in the size of the dominant follicle at the commencement of the treatment, since these were similar in CL positive and negative cows.

Comparison between treatment regimens

Across cows with a CL on Day 0, cows without a CL on Day 0, and all cows, no effect of treatment on follicle size was observed in any model. Based on a post-hoc power analysis for the individual groups, separated by CL status, the differences in dominant follicle size that could be detected were around 3–4 mm in size; such differences could be biologically important. However, as there was no significant effect of CL status on Day 0; it is the power of the combined data that is more relevant to this situation – post-hoc analysis for this group showed that a difference of 1.3 mm could have been detected with 80% power ($\alpha = 0.05$). So the finding that removal of either the first GnRH injection or the progesterone device had no impact on dominant follicle size on Day 7 is robust.

The situation in regard to CL volume is more complex. In CL negative cows, Day 7 CL volume was smaller in the P+G+P4 group compared with the other two groups (GPG: $3357 \pm 549 \text{ mm}^3$, GPG+P4: $2919 \pm 387 \text{ mm}^3$, P+G+P4: $1670 \pm 485 \text{ mm}^3$), but in CL positive cows, Day 7 CL volume was smaller in the GPG group than the other two treatment groups (GPG: $2577 \pm 345 \text{ mm}^3$, GPG+P4: $4314 \pm 830 \text{ mm}^3$, P+G+P4: $3454 \pm 1003 \text{ mm}^3$). These comparisons were not significant when analysed using the univariable ANOVA ($P>0.26$), but treatment alone was almost significant at the 5% level in model 1 ($P = 0.06$) and interaction between treatment was significant ($P=0.03$). The difference between the two groups suggest that when CL are present, GnRH may not be necessary to support those CL when progesterone is also supplied exogenously (in contrast to previous studies where GnRH was used and was shown to improve the duration of luteal activity: MacMillan *et al.*, 1985; Thatcher *et al.*, 1989; Peters *et al.*, 1999a). Indeed our data suggest that exogenous progesterone may directly support CL size. In contrast, when CL are absent, the lack of GnRH reduces the chance of CL being present on Day 7, and, perhaps more importantly, if present these may be younger, smaller CL which are less responsive to PGF_{2a}. Further data is required to confirm these suggestions, as at the univariable

level the study lacked power, across the combined data a difference of 764 mm³ in CL volume could have been detected with 80% power ($\alpha = 0.05$).

In addition, to mean CL volume, whether a CL is present on Day 7 may also be an important outcome. In the overall data set (with both CL positive and negative cows on Day 7), the absence of the first GnRH was associated with an increased risk of no CL on Day 7; most of this difference in risk of no CL was seen in the CL negative group, but the difference in this group was not significant as the study had insufficient power to detect such a difference (the study was designed to detect differences in the continuous variables CL volume and dominant follicle size rather than categorical variables such as presence or absence of CL). Further research with larger numbers of cows is required to confirm the suggestion that in CL negative cows, the absence of the first GnRH is likely to lead to an increased number of CL on Day 7 and to establish whether the absence of a CL on this day is significant in terms of reproductive outcomes.

Effects of energy balance

This analysis has shown that nutritional status also exerts an influence upon treatment response, as Model 2 showed significant effects of Day 0 insulin concentration, interaction of treatment and days postpartum and treatment by Day 7 NEFA concentration upon Day 7 CL volume.

Insulin and IGF-I are described as having direct effects on ovarian cells *in vitro*, including stimulation of proliferation of granulosa cells and progesterone production from bovine granulosa and luteal cells (Poretsky and Kalin, 1987; Spicer and Echternkamp, 1995). *In vivo*, their concentrations have been related to postpartum ovarian follicular development (Adashi *et al.*, 1985; Hammond *et al.*, 1988). In the present study, concentrations of insulin on Day 0 were unrelated to those of IGF-I (Table 2.3). Changes in insulin concentrations during the post-partum period are complex, as they reflect both short- and long-term responses to nutrition and energy status (Villa-Godoy *et al.*, 1990; Vandehaar *et al.*, 1995). Moreover, the direct role of circulating insulin upon luteal activity is not very clear. However, Judson *et al.* (1985) suggested that many of the previously recognised effects of very low doses of the insulin could be due to its interaction with type-I IGF-I receptors, and IGF-I has been shown to cause the same responses at much smaller doses as compared to insulin. This needs further research at the cellular level as to how insulin concentration affects the CL development and modulates the hormonal treatment. There was a significant interaction effect of treatment with Day 7 NEFA concentration. Previous *in vitro* studies showed that the elevated NEFA had a detrimental effect on follicle cell viability and function (Leroy *et al.*, 2005; Vanholder *et al.*, 2006) and retardation of luteal growth due to a decline in propagation of the granulosa cells caused by the elevated

NEFAs (Zulu *et al.*, 2002b; Jorritsma *et al.*, 2004). However in the present study the interaction effect of Day 7 NEFA and the treatment on Day 7 CL volume was not in the same direction, as a higher Day 7 NEFA concentration led to a larger Day 7 CL volume in both the GPG and P+G+P4 groups whereas it decreased the CL volume in the GPG+P4 group. This finding needs further investigation and could be attributed to small sample size.

The interval between calving and the onset of treatment had a significant interaction in both of the statistical models, which shows that time postpartum at which hormonal treatment begins may be of importance in considering the optimum response. The period of negative energy balance lasts for 4 to 14 weeks postpartum, and varies in depth such that anoestrus cows are typically in lower energy balance than cycling cows (McDougall *et al.*, 1993). Because negative energy balance can impair the activity of the reproductive axis during the postpartum period (Beam and Butler, 1997), hormonal treatments for anovulatory anoestrus may not work as well as in the later postpartum period when the energy balance shifts from negative to positive. It seems that the length of the postpartum period plays an important role in the effectiveness of hormonal treatment of the anoestrous cow. A negative effect of treating anoestrous cows within three weeks postpartum was reported in New Zealand dairy cows (Nation *et al.*, 1997), shown by poor conception and oestrous response following treatment. A potential cause was the interference of the hormonal treatment with the process of uterine involution. Nonetheless, the present finding suggests that the postpartum period significantly affected the treatment response but not always in the same direction e.g. a longer postpartum period was associated with a larger CL volume in both the GPG+P4 and P+G+P4 groups whereas in the GPG group it was associated with a smaller CL volume. This finding needs further clarification with a greater number of animals.

The mean BCS of cows used in this study was significantly less (3.4–3.7) than the industry target of ≥ 4 at the time of mating (Dairy NZ, 2007) and was in agreement with the median BCS (3.5) for the postpartum anoestrous dairy cows in New Zealand (McDougall, 2010a). Literature under New Zealand managemental conditions shows a negative impact of lower BCS on reproductive performance (McDougall, 1992; Roche *et al.*, 2007). It would be interesting for further study to see the effects of BCS on response to treatment or whether the similar results are repeatable in cows with a BCS of ≥ 4 at the time of mating.

Conclusions

Overall, in CL negative cows, Day 0 GnRH treatment of a GPG programme still seems to be essential; however no conclusion can be drawn for the treatment difference between the GPG and GPG+P4 programmes, whereas in CL positive cows, inclusion of a progesterone device led

to a higher CL volume. Postpartum duration and negative energy balance can significantly affect the treatment response to an oestrus synchronisation programme. Hormonal treatment of the postpartum anoestrus can be modulated by the plasma insulin and NEFA concentrations and days postpartum at the time of treatment. Further research involving frequent ultrasound to study the CL size and blood sampling to study the progesterone concentration, or comparing the treatment response between cows with artificially induced negative and positive energy balances would clarify the correlation of energy balance and the response to oestrus synchronisation.

Acknowledgements

This research was supported by a New Zealand International Doctoral Research Scholarship and a Massey University Doctoral Research Scholarship. The product provided by Bomac Laboratories is gratefully acknowledged. The contributions of the farm manager of Massey University No. 4 Dairy Farm and undergraduate veterinary students of Massey University are acknowledged.

CHAPTER 4

The effect of modifications to a GPG+Progesterone programme and negative energy balance on the response to oestrus synchronisation of anoestrous dairy cows kept at pasture

Abstract

The effects of a GPG (Day 0: 100 µg GnRH, Day 7: 500 µg PGF_{2α}, Day 9: 100 µg GnRH, fixed-time artificial insemination 16–20 h later; n = 21) programme upon follicular and luteal dynamics (principally, changes in size in relation to time and ovulation synchronisation), and patterns of oestradiol and progesterone secretion in postpartum anoestrous dairy cows were compared with (i) a GPG programme plus a progesterone insert from Days 0–7 (GPG+P4: progesterone-releasing intravaginal device from Days 0–7; n = 18) and (ii) a GPG+P4 programme from which the first GnRH treatment had been omitted (P+G+P4; n = 22). Interactions of each treatment with energy balance, as determined by NEFA, IGF-I and insulin concentrations, were also studied. Friesian and Friesian x Jersey cows were first blocked into two groups based on presence or absence of a corpus luteum at the outset of the study (CL negative and CL positive) and animals in each group were thereafter randomly allocated to the three programmes.

There was a significant effect of treatment and time on follicular dynamics ($P < 0.001$) in CL negative cows. Compared with GPG treatment alone, treatment with P+G+P4 resulted in significantly larger dominant follicles, particularly a larger preovulatory follicle. Although the proportion of cows ovulating within 48 h after the second GnRH injection was not different between the groups (GPG: 85.7%, GPG+P4: 88.9%, P+G+P4: 81.8%; $P = 0.79$), the timing of this ovulation was significantly different between the three treatments ($P = 0.04$), with the GPG treatment resulting in more concentrated ovulations than the other two groups. There was a significant ($P = 0.01$) negative effect of Day 7 non-esterified fatty acids (NEFA) concentration on the probability of ovulation after the Day 9 GnRH injection.

In summary, this study found no evidence that differences in pre-ovulation follicular dynamics were responsible for the previously reported significant improvement in conception rate seen in cows with anovulatory anoestrus which are treated with GPG+P4 rather than GPG alone. Indeed, GPG alone resulted in a better synchronisation of ovulation with a smaller spread of ovulation after the end of the treatment. For all three treatments cows that ovulated in response

to the Day 9 GnRH injection had improved energy status compared to those did not (as determined by NEFA concentration).

(Key words: anovulatory anoestrus, NEFA, insulin, IGF-I, GPG)

Introduction

Cows with anovulatory anoestrus pose a significant problem to the New Zealand dairy industry, as these cows have lower conception and pregnancy rates than cycling cows (Xu and Burton, 2000; McDougall, 2001). Cows that have not ovulated by one week before the planned start of mating have a lower probability of being pregnant within 21 days and a longer interval between the planned start of the seasonal breeding programme and conception than those that have ovulated (McNaughton *et al.*, 2007). Typically, approximately 20% of cows fail to show oestrus behaviour between calving and the start of mating (Rhodes *et al.*, 2003). These cows represent a significant cost, not only in terms of actual treatment costs, but also in the loss of genetic material due to the high culling rates (Rhodes *et al.*, 2000) that are needed to maintain a compact seasonal calving pattern (Harris and Kolver, 2001).

Type II anoestrus has been defined as deviation and growth of a dominant follicle, followed by either atresia or regression. In certain cases, the regression or atresia occurs only after a follicle has reached dominant status (Peter *et al.*, 2009) . However despite the presence of these large follicles and a low concentration of progesterone, many cows fail to ovulate for an extended period (McDougall *et al.*, 1995). A failure of the preovulatory surge of gonadotrophins, perhaps due to insufficient production of oestradiol by these follicles, may be the key mechanism preventing the resumption of cyclical activity (Chenault *et al.*, 1975; Beck and Convey, 1977; Kesler *et al.*, 1977).

A number of different methods of oestrous cycle control have been developed for the treatment of prolonged postpartum anoestrus in New Zealand. Before the European Union prohibited the use of oestradiol esters (such as oestradiol benzoate [ODB]) in food producing animals (European Union, 2003), ODB/progesterone programmes were the mainstay of protocols for oestrus synchronisation and induction of oestrus in anoestrous cows in New Zealand (Lucy *et al.*, 2004; McDougall, 2010a), with 13% of the national dairy herd being treated in the year 2000–2001 (Rhodes *et al.*, 2003). This protocol was extensively evaluated in New Zealand, and typically resulted in 87% of the cows being detected in oestrus within 7 days and 42% of cows conceiving to insemination during this period (Rhodes *et al.*, 2003). Use of this treatment protocol resulted in improved four week in-calf rates and reduced the interval from start of breeding to conception, compared to untreated controls (McDougall and Compton, 2005).

In 2007, the use of oestradiol in dairy cows was prohibited in New Zealand. Treatment protocols were revised, with the most noticeable difference being the use of gonadotrophin-releasing hormone (GnRH) instead of oestradiol, although progesterone remained as the basis of treatment (McDougall, 2010a). In 2008, the recommended programme for treating anoestrous dairy/beef cows in New Zealand was a GPG programme supplemented by intravaginal progesterone (Laven, 2008). This programme involves two treatments of GnRH (9 days apart), PGF_{2α} injection on Day 7 and intravaginal progesterone between Days 0 and 7. Fixed-time artificial insemination (FTAI) is performed 16–20 h after the second GnRH treatment.

Field studies have generally shown that the addition of progesterone to a GnRH-based synchronisation programme in both cycling and anoestrous dairy cows significantly improves conception rates. For example, increases in conception rate of 10 to 20% have been reported in several North American studies (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b). However, not all studies have shown such an effect; Stevenson *et al.* (2006) reported no improvement in conception rate from the addition of progesterone in cycling (GPG = 39%, GPG + P4 = 43%; n = 357) or noncycling (GPG = 39%, GPG + P4 = 34%; n = 105) lactating dairy cows.

In New Zealand, the published data strongly suggest that addition of progesterone to a GnRH-based synchronisation programme improves the response of cows in anoestrus. In a large scale field trial with 2,222 cows from 12 herds, McDougall (2010a) reported that addition of progesterone to a standard GPG programme resulted in a higher conception rate than GPG alone ($45.7 \pm 2.6\%$ *versus* $33.9 \pm 2.4\%$). However, the exact mechanism underlying this increase in conception rate is unclear: it may be due to the inhibition of premature oestrus (between the first GnRH and PGF_{2α} injection; Roy and Twagiramungu 1999; DeJarnette *et al.* 2001), better synchronisation of ovulation after the GnRH, or a reduction in the proportion of short cycles after the induced oestrus. Better identification of the means by which progesterone treatment improves the response to synchronisation could be valuable in identifying alternatives to progesterone, as the progesterone device is expensive and accounts for almost half the cost of synchronisation programme.

In contrast to the data on progesterone, the benefits of using GnRH at the start of such programmes have not been fully evaluated in anoestrous cattle. The aim of GnRH treatment on Day 0 is to stimulate the ovulation of large dominant follicles. In cyclic dairy cattle, a lower response rate to first GnRH may result in reduced pregnancy rates following FTAI due to asynchronous ovulation (Vasconcelos *et al.*, 1999).

Another constraint in the seasonal calving systems of New Zealand is the adverse effect of negative energy balance on reproduction. This problem is accentuated because the planned start of mating coincides with peak lactation, such that many cows are still in negative energy balance (Kay *et al.*, 2009) before the flush of spring pasture growth occurs (Clark *et al.*, 2000). A significant degree of negative energy balance can result in impaired reproductive as well as productive performance (Clark *et al.*, 2000; Harris and Kolver, 2001). The mechanisms that regulate energy and nutrient distribution in the somatotropic system may also affect reproduction at different levels of the hypothalamo-pituitary-ovarian axis (Lucy *et al.*, 1991; Lucy *et al.*, 1992; Roche, 2006; Chagas *et al.*, 2007). Several studies have shown that high postpartum concentrations of non-esterified fatty acids (NEFAs), and low concentrations of insulin and insulin-like growth factors I (IGF-I) negatively affect the ovarian activity of lactating dairy cows (Beam and Butler, 1997; Butler *et al.*, 2004; Leroy *et al.*, 2005; Vanholder *et al.*, 2006; Kawashima *et al.*, 2007; Ortega *et al.*, 2008). It is thus likely that the metabolic status of anoestrous cows will affect their response to treatment.

The aim of the present study was to assess three oestrus synchronisation protocols for anoestrous cows. The present study was undertaken to investigate whether:

- 1) The removal of progesterone from a standard GPG+P4 protocol would affect the synchrony of follicular development and ovulation;
- 2) Exclusion of the Day 0 GnRH injection from a GPG+P4 protocol would affect the overall synchronisation of ovulation after Day 9 GnRH injection;
- 3) Indicators of postpartum energy balance such as IGF-I, insulin, NEFA, milk yield and changes in body weight could affect the response to synchronisation of ovulation.

Materials and methods

All animal use was approved by Massey University Animal Ethics Committee, Palmerston North.

Animals

This study was conducted at No. 4 Dairy Farm, a spring-calving, pasture-based, 460-cow dairy farm in Palmerston North (latitude 40°2'S, longitude 175°4'E), New Zealand, during spring (September to November) 2009. Nine days before the planned start of breeding season, all lactating cows which had been calved for >30 days, had not had an assisted calving or a diagnosis of endometritis, and which had not been recorded in oestrus ($n = 61$) were examined.

Oestrus detection had been undertaken by farm staff using a combination of tail paint and observation from 40 days prior to the planned start of mating. The reproductive tracts of all the

selected animals were palpated *per rectum* and examined by ultrasonography to confirm the absence of detectable utero-ovarian pathology. Body condition score (BCS: Scale 1–10; Macdonald and Roche, 2004) of all animals was also recorded at the time of enrolment. The date of birth and calving date were retrieved from the electronic database of the farm. The body weight for each cow was recorded twice daily using the Walkover Weighing System (Gallagher, Hamilton, New Zealand). Milk yield of individual cows was measured twice daily using a DeLaval ALPRO milk-metering system (DeLaval International, Tumba, Sweden). These data were used to calculate the age, days postpartum, average milk production and changes in body weight before, during and after treatments.

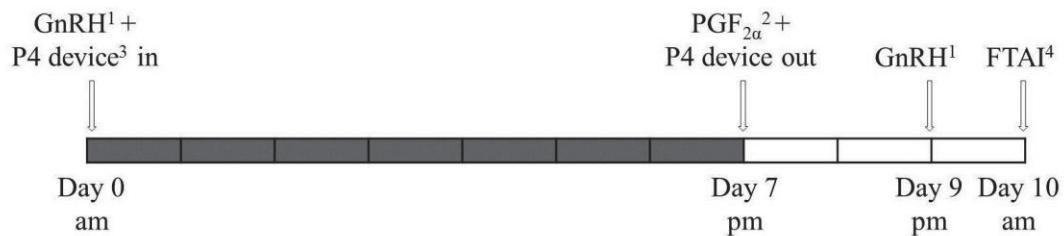
The presence or absence of a visible CL was also determined using transrectal ultrasonography and cows were blocked according to CL status, before being allocated to treatment. All the selected cows were randomly allocated using a random allocation sheet based on their order through the race and CL status on Day 0 to one of three treatments (Figure 1.4): (1) GnRH (100 µg i/m: Ovurelin, Bomac Laboratories Ltd, Auckland, New Zealand) on Day 0 (morning), a progesterone-releasing intravaginal device (1.56 g progesterone: Cue-Mate, Bomac Laboratories Ltd) from Days 0 to 7, cloprostenol, (500 µg i/m: Ovuprost, Bomac Laboratories Ltd) on Day 7 (late afternoon) and a second dose of GnRH on Day 9 (late afternoon), followed by FTAI on Day 10 (16–20 h after second GnRH treatment) (GPG+P4; n = 18); (2) As for Group 1, with the exclusion of the progesterone-releasing intravaginal device (GPG; n = 21); and (3) as for Group 1, but omitting the GnRH treatment on Day 0 (P+G+P4; n = 22).

Ultrasonography

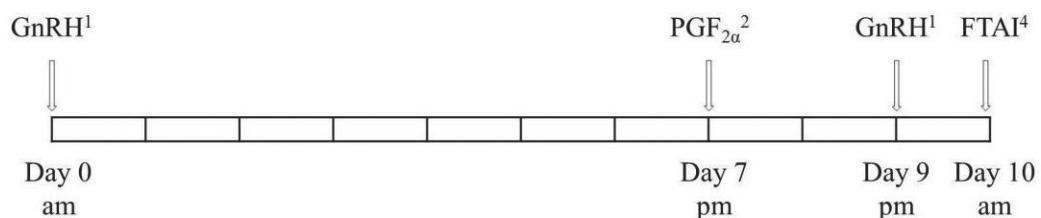
Ovarian structures of all cows were monitored and studied as previously described (Pierson and Ginther, 1984) using a real time B-mode ultrasound scanner (DP-6600 Vet, Mindray, Szechuan, China), equipped with a variable linear transducer set at 7.5 MHz. Digital ultrasound images of both ovaries were recorded on all occasions and a corresponding ovarian map was also drawn manually on the recording sheet to locate and identify the structures on the ovary, particularly the presence or absence of a CL.

The size of the largest dominant follicle was measured using ImageJ (v1.46d, National Institutes of Health, USA) by taking the average of two measurements: (i) size at the widest point and (ii) size at right angles to the first measurement. In cows which were CL negative on Day 0 (n = 40), ultrasonography was undertaken on Days 0, 1, 2, 3, 4, 7 and 9, whilst in the CL positive group (n = 21), ultrasonography was performed on Days 0, 7 and 9.

GPG+P4 (n = 18)



GPG (n = 21)



P+G+P4 (n = 22)

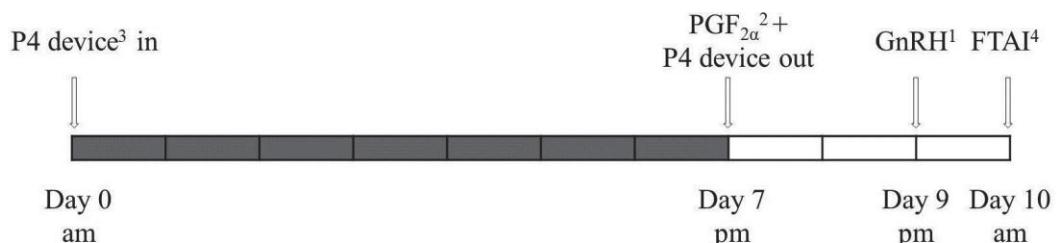


Figure 1.4 Synchronisation protocol for three groups (grey shading indicates progesterone releasing intravaginal device).

¹ 100 µg GnRH, i/m.

² 500 µg PGF_{2α} i/m.

³ Progesterone releasing intravaginal device containing 1.56 g of progesterone.

⁴ Fixed-time artificial insemination.

For CL negative cows, the response of the dominant follicle between Day 0 and Day 7 to treatment (i.e. persistence, ovulation or atresia) was recorded. Ovulation was defined as the disappearance of a dominant follicle followed by the development of a CL (or accessory CL), whereas atresia was defined as the disappearance of a dominant follicle without subsequent CL development. In CL negative cows, these data were also used to measure the interval to the emergence of a new follicular wave. This day was the first on which the new dominant follicle was retrospectively identified to have had a diameter of ≥ 4 mm. If the dominant follicle was not detected until it reached 6 or 7 mm, the previous day was taken as the first day (Ginther *et al.*, 1989b). The growth rate of the preovulatory follicle was established from the diameter it reached on Day 9, minus its diameter the day of its detection divided by the number of the days. In CL positive cows no measurements of dominant follicle for persistence, ovulation or atresia was recorded from Day 0 to 7, however measurements of dominant follicle diameter were made on Days 0, 7 and 9.

After Day 7, timing of ovulation in all cows (both CL negative and positive) was determined by ultrasound examination of the ovaries every 12 h from the morning of Day 9 until the afternoon of Day 11, or ovulation, whichever was sooner. Ovulation was defined as the disappearance of a previously identified dominant follicle ≥ 9 mm. Persistence was defined as no disappearance of the dominant follicle.

Blood samples and hormone assays

Blood samples (10 mL) were collected via coccygeal venepuncture into heparinised Vacutainers (Becton Dickinson New Jersey, USA) on Days 0, 7, 9, 10, 12, 14, 16, and 22 of the study. Plasma was separated by centrifugation at 1500 g for 20 min at 4°C within 2 h of collection. The plasma was then stored at -20°C until assay. Plasma progesterone concentrations were measured in CL positive and CL negative cows as described below. All other hormone measurements were undertaken in CL negative cows only.

Progesterone assay

Plasma progesterone concentrations were measured in samples taken from CL negative cows on Days 0, 7, 10, 12, 14, 16 and 22, and from CL positive cows on Days 7, 10 and 16 only. Concentrations were measured by radioimmunoassay using the ImmuChem Double Antibody Progesterone ^{125}I RIA kit for *in vitro* diagnostic use (MP Biomedicals, USA), in duplicate 10 μL aliquots (see Appendix 1). The sensitivity of the assay was 0.14 ng progesterone/mL. The intra-assay coefficients of variation at 80, 50 and 20% binding on the standard curve were 16.1, 8.4 and 9.9% respectively; the inter-assay coefficients of variation were 19.1, 14.4 and 15.7% for

low, medium and high solutions, respectively. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer.

Oestradiol assay

Plasma oestradiol concentrations were measured by radioimmunoassay using the Ultra-Sensitive Estradiol ^{125}I RIA kit, (Immunotech, Czech Republic) in duplicate 40 μL aliquots of samples collected on Days 0 and 12 (see Appendix 2). The sensitivity of the assay was 3.3 pg oestradiol/mL. The intra-assay coefficients of variation at 60 and 20% binding on the standard curve were 10.8% and 7.1% respectively; the inter-assay coefficients of variation were 14.8 and 4.0% for low and high solutions, respectively.

Insulin assay

Plasma insulin concentrations were measured by radioimmunoassay using the INSIK-5 kit (DiaSorin Inc. USA) in duplicate 20 μL aliquots of samples collected on Days 0, 9 and 16 (see Appendix 3). The sensitivity of the assay was 4.4 μU of insulin/mL. The intra-assay coefficients of variation at 80 and 50% binding on the standard curve were 11.2 and 15.5%, respectively; the inter-assay coefficients of variation were 12.9 and 11.3% for low and high solutions, respectively.

Analysis of IGF-I

Plasma IGF-I concentrations were measured by ELISA, using the DSL-10-2800 ACTIVE IGF-I ELISA kit (Diagnostic Systems Laboratories, USA), in duplicate 20 μL aliquots of samples collected on Days 0, 9, 16 and 22 (see Appendix 4 for a description of the method). The limit of sensitivity of the assay was 1.5 ng/mL. Control solutions of IGF-I were used as low and high quality controls in every plate. The mean concentrations of IGF-I in these solutions were 188.9 ± 8.7 and 353.9 ± 10.8 ng/mL, respectively. The intra-assay coefficients of variation for low (~ 200 ng/mL) and high (~ 350 ng/mL) control solutions were 8.1 and 2.5% respectively, whilst the inter-assay coefficients of variation were 6.5 and 4.3%. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer.

Non-esterified fatty acid (NEFA)

Plasma samples for measurement of NEFA concentrations were sent to New Zealand Veterinary Pathology (Palmerston North) immediately after collection. Samples collected on Days 0, 7, 9 and 16 were analysed in duplicate 50 μL aliquots using the commercial Wako NEFA C test kit (Wako Chemicals GmbH, Neuss, Germany). Optical density was read using the Hitachi

Modular P800 analyser (Roche Diagnostics, Mannheim, Germany). The sensitivity of the method expressed as absorptivity (using Hitachi 556 spectrometer at 550 nm) was 52 I/mEq^{*}cm. Within-run CV of three repeated assays ($n = 20$) was 2.7% or less.

Statistical analyses

Except where noted all statistical analysis was performed using the Prism statistical package (v 5.03, GraphPad Software, Inc. La Jolla, USA). Data were separated into two groups for analysis: 1) Data from cows which had no observable CL on Day 0 (CL negative cows); and 2) data from cows which had an observable CL on Day 0 (CL positive cows).

CL negative cows

The key outcome variables in this group were the diameter of the dominant follicle from Day 0 to 9, diameter of the preovulatory follicle, and growth rate of preovulatory follicle after Day 0. Secondary objectives were measurement of the proportion of cows ovulating in response to GnRH injection on Day 0 and Day 9; proportion with atresia of dominant follicle between Day 0 and Day 7; interval from Day 0 to the emergence of the follicular wave producing the dominant follicle on day 9; presence or absence of CL on Day 7, timing of ovulation after GnRH injection on Day 9; and plasma concentration of progesterone and oestradiol.

The effect of treatment on the proportion of cows with atresia or ovulation and new follicular wave emergence between Day 0 and Day 7, the effect of treatment on the presence of a CL on Day 7, the proportion of cows with synchronised ovulation within 48 h after the second GnRH treatment and the proportion of cows with an ovulation (within 12, 24, 36, 48 h) or no ovulation after the second GnRH injection, were compared by chi-squared test.

The effect of treatment on the interval to new wave emergence was analysed using one way analysis of variance. Student's t-test was used to compare the body weight change, average milk yield, Day 9 dominant follicle size, progesterone, NEFA, insulin and IGF-I concentrations between the CL negative cows that ovulated or did not ovulate after receiving GnRH on Day 9. Areas under the curves (AUC) of NEFA, insulin, IGF-I and progesterone concentrations (calculated using the trapezoid rule) were compared, using Student's t-test, between the cows that ovulated and those that did not.

The effect of treatment on dominant follicle size was analysed using a repeated measures mixed model analysis of variance, including treatment and day of measurement as fixed effects, cow as a random effect, and using a compound symmetric structure based on the lowest Akaike Information Criterion (Littell *et al.*, 1996). This analysis was undertaken using the MIXED

procedure of SAS (v 9.3, SAS Institute Inc., Cary, North Carolina, USA). The effect of treatment on serum progesterone concentration was analysed using a similar mixed model.

Effects of continuous variables (NEFA, insulin, IGF-I, milk yield and changes in body weight) on ovulation rate after administration of GnRH injection in CL negative cows were determined using binary logistic regression in Minitab (Logit Link function: Minitab v16, Minitab Inc., State College, PA, USA). The dependent variable was ovulation in response to the second GnRH injection, which was treated as a binomial variable with a logit transformation. The independent variables were NEFA, insulin, IGF-I, milk yield and changes in body weight and all two-factor interactions. To avoid covariance due to two similar variables, a matrix plot of all the independent variable was first generated. Those with a P value of <0.2 were kept in the model, unless there was a significant correlation ($r>0.7$) between the predictor variables when the variable with the smallest P value was kept in the model. A backward elimination of nonsignificant factors were done one by one until all the variables were significant ($P\leq0.05$) in the model. The preliminary final model was checked using diagnostic plots involving event probability (delta chi square vs. probability) and leverage (delta chi-square vs. leverage) and goodness of fit tests and if there were no any unusual observations and P value of goodness of fit test was >0.05 then the model was declared final.

CL positive cows

The outcome variables measured in this group were dominant follicle diameter on Day 0, 7 and 9, presence or absence of corpus luteum on Day 7 and plasma progesterone concentration on Day 7, 10 and 16. The effect of treatment on dominant follicle size (Days 7 and 9) was analysed using a repeated measures mixed model similar to that used for CL negative cows. This analysis was also undertaken using the MIXED procedure of SAS. The effect of treatment on serum progesterone concentration (Days 7, 10 and 16) was analysed using a similar mixed model to that used for CL negative cows. The proportion of cows with synchronised ovulation within 48 h after the second GnRH treatment and the proportion of cows with an ovulation (within 12, 24, 36, 48 h) or no ovulation after the second GnRH injection, were compared by chi-squared test.

Combined data from CL positive and CL negative cows

Where assessments had been made on the same day for both CL positive and negative cows the two datasets were amalgamated for the final analysis. Follicle diameter (Days 7 and 9 only) and progesterone concentrations (Days 7, 10 and 16) were analysed using the same repeated measures mixed models as used for CL positive cows except that Day 0 CL status was also included as a fixed effect.

Results

There were no significant differences in age, BCS, days postpartum, mean milk production (L/day) (Table 1.4) and changes in body weight (Table 2.4) between CL negative and CL positive cows or between treatment groups, indicating that the treatment groups were balanced for these factors. For CL negative cows, there were no significant differences in mean NEFA, insulin and IGF-I concentrations between treatment groups at any time (Table 3.4).

CL negative cattle

Follicular dynamics between Days 0 and 9

Changes in mean diameters of dominant follicles are summarised in Table 4.4 and Figure 2.4. Mean dominant follicle size on Day 0 was not significantly different between groups (GPG: 12.7 ± 0.7 mm, GPG+P4: 12.6 ± 1.0 mm, P+G+P4: 12.4 ± 0.8 mm; $P = 0.96$). There were no significant differences between treatments in the proportion of cows with atresia or ovulation, mean days to the emergence of new follicular wave, or growth rate of the preovulatory follicle after GnRH administration on Day 0.

However, there was a significant effect of treatment and time (Day 1 to Day 9; $P < 0.001$ for both) on follicular size (Figure 2.4). Cows treated with P+G+P4 had significantly larger dominant follicles than those treated with GPG on Days 2, 3, 7 and 9 ($P < 0.04$) and those treated with GPG+P4 on Day 3 ($P = 0.01$). There were no significant differences at any time point between cows treated with GPG and GPG+P4.

Presence of a CL on Day 7

The percentage of cows that had developed a CL by Day 7 is shown in Figure 3.4 for each treatment. The number of cows with a CL by Day 7 was significantly higher in the GPG+P4 ($P = 0.01$) and GPG ($P = 0.001$) groups compared with the P+G+P4 group, whereas there was no difference between the GPG+P4 and GPG groups ($P = 0.66$). There was a significant effect of treatment ($P = 0.007$), such that the probability of having a CL present on Day 7 was 92%, 643.6% and 14% for GPG, GPG+P4 and P+G+P4 treatments, respectively.

Table 1.4 Descriptive data of the cows enrolled in the experiment.

Parameters	Treatment			P value
	GPG	GPG+P4	P+G+P4	
N (<i>CL negative</i>)	14	11	15	
N (<i>CL positive</i>)	7	7	7	
Age (months) (range)	34.7 (24.7–73.9)	43.8 (25.0–86.0)	39.4 (24.4–74.0)	0.24
BCS (1–10) (range)	3.5 (3–4)	3.5 (3–4)	3.4 (3–4)	0.66
Days postpartum (range)	68.4 (53–87)	64.1 (53–79)	66.3 (41–84)	0.41
Average milk production (L/day)	15.0 ± 0.7	16.3 ± 0.6	15.5 ± 0.8	0.47

CL negative: cows without an observable corpus luteum on Day 0; CL positive: cows with observable corpus luteum on Day 0.

Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

Table 2.4 Changes in body weight (mean \pm SEM) corpus luteum negative ($n = 40$) and corpus luteum positive ($n = 21$) cows before[†], during[†] and after[†] the synchronisation protocol.

Group	Body weight change (kg)								
	GPG		GPG+P4		P+G+P4				
	Before	During	After	Before	During	After	Before	During	
CL negative	9.9 \pm 5.0	0.4 \pm 3.7	-8.7 \pm 3.0	-1.9 \pm 4.8	1.4 \pm 6.4	4.6 \pm 5.4	2.1 \pm 4.7	5.1 \pm 3.5	-11.7 \pm 4.6
CL positive	-8.2 \pm 7.5	4.7 \pm 12.2	-16.2 \pm 6.7	5.9 \pm 3.9	3.3 \pm 3.2	-8.6 \pm 5.5	22.1 \pm 7.4	-9.4 \pm 6.6	5.7 \pm 6.8

[†] Average changes in body weight one month before the commencement of the treatment, during the observation period (approximately one month) and one month after the end of the experiment (Not significant: P>0.05).

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 3.4 Plasma NEFA, insulin and IGF-I concentrations (mean \pm SEM) in the CL negative group during and after the synchronisation protocol.

Day	Treatment						P+G+P4 (n = 15)		
	GPG (n = 14)			GPG+P4 (n = 11)			NEFA (mMol/L)	Insulin (μ U/mL)	IGF-I (ng/mL)
	NEFA (mMol/L)	Insulin (μ U/mL)	IGF-I (ng/mL)	NEFA (μ Mol/L)	Insulin (μ U/mL)	(ng/mL)			
0	0.6 \pm 0.1	16.5 \pm 1.2	49.2 \pm 4.3	0.5 \pm 0.1	18.4 \pm 2.1	52.2 \pm 5.5	0.6 \pm 0.1	18.7 \pm 1.5	47.9 \pm 4.8
7	0.9 \pm 0.1	-	-	0.8 \pm 0.2	-	-	0.8 \pm 0.1	-	-
9	0.9 \pm 0.1	15.0 \pm 1.2	43.9 \pm 4.5	0.6 \pm 0.1	15.0 \pm 1.4	45.7 \pm 6.1	0.9 \pm 0.1	12.5 \pm 1.1	40.0 \pm 5.3
16	0.1 \pm 0.0	18.0 \pm 1.6	58.6 \pm 4.5	0.1 \pm 0.0	17.4 \pm 1.1	57.9 \pm 5.6	0.1 \pm 0.0	21.3 \pm 2.7	53.5 \pm 5.5
22	-	-	56.8 \pm 5.9	-	-	53.1 \pm 8.3	-	-	44.5 \pm 6.0

Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

NEFA, non-esterified fatty acids (Not significant between treatments, P>0.05).

IGF-I, insulin like growth factor-I (Not significant between treatments, P>0.05).

Table 4.4 Ovarian dynamics in three groups of CL negative cows during the synchronisation protocol (Day 0–9).

Parameter	Treatment				P value
	GPG	GPG+P4	P+G+P4		
	(n = 14)	(n = 11)	(n = 15)		
Ovulation ¹	36%	27%	21%	0.70	
	(5/14)	(3/11)	(3/14)		
Atresia ²	64%	73%	79%	0.70	
	(9/14)	(8/11)	(11/14)		
Interval to follicular wave emergence ³ (days)	1.9± 0.3	1.9 ± 0.2	2.6 ± 0.3	0.09	
Growth rate of the preovulatory follicle (mm/day)	1.3 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	0.28	

¹ Defined as the disappearance of a dominant follicle >9 mm, followed by the formation of a CL.

² Proportion of cows with disappearance of a dominant follicle without the development of a CL within 7 days after the first GnRH treatment.

³ Identified as the day on which the dominant follicle was retrospectively identified to have a diameter of 4–5 mm within 7 days of treatment period.

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

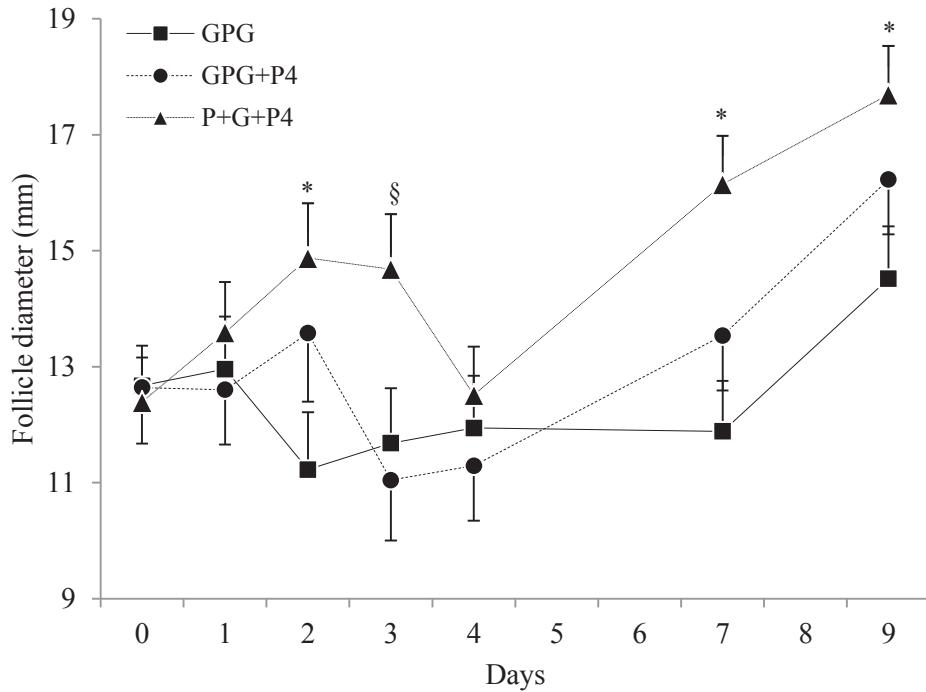


Figure 2.4 Changes in dominant follicle size (least square mean \pm SEM) in CL negative cows during the synchronisation protocol (Days 0 to 9) in GPG, GPG+P4 and P+G+P4 treatment groups. Effect of treatment and day was significant ($P<0.001$).

*: Significantly larger in the P+G+P4 than the GPG group (Day 2: $P = 0.01$, Day 7: $P = 0.04$, Day 9: $P = 0.01$).

§: P+G+P4 significantly larger than other two groups group (Day 3: $P<0.03$).

Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

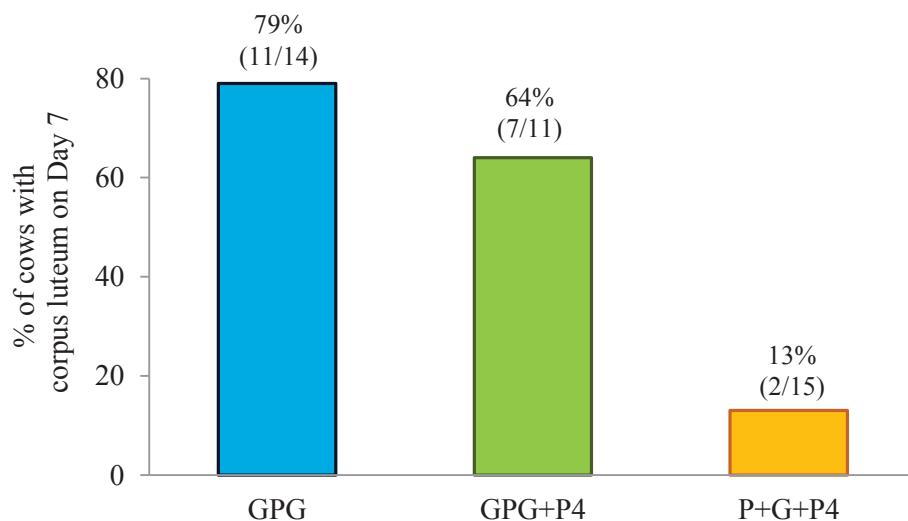


Figure 3.4 Proportions of CL negative cows that had developed an observable CL by Day 7. Differences between treatment groups are significant ($P = 0.007$). Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

Progesterone concentrations

There were no differences in progesterone concentrations between treatment groups on Day 0 (GPG: 0.7 ± 0.5 , GPG+P4: 1.9 ± 0.6 , P+G+P4: 2.7 ± 0.5 ng/mL, $P = 0.07$, Figure 4.4) in CL negative cows. However, some animals diagnosed as CL negative had progesterone concentrations >1 ng/ mL (GPG: 1/14, GPG+P4: 6/11, P+G+P4: 9/15) on Day 0. In the GPG and GPG+P4 groups there was a significant rise in mean progesterone concentration between Days 0 and 7 ($P < 0.001$ and $P = 0.02$ respectively), whereas there was no such change in the P+G+P4 group ($P = 0.64$). However, Day 7 progesterone concentrations did not differ between any of the groups (GPG: 3.7 ± 0.5 ng/mL, GPG+P4: 4.1 ± 0.6 ng/mL, P+G+P4: 3.1 ± 0.5 ng/mL, $P > 0.05$).

Between Days 10 and 22, there was a significant ($P < 0.001$) effect of time on progesterone concentrations, but there was no effect of treatment ($P = 0.22$). However, the treatment by time interaction approached significance such that concentrations in the P+G+P4 group tended to be highest, and those in the GPG group lowest($P = 0.06$, Figure 4.4).

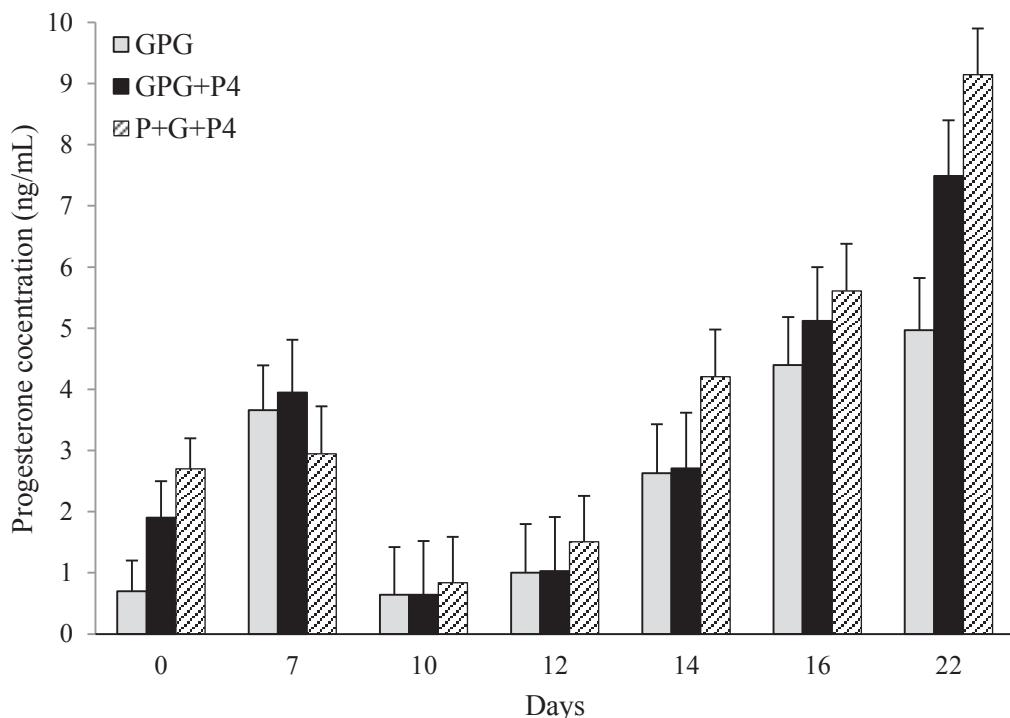


Figure 4.4 Least square means of plasma progesterone concentration (\pm SEM) in CL negative cows treated with GPG, GPG+P4 or P+G+P4 synchronisation protocols. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Progesterone concentrations on Days 14, 16 and 22 (Figure 5.4) were significantly higher in cows that ovulated compared with those that did not (Day 14: 3.4 ± 0.3 ng/mL *versus* 1.6 ± 1.1 ng/mL, $P = 0.05$, Day16: 5.5 ± 0.5 ng/mL *versus* 2.7 ± 1.5 ng/mL, $P = 0.04$, Day 22: 8.4 ± 1.0

ng/mL versus $2.3 \pm 1.8 \text{ ng/mL}$, $P = 0.01$). The area under the curve of progesterone concentrations for the cows that did not ovulate was not different ($P = 0.28$) from those that did.

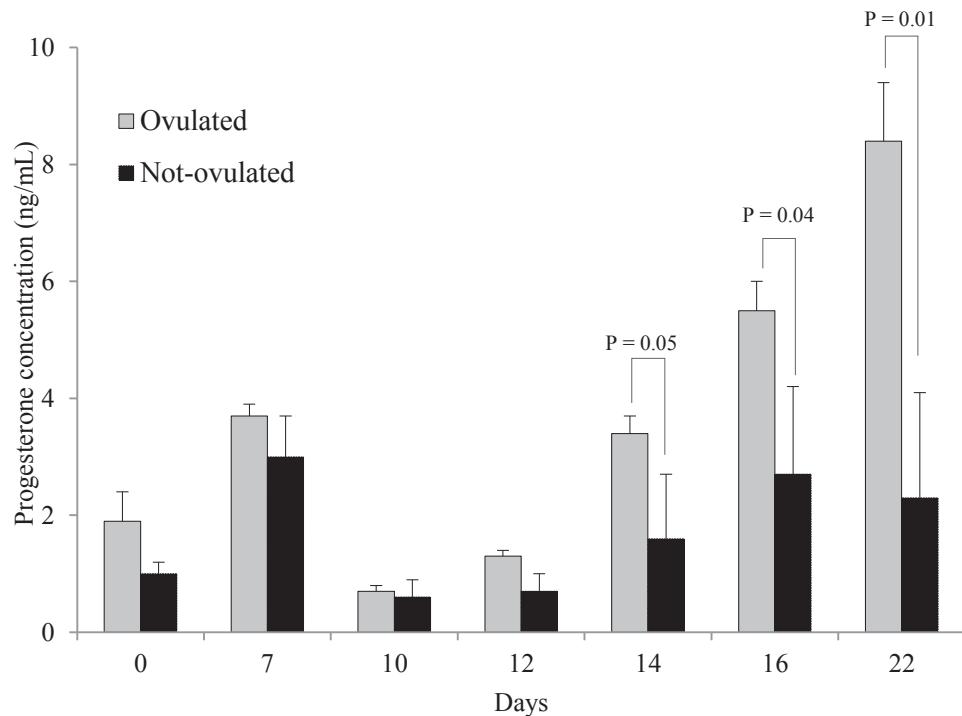


Figure 5.4 Mean progesterone concentrations ($\pm \text{SEM}$) at different times in CL negative cows which either ovulated ($n = 34$) or did not ($n = 6$) in response to administration of GnRH on Day 9.

Oestradiol concentrations

There were no differences in oestradiol concentration between treatment groups on Day 0 (GPG: $2.7 \pm 0.6 \text{ pg/mL}$, GPG+P4: $3.5 \pm 1.0 \text{ pg/mL}$, P+G+P4: $4.7 \pm 0.5 \text{ pg/mL}$; $P = 0.11$). However on Day 12, cows in the P+G+P4 group had significantly higher mean oestradiol concentrations than the GPG group (GPG: $1.7 \pm 0.6 \text{ pg/mL}$, P+G+P4: $3.7 \pm 0.6 \text{ pg/mL}$; $P = 0.05$). Values in the GPG+P4 group fell between the other two groups ($2.1 \pm 0.7 \text{ pg/mL}$).

Development of the ovulatory follicle

The mean size of the dominant follicle in the P+G+P4 group on Day 9 was significantly greater than that of the GPG group ($17.7 \pm 0.8 \text{ mm}$ versus $14.5 \pm 0.9 \text{ mm}$, $P = 0.01$; Figure 6.4), as was the size of the preovulatory follicle after the second GnRH injection ($18.6 \pm 0.9 \text{ mm}$ versus $15.7 \pm 1.1 \text{ mm}$, $P = 0.03$; Table 5.4). Inclusion of Day 0 progesterone concentration as a factor in this model (divided into $0 = <1 \text{ ng/mL}$ and $1 = \geq 1 \text{ ng/mL}$) did not alter this effect, indicating that there was no effect of Day 0 progesterone status on follicle size on Day 9 ($P = 0.72$).

Irrespective of treatment, the size of the dominant follicle on Day 9 was greater in cows that subsequently ovulated within 48 h of the administration of GnRH on Day 9 than in those that did not (16.9 ± 0.5 mm *versus* 12.8 ± 0.9 , $P = 0.004$).

Table 5.4 Activity of the ovulatory follicle in CL negative cows undergoing synchronisation protocols with GPG, GPG+P4 or P+G+P4.

Parameter	Treatment		
	GPG	GPG+P4	P+G+P4
	(n= 14)	(n= 11)	(n= 15)
Did not ovulate	3	1	2
Ovulated within 48 h of Day 9 GnRH (synchronised ovulation)	11	10	13
Mean maximal preovulatory follicle size of cows that ovulated within 48 h of Day 9 GnRH	15.7 ± 1.1^a	16.5 ± 1.0^{ab}	18.6 ± 0.9^b

^{a, b} Means with different superscripts within a row differ at $P \leq 0.05$.

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

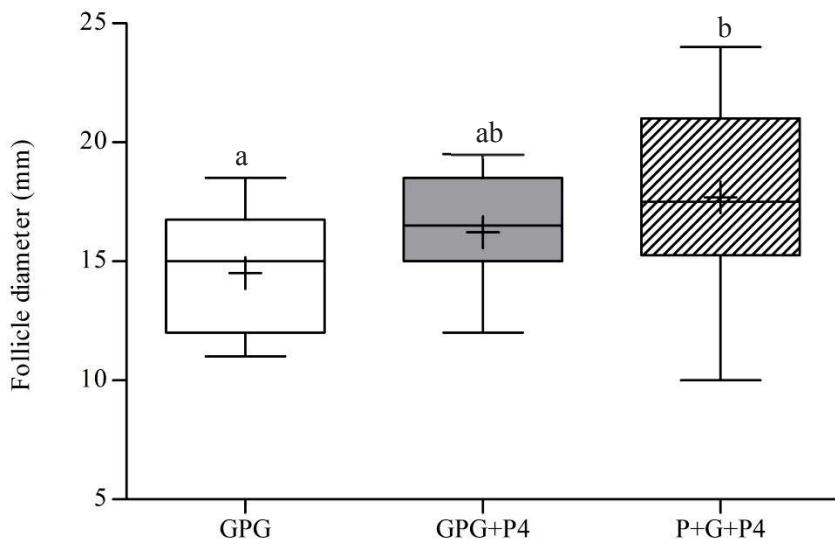


Figure 6.4 Comparison of Day 9 follicular diameter in CL negative cows undergoing synchronisation protocols with GPG, GPG+P4 or P+G+P4. Boxplots without a common superscript are different from each other ($P < 0.05$). '+' shows mean value in each group. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Synchronisation of ovulation after giving GnRH on Day 9

The proportions of cows which ovulated within 48 h of the Day 9 GnRH injection was 78.6, 90.9 and 86.7% for GPG, GPG+P4 and P+G+P4 groups, respectively ($P = 0.69$). Progesterone concentration on Day 0 had no significant effect on whether ovulation was synchronised after the Day 9 GnRH ($P = 0.90$). There was no difference in the proportions of ovulations at different time intervals after the Day 9 GnRH treatment ($P = 0.33$; Figure 7.4, Table 6.4).

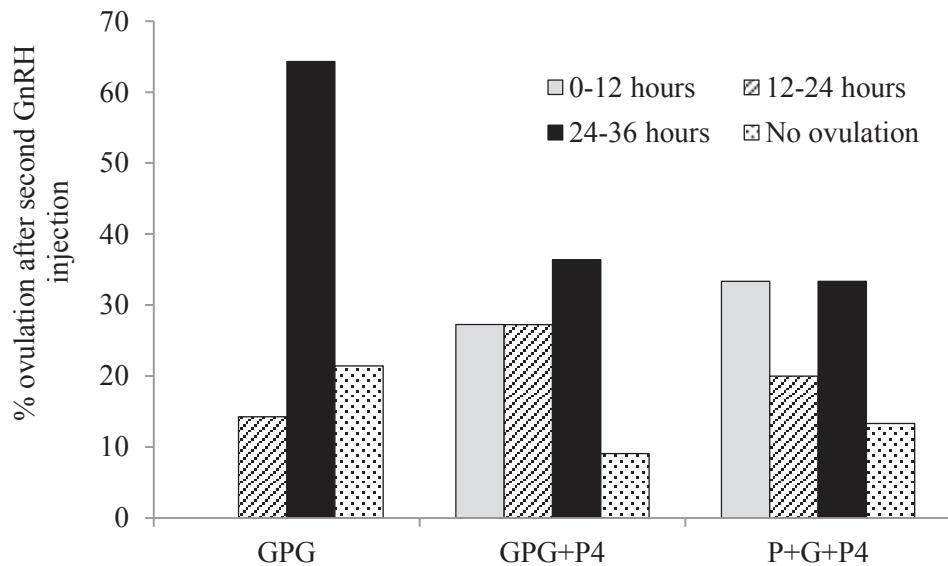


Figure 7.4 Proportions of CL negative cows treated with GPG, GPG+P4 or P+G+P4 that ovulated at different time intervals or failed to ovulate after receiving GnRH on Day 9 ($P = 0.33$). Abbreviations: G, GnRH; P, PGF2 α ; P4, progesterone.

Table 6.4. Distribution of ovulations at different time intervals after receiving GnRH on Day 9 in CL negative cows treated with GPG, GPG+P4 or P+G+P4.

Treatment	Early (0–12 h)	Mid (12–24 h)	Late (24–36 h)	No ovulation within 48 h	Total
GPG	0	2	9	3	14
GPG+ P4	3	3	4	1	11
P+G+P4	5	3	5	2	15
Total	8	8	18	6	40

Abbreviations: G, GnRH; P, PGF2 α ; P4, progesterone.

Relationships between energy balance and ovulatory response to administration of GnRH on Day 9

Concentrations of NEFA were significantly lower ($P<0.001$ to 0.003) on Days 0, 7 and 9 in cows that ovulated than in those that failed to do so, whereas insulin concentrations were higher ($P = 0.01$) on Day 0 in cows that ovulated compared to those that did not, but there were no differences on other days ($P>0.05$). Insulin-like growth factor-I concentration (at any time point), milk yield, body weight (actual or change) did not differ significantly between cows that ovulated and those that did not. (Table 7.4).

Multiple correlations were present between the variables used to assess energy balance, including between NEFA, insulin and IGF-I. Although milk yield was not correlated to any other variable, body weight changes were significantly correlated with each other, as well as with Day 9 insulin and IGF-I concentrations (Table 8.4).

Concentrations of NEFA on Days 0, 7 and 9, and insulin concentrations on Day 0 were related ($P<0.2$) to ovulation rate after giving GnRH on Day 9 in the univariate logistic regression analyses. Concentrations of NEFA on Days 0 and 7 were correlated with each other ($r^2 = 0.7$), so Day 7 values were used in preference to those from Day 0. Thus, in the first multivariate analysis after backwards elimination of non-significant factors, only Day 7 NEFA concentrations were significant ($P = 0.01$) in the logistic regression model. The probability of ovulation was inversely proportional to Day 7 NEFA concentration (odds ratio 0.001; i.e. for every increase of 1 mMol/L above mean concentrations of NEFA on Day 7 the odds of ovulation reduced by 1000).

There was no difference in the AUC of NEFA ($P = 0.26$), insulin ($P = 0.38$) or IGF-I ($P = 0.58$) concentrations (Figure 8.4) in cows that ovulated after administration of GnRH on Day 9 compared with those that did not.

Table 7.4 Comparison of concentrations of NEFA, insulin, IGF-I, milk yield and body weight change (mean \pm SEM) between CL negative cows that ovulated or failed to ovulate within 48 h of GnRH treatment on Day 9 of the synchronisation protocol.

Parameters	Day	Ovulated	Not-ovulated	P value
		(n = 34)	(n = 6)	
NEFA (mMol/L)	0	0.5 \pm 0.0 ^a	1.0 \pm 0.1 ^b	< 0.001
	7	0.7 \pm 0.1 ^a	1.5 \pm 0.2 ^b	< 0.001
	9	0.8 \pm 0.1 ^a	1.2 \pm 0.2 ^b	0.003
	16	0.1 \pm 0.0	0.2 \pm 0.0	0.12
Insulin (μ U/mL)	0	18.8 \pm 1.0 ^a	12.9 \pm 1.0 ^b	0.01
	9	14.0 \pm 1.0	14.3 \pm 2.1	0.90
	16	19.7 \pm 1.4	15.6 \pm 1.1	0.20
IGF-I (ng/mL)	0	49.8 \pm 3.0	50.9 \pm 7.7	0.89
	9	43.4 \pm 3.3	40.6 \pm 7.2	0.73
	16	57.7 \pm 3.2	49.8 \pm 8.3	0.33
	22	51.3 \pm 4.6	46.6 \pm 7.2	0.64
Milk yield (L/day)		15.21 \pm 0.5	17.06 \pm 1.7	0.19
Body weight change [†] (kg)		5.0 \pm 3.2	- 3.3 \pm 5.2	0.30

^{a, b} Values with different superscript in the same row differ significantly.

[†] Overall changes in body weight from one month before to one month after the end of the experimental period).

Table 8.4 Correlations between milk yield (L/day), body weight change (kg) (BWT_b , before; BWT_d , during and BWT_a , after the end of treatment period), NEFA (mMol/L), insulin (μ U/mL) and IGF-I (ng/mL) concentrations on different days in CL negative cattle. Significant correlations are in bold and marked with an asterisk

	Pearson correlation (P values)											
	Milk yield	BWT_b	BWT_d	BWT_a	Day 0 NEFA	Day 7 NEFA	Day 9 NEFA	Day 0 insulin	Day 7 insulin	Day 9 insulin	Day 0 <i>IGF-I</i>	Day 7 <i>IGF-I</i>
BWT_b	-0.06 (0.73)											
BWT_d	0.20 (0.21)	-0.41 (0.01)*										
BWT_a	-0.15 (0.34)	-0.37 (0.02)*	-0.37 (0.02)*									
Day 0 NEFA	0.15 (0.36)	0.15 (0.37)	-0.12 (0.47)	-0.21 (0.23)								
Day 7 NEFA	0.05 (0.76)	-0.02 (0.92)	0.06 (0.71)	-0.28 (0.11)	0.7 (0.00)*							
Day 9 NEFA	0.08 (0.66)	-0.17 (0.34)	0.11 (0.53)	-0.30 (0.08)	0.44 (0.01)*	0.45 (0.01)*						
Day 0 insulin	-0.17 (0.33)	-0.02 (0.93)	0.06 (0.75)	-0.06 (0.75)	-0.41 (0.01)*	-0.41 (0.02)*	-0.04 (0.83)					
Day 9 insulin	0.01(0.93)	-0.33 (0.05)*	0.08 (0.66)	0.15 (0.39)	-0.17 (0.32)	-0.05 (0.79)	0.14 (0.42)	0.37 (0.03)*				
Day 0 <i>IGF-I</i>	0.10 (0.58)	-0.30 (0.08)	0.25 (0.14)	-0.19 (0.27)	-0.08 (0.66)	0.22 (0.23)	0.13 (0.49)	0.18 (0.33)	0.10 (0.58)			
Day 9 <i>IGF-I</i>	0.03 (0.85)	-0.38 (0.03)*	0.19 (0.27)	0.01 (0.94)	-0.08 (0.65)	0.18 (0.32)	0.12 (0.50)	0.06 (0.75)	0.18 (0.30)	0.94 (0.00)*		

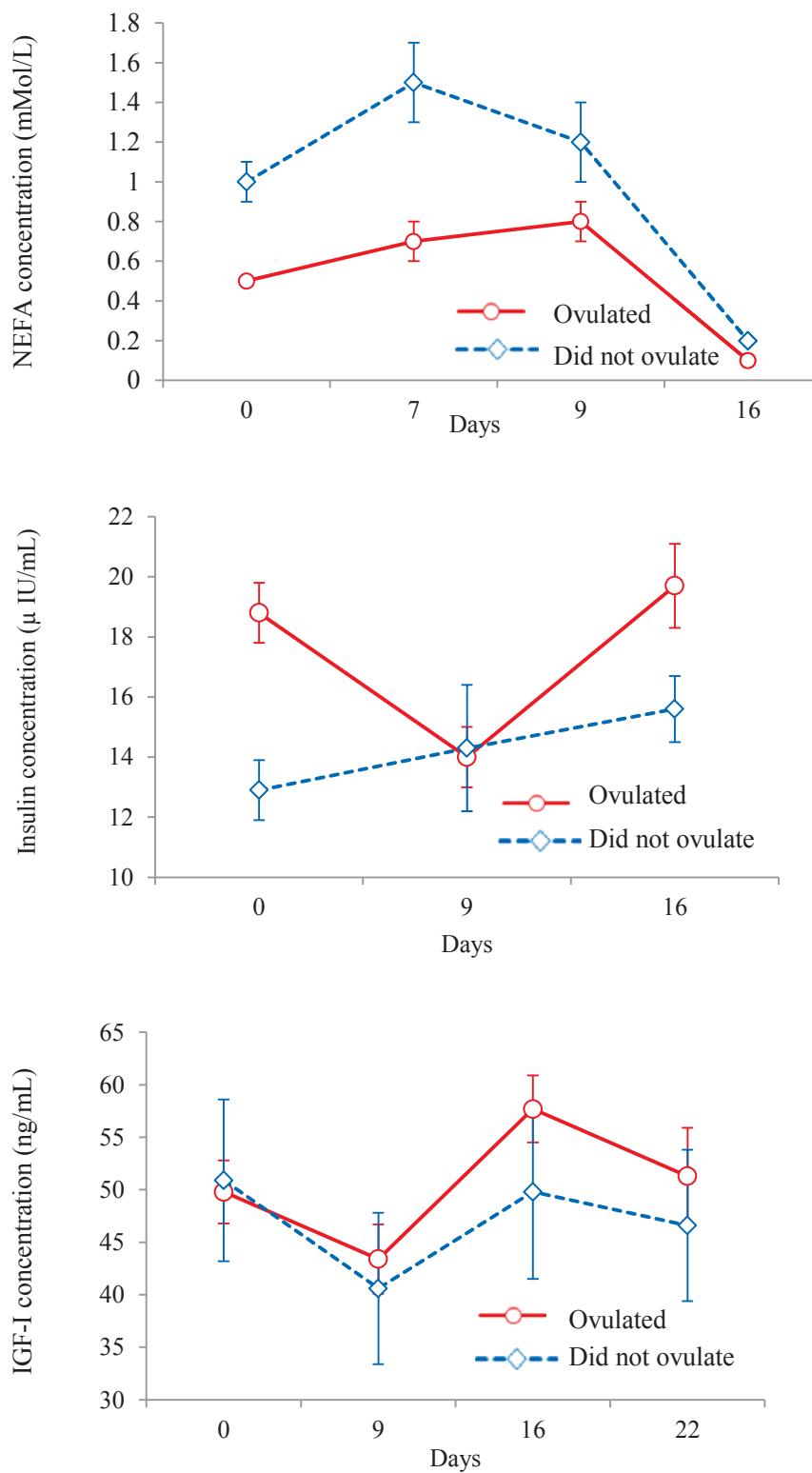


Figure 8.4 Mean NEFA (mMol/L \pm SEM), insulin (μ U/mL \pm SEM) and IGF-I (ng/mL \pm SEM) concentrations at different times in CL negative cows which either ovulated (n = 34) or did not (n = 6) in response to administration of GnRH on Day 9.

CL positive cattle

Follicular dynamics between Days 0 and 9

The size of the dominant follicle on Day 0 did not differ between treatment groups (GPG: 9.3 ± 1.1 mm, GPG+P4: 10.6 ± 1.1 mm, P+G+P4: 13.2 ± 1.5 mm; $P = 0.12$). There was no effect of treatment, time or treatment by time interaction on the dominant follicle size on Days 7 or 9 ($P = 0.21$, 0.15 and 0.62 respectively, Figure 9.4 and 12.4). However, the small number of animals in each group meant that this part of the analysis lacked power. Based on post-hoc power analysis of the present data, repeating the study with CL-positive cows, 7 cows per group (and an α of 0.05) would result in a power of 80% for detecting a difference of 5 mm ($\sigma = 3.8$) in dominant follicle size.

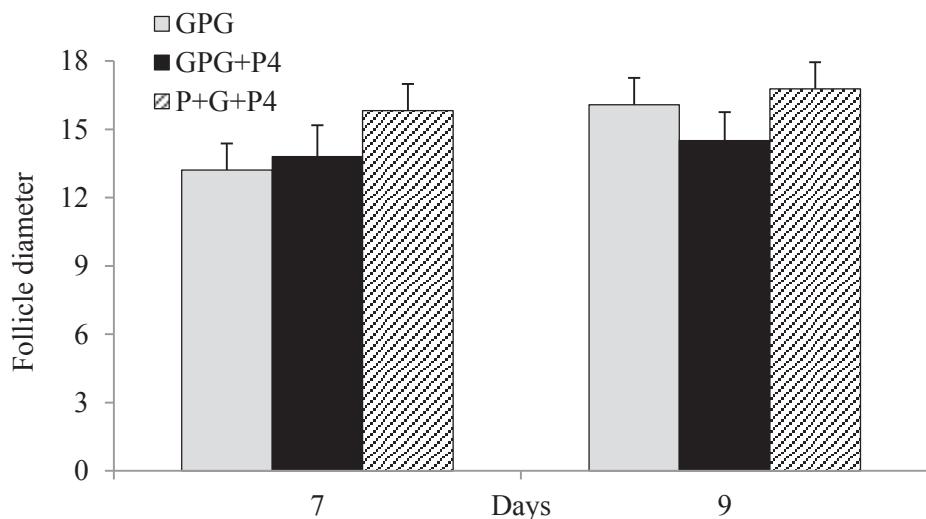


Figure 9.4 Effect of treatment on dominant follicle size (least square mean \pm SEM) on Days 7 and 9 in CL positive cows. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Effect of treatment group on the presence of a CL on Day 7

This study found no effect of treatment on the presence of a CL on Day 7 ($P = 0.85$); CL were observed on Day 7 in 71%, 57% and 57% of GPG, GPG+P4, and P+G+P4 cows, respectively (Figure 10.4).

Progesterone concentrations

Progesterone concentrations on Days 7, 10 and 16 are shown in Figure 11.4. There was a significant effect of treatment ($P = 0.03$) and time ($P < 0.001$), but no treatment by time

interaction ($P = 0.44$). In all the treatment groups, there was a significant drop in progesterone concentration from Day 7 to 10, followed by a significant rise by Day 16.

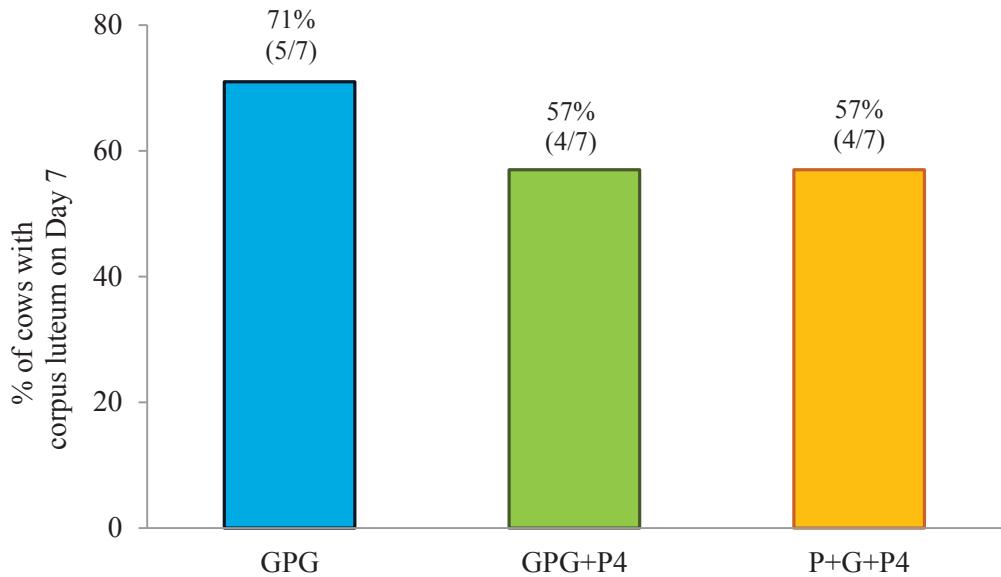


Figure 10.4 Comparison of the proportions of CL positive cows undergoing different synchronisation protocols with an observable CL on Day 7 ($P = 0.85$). Abbreviations: G, GnRH; P, PGF2 α ; P4, progesterone.

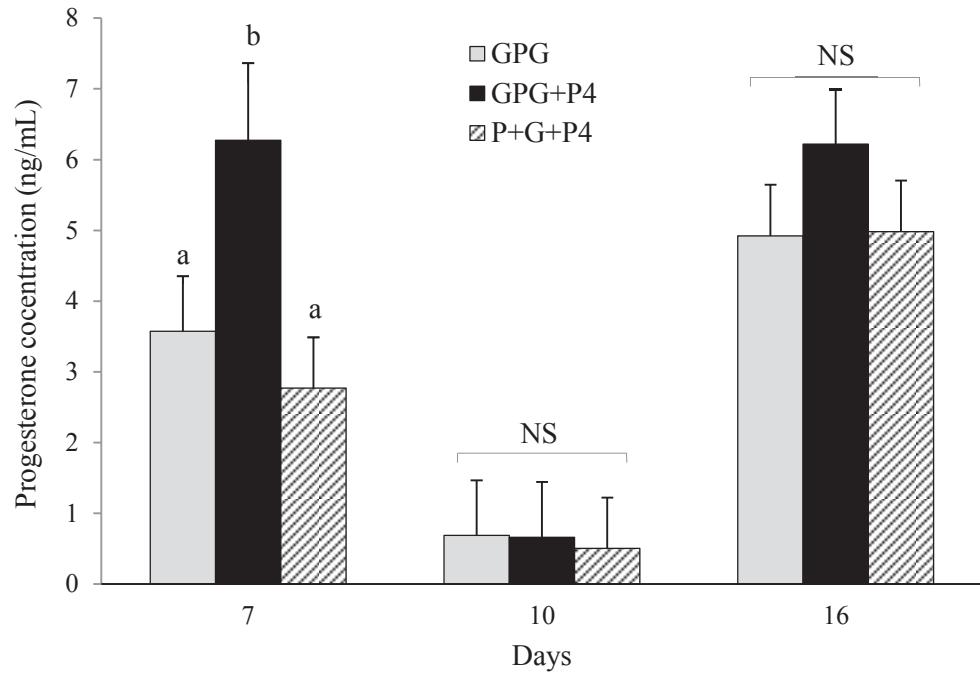


Figure 11.4 Least square means \pm SEM (treatment*day) of progesterone concentrations in CL positive cows treated with GPG, GPG+P4 and P+G+P4. Bars without a common superscript are different from each other ($P < 0.05$); NS: Non-significant. Abbreviations: G, GnRH; P, PGF2 α ; P4, progesterone.

Table 9.4 Activity of the ovulatory follicle in CL positive cows undergoing synchronisation protocols with GPG, GPG+P4 or P+G+P4.

Parameter	Treatment		
	PGP	GPG+P4	P+G+P4
	(n= 7)	(n= 7)	(n= 7)
Did not ovulate	0	1	2
Synchronised ovulation [†]	7	6	5
Mean (\pm SEM) ovulatory follicle size (mm)	16.7 \pm 0.0	14.9 \pm 1.4	16.9 \pm 1.8

[†]Ovulation by 48 h after second GnRH injection.

Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

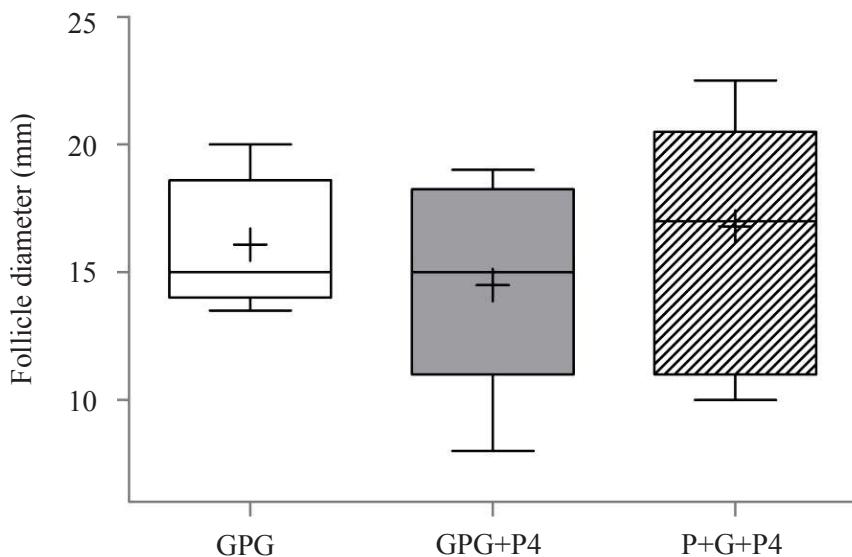


Figure 12.4 Comparison of Day 9 follicular diameters in CL: positive cows treated with GPG, GPG+P4 and P+G+P4. Differences between groups were not significant ($P>0.05$). '+' shows the mean value in each group. Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone.

Synchronisation of ovulation after giving GnRH on Day 9

The proportion of cows which ovulated within 48 h after receiving GnRH on Day 9 was 100, 85.7 and 71.4% for the GPG, GPG+P4 and P+G+P4 groups, respectively (Figure 13.4 and Table 10.4). Likewise, there was no difference ($P = 0.88$) in the proportion of ovulations at different time intervals after the Day 9 GnRH treatment (Figure 13.4).

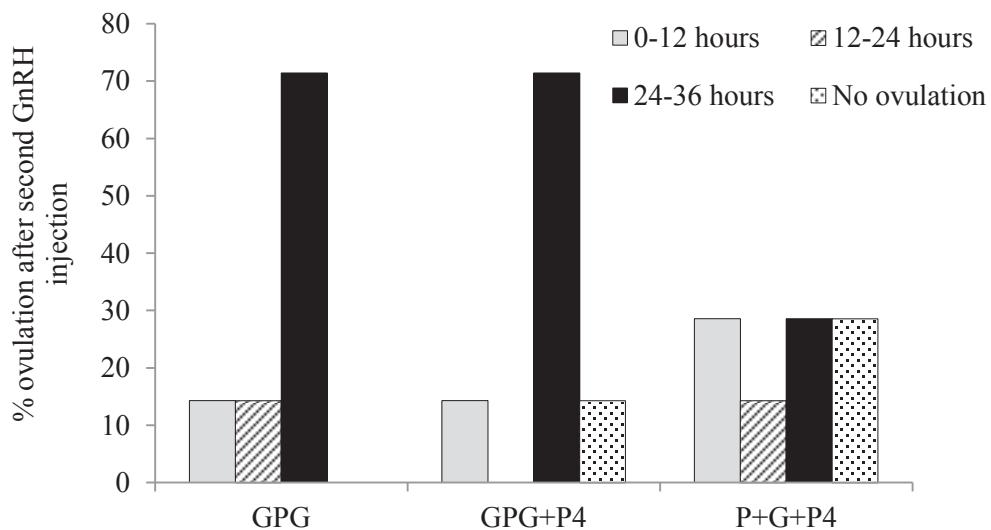


Figure 13.4 Proportions of CL positive cows treated with GPG, GPG+P4 or P+G+P4 that ovulated at different time intervals or failed to ovulate after receiving GnRH on Day 9 ($P = 0.82$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 10.4 Distribution of ovulations in CL positive cows at different time intervals after administration of GnRH on Day 9.

Treatment	Early (0–12 h)	Mid (12–24 h)	Late (24–36 h)	No ovulation within 48 h	Total
GPG	1	1	5	0	7
GPG+ P4	1	0	5	1	7
P+G+P4	2	1	2	2	7
Total	4	2	12	3	21

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Combined data from CL negative and positive cows

Development of the ovulatory follicle

The dynamics of the ovulatory follicle for the combined data is shown in Table 11.4. There was no difference in the size of ovulatory follicle between the groups ($P = 0.09$).

Effect of treatment group on the presence of a CL on Day 7

There was a significant effect of treatment on the presence of a CL on Day 7 ($P = 0.02$), such that the mean probabilities for the presence of a CL on Day 7 were 84, 65 and 32% for the GPG, GPG+P4, and P+G+P4 groups, respectively (Figure 14.4). However, the effect of CL status on Day 0 was not significant, nor was there any significant interaction between treatment and CL status ($P = 0.72$ and 0.12, respectively).

Table 11.4 Activity of the ovulatory follicle in CL positive and negative cows together undergoing synchronisation protocols with GPG, GPG+P4 or P+G+P4.

Parameter	Treatment		
	GPG (n= 21)	GPG+P4 (n= 18)	P+G+P4 (n= 22)
Did not ovulate	3	2	4
Synchronised ovulation [†]	18	16	18
Mean (±SEM) ovulatory follicle size (mm)	16.1 ± 0.7	15.9 ± 0.8	18.2 ± 0.8

[†]Ovulation by 48 h after second GnRH injection ($P = 0.79$).

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

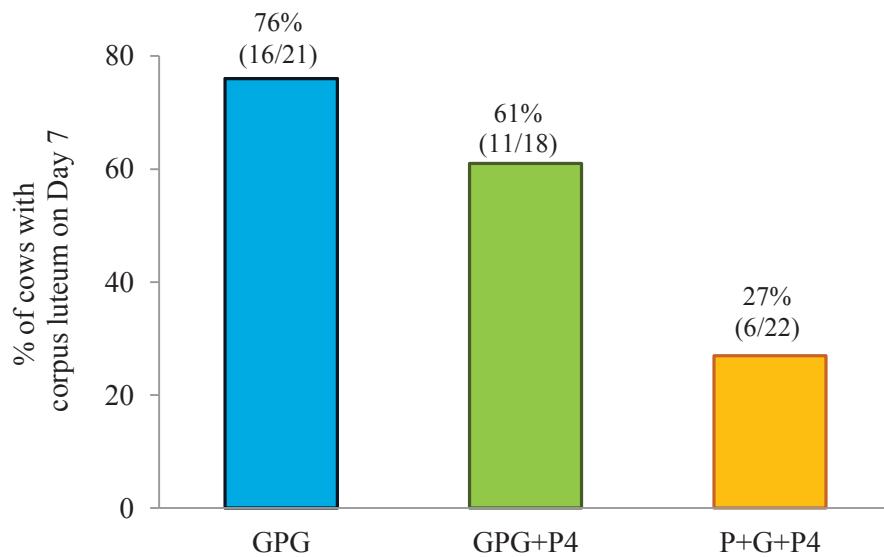


Figure 14.4 Comparison of the proportions of cows (CL negative and CL positive groups together) undergoing different synchronisation protocols with an observable CL on Day 7 ($P = 0.02$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Progesterone concentrations

There was a significant time effect ($P < 0.001$) on progesterone concentration, but there were no effects of treatment, Day 0 CL status or their interaction ($P > 0.05$ for all) on progesterone concentrations on Days 7, 10 and 16 (Figure 15.4). Progesterone concentrations on Day 7 were significantly higher in the GPG+P4 treated CL positive group compared with both the CL positive and negative P+G+P4 groups. There was a significant drop in progesterone concentrations from Day 7 to 10, followed later by a significant rise on Day 16, in all the treatment groups ($P < 0.001$ for all).

Synchronisation of ovulation after giving GnRH on Day 9

The proportion of cows which ovulated within 48 h after the administration of GnRH on Day 9 was 85.7, 88.9 and 81.8% for the GPG, GPG+P4 and P+G+P4 groups, respectively. There was no effect of treatment ($P = 0.79$) or CL status on Day 0 ($P = 0.97$) or interaction between treatment and Day 0 CL status ($P = 0.97$) on the final ovulation risk. There was a significant difference between the treatment groups in the proportion of animals that ovulated at different time intervals ($P = 0.04$; Figure 16.4). Overall, cows treated with GPG had a higher proportion of ovulations in the period between 24 and 36 h after GnRH injection than did the other groups (GPG: 67%, GPG+P4: 50%, P+G+P4: 39%; $P = 0.06$).

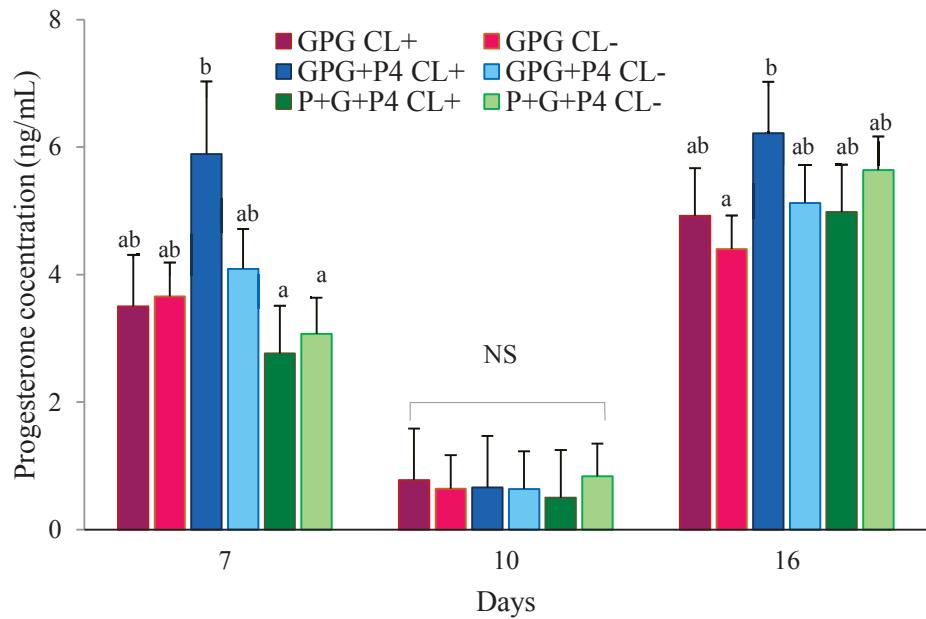


Figure 15.4 Mean plasma progesterone concentrations (\pm SEM) of in both CL negative and CL positive cows treated with GPG, GPG+P4 or P+G+P4 synchronisation protocols. Bars without a common superscript on a specific day are different from each other (Day 7, $P < 0.05$; Day 16, $P = 0.06$); NS: Non-significant. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

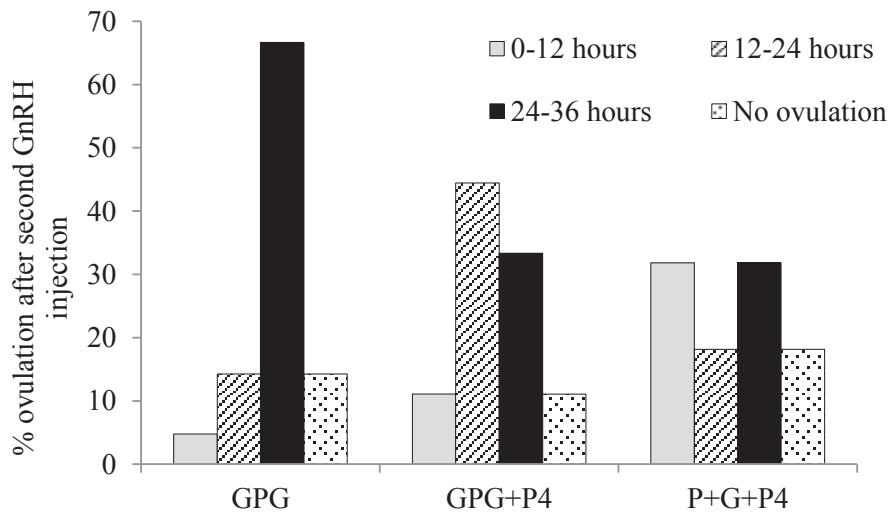


Figure 16.4 Proportions of combined CL negative and positive cows treated with GPG, GPG+P4 or P+G+P4 that ovulated at different time intervals or failed to ovulate after receiving GnRH on Day 9 ($P = 0.04$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 12.4 Distribution of ovulations at different time intervals ($P = 0.04$) after receiving GnRH on Day 9 in combined CL negative and positive cows treated with GPG, GPG+P4 or P+G+P4.

Treatment	Early (0–12 h)	Mid (12–24 h)	Late (24–36 h)	No ovulation within 48 h	Total
GPG	1	3	14	3	21
GPG+ P4	4	3	9	2	18
P+G+P4	7	4	7	4	22
Total	12	10	30	9	61

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Discussion

The aim of the present study was to evaluate three oestrus induction and synchronisation protocols for anoestrous cows. It was postulated firstly, that the inclusion of progesterone in a standard GPG protocol would result in more synchronous follicular development and ovulation and secondly, that omission of the first (Day 0) administration of GnRH from a GPG+P4 protocol would lead to poorer synchrony of ovulation. The first hypothesis is not supported by the present study, inasmuch as neither the proportion of cows that ovulated nor the synchrony of those ovulations differed between the GPG and GPG+P4 groups. Surprisingly, given the plentiful literature that shows the importance of the first GnRH treatment in a GPG programme (Pursley *et al.*, 1995; Pursley *et al.*, 1997; Stevenson *et al.*, 1999; Vasconcelos *et al.*, 1999; Gümen *et al.*, 2003) (i.e. one in which progesterone is not also administered), the present study also showed that there was no difference in the proportion of cows that ovulated between the GPG+P4 and the P+G+P4 groups. Importantly, these results were achieved in the face of a remarkable difference in the proportions of CL negative cows that had developed active corpora lutea by Day 7 of the protocol (GPG: 76%, GPG+P4: 61%, P+G+P4: 27%). The third hypothesis, that postpartum energy balance could affect the response to oestrus synchronisation, was supported by the fact that cows which failed to ovulate in response to GnRH injection on Day 9 had higher circulating concentrations of NEFA on Day 7 compared with those that did ovulate a response that was independent of synchronisation protocol.

The final ovulation risk of 85.2% across all groups in response to GnRH given on Day 9 in the current study compares with previous reports of 73–95% (Fricke and Wiltbank, 1999;

Vasconcelos *et al.*, 1999; Moreira *et al.*, 2001; Stevenson *et al.*, 2006) in cycling cows and 94% in CL negative cows (Gümen *et al.*, 2003), and is a little higher than the 51% reported by Cartmill *et al.* (2001). The small differences are possibly attributed to relatively small numbers of animals and a consequent lack of power in each of the studies.

The lack of any differences between the ovulation responses to different treatment regimens in cows of different initial luteal status in the present study cannot readily be attributed to the characteristics of the dominant follicle at the start of the study, since, on Day 0, there were no differences in the size of the dominant follicles either between cows in different treatment groups, or between those that were CL positive or CL negative. Indeed, it was clear that, because the dominant follicle at this time in the ovary was >9 mm in all animals, they all had follicles that would be expected to have been responsive to gonadotrophin. This notion is supported by the finding that oestradiol concentrations on Day 0 in CL negative cows were similar between all three synchronisation protocols, as circulating oestradiol concentrations increase in parallel with the growth of dominant follicles during both follicular and early luteal phases (Kaneko *et al.*, 1991). Conclusions in regard to CL positive cows need to be guarded as they were not the primary focus of this study and the small number of animals meant that, particularly for dominant follicle size the study lacked sufficient power to detect biologically important differences

Interestingly, there was no difference in the time to emergence of a new follicular wave between the GPG and the GPG+P4 groups (GPG: 1.9 ± 0.3 days, GPG+P4: 1.9 ± 0.2 days); these intervals are one day earlier than the previous reports of 2.9 days in cycling dairy cows after GnRH treatment (Kim *et al.*, 2005). From these data, it can be concluded that neither the process of ovulation in response to the Day 0 GnRH treatment, nor the interval to follicle emergence, were affected by any negative feedback effects of administered progesterone upon the gonadotrophic axis. Thus, although LH concentrations were not measured, it would seem likely that animals in the GPG and GPG+P4 groups were both similarly competent at developing an LH surge in response to exogenous GnRH. Likewise, the similar, and normal, interval to emergence of a new follicular wave also indicates that the FSH-based stimulation of the follicular wave was unaffected by the presence or absence of progesterone. Again, this notion is supported by the lack of a significant difference in follicle diameter and the similar growth rates of the dominant follicle between the GPG and GPG+P4 groups (GPG: 1.3 ± 0.2 mm/day, GPG+P4: 1.5 ± 0.2 mm/day), values that are also compatible with previous reports of 0.9 to 1.3 mm/day in GPG treated dairy (Pursley *et al.*, 1995) and beef (Perry *et al.*, 2005) cows.

As expected, there were significant increases in progesterone concentration from Day 0 to Day 7 in all groups of animals. In the CL negative cows, progesterone concentrations on Day 7 did not differ significantly between groups, which suggests that the presence of a progesterone insert (GPG+P4 and P+G+P4 groups) did not elevate progesterone concentrations above those produced by an induced endogenous CL alone (i.e. in the GPG group) nor, in the GPG+P4 group, did the insert raise concentrations above those in cows that had not received an insert (GPG). The result in the P+G+P4 group is curious, as some studies of beef (Kojima *et al.*, 1992; Stevenson *et al.*, 2003) and dairy (Nation *et al.*, 2000) cows indicate that the inclusion of a progesterone device provides relatively small increases (0.5 to 2.8 ng/mL) in serum progesterone concentrations. However, no other source of progesterone was identified, so it is difficult to account for the unexpectedly high concentrations in this group. In contrast, in the CL positive group, progesterone concentrations on Day 7 were significantly higher in GPG+P4 cows than in the P+G+P4 group. Again, this result is difficult to explain in terms of combinations of pre-existing or induced corpora lutea, even if there was a small augmentation of progesterone concentrations due to the insert. Regardless, the animals with markedly elevated progesterone concentrations on Day 7 did not differ from other animals in terms of their ovulation response on Day 9, so it is difficult to attribute biological significance to these data. All cows, regardless of treatment group or CL status, had basal (<1 ng/mL) progesterone concentrations on Day 10, which indicates that complete luteolysis had occurred in all cows with active progesterone-secreting structures. This finding agrees with the work of Pursley *et al.* (1995), who also reported a 100% response to the PGF_{2α} administration on Day 7 in GPG treated cows.

There were, however, significant differences in the distribution of the time of ovulation between groups. Overall, cows treated with GPG, regardless of their CL status at the start of the experiment, had a significantly ($P = 0.06$) higher proportion of ovulations in the period between 24 and 36 h after GnRH administration (66.7%) than did other groups (GPG+P4: 50%, P+G+P4: 38.9%). These data closely agree with the findings of Pursley *et al.* (1995), who reported that the highest proportion of ovulations (60%) occurred at 28h after final GnRH administration. (Pursley *et al.*, 1998).

The first administration of GnRH treatment in a GPG programme is intended to induce atresia/ovulation of the dominant follicle, thereby resulting in the emergence of a new follicular wave within approximately 2 days (Macmillan and Thatcher, 1991; Twagiramungu *et al.*, 1995; Martinez *et al.*, 1999). In the present study, there was no difference between treatment groups in the percentages of CL negative cows which underwent either ovulation or atresia of the original dominant follicle after the start of the synchronisation protocol (Table 4.4). However,

emergence of the new follicular wave tended ($P = 0.09$) to be 0.7 days later in cows treated with the P+G+P4 protocol than in those that had received GnRH on Day 0. In the GPG+P4 and GPG groups, the new dominant follicle reached a diameter of >11mm by Day 2–3, after which its size underwent only a slow increase until around Day 7. By contrast, the size of the dominant follicles that did not ovulate at the start of treatment in the P+G+P4 group increased between Days 0 and 2, thereafter regressing between Days 3 and 4. The new wave that emerged on Day 2.6 ± 0.3 in this group grew rapidly between Days 4 and 9, such that it was significantly larger by Day 9. It is difficult to explain these differences in growth rate with respect to progesterone, as, between Days 0 and 9, its concentration did not differ between groups. Hence, it appears that the persistence of the dominant follicle in the P+G+P4 group, together with a largely unregulated emergence of a new follicular wave in the middle of the synchronisation protocol allowed for a greater rate of growth in the dominant follicle of that group compared to the GPG or GPG+P4 groups. Regardless, the size of the dominant follicle on Day 9 did not affect its ability to ovulate in response to the final GnRH treatment. Whether the fertility of the larger follicle in the P+G+P4 group would have been affected is unclear: on one hand, some studies show that larger follicles result in higher conception rates than do smaller ones (Perry *et al.*, 2005; Bello *et al.*, 2006; Lopes *et al.*, 2007) whilst, on the other hand, the age of the dominant follicle appears to be of greater significance than does size *per se* in both dairy and beef cows (Mihm *et al.*, 1994; Ahmad *et al.*, 1997; Austin *et al.*, 1999; Townson *et al.*, 2002).

The interval between calving and the start of the synchronisation protocols (9–10 weeks), together with the small overall change in weight before, during and after the experiment (Table 2.4), indicates that the animals used in this experiment had probably passed through the peak period of negative energy balance. McDougall *et al.* (1993) showed that the period of negative energy balance typically lasts for 4 to 14 weeks postpartum, and that anoestrous cows tend to have a longer, and more significant negative energy balance than do those cows that resume oestrous cycles by the start of mating. Hence, it was notable that NEFA concentrations were significantly higher on Days 0, 7 and 9, and insulin concentrations were significantly lower on Day 0 in cows that failed to ovulate in response to the synchronisation protocol compared with cows that did ovulate. These findings are supported by previous *in vitro* studies in which the elevated NEFA had a detrimental effect on follicle cell viability and function (Leroy *et al.*, 2005; Vanholder *et al.*, 2006) and resulted in retardation of luteal growth due to a decline in propagation of the granulosa cells by the elevated NEFA (Zulu *et al.*, 2002b; Jorritsma *et al.*, 2004). Similarly, the areas under the curves of insulin and IGF-I concentrations were numerically lower in cows that did not ovulate than in cows that did. Insulin and IGF-I are well known to have positive effects on ovarian cells, including the stimulation of granulosa cell proliferation, production of progesterone and enhancing luteal cell steroidogenesis

(Echternkamp *et al.*, 1994; Beam and Butler, 1998; Butler *et al.*, 2004; Sartori *et al.*, 2013). It is also well recognised that low postpartum insulin concentrations suppress hypothalamic GnRH secretion and concurrent pituitary LH release (Diskin *et al.*, 2003). Whilst it is feasible that inadequate insulin concentrations could have affected the development of follicles in a way that resulted in anovulation in these animals, neither the endocrine data (oestradiol and progesterone) nor the size of the Day 9 follicle provide any clear indication that this was the case. Nonetheless, the clear and significant relationship between NEFA concentrations and ovulation in response to all synchronisation protocols is a key finding of the present study, which shows that, regardless of the regimen that is used to treat anoestrus, the response will be moderated and limited by the degree of negative energy balance.

Conclusions

The present study has shown that the previously reported beneficial effects of the inclusion of a progesterone device in a GPG programme might not be mediated via the preovulatory follicular dynamics or synchronised ovulation in postpartum anoestrous dairy cattle. Moreover, the exclusion of Day 0 GnRH treatment from the GPG+P4 programme had no impact on synchronisation of ovulation after Day 9 GnRH and it was not different from a GPG and a GPG+P4 programme. The standard GPG programme resulted in a better synchronisation of ovulation and a smaller spread of ovulations after the end of the treatment than did the other treatments. The energy balance of the postpartum cows could be an important factor in getting an optimum treatment response in anoestrous cows, as those cows that did ovulate in response to Day 9 GnRH treatment had a relatively better energy status than those that did not. Further research into the effects of negative energy balance on conception rate will give a better understanding of the role of energy status in determining the outcome of any synchronisation programme. Furthermore, studies of several other factors determining the energy balance and affecting the reproductive axis could help in better understanding the relationship of the response to hormonal treatment and energy balance.

Acknowledgements

This research was supported by a New Zealand International Doctoral Research scholarship and a Massey University Doctoral Research Scholarship. The product provided by Bomac Laboratories is gratefully acknowledged. The contributions of the farm manager of Massey University No.4 Dairy Farm and undergraduate veterinary students of Massey University are acknowledged.

CHAPTER 5

Comparison of two oestrus synchronisation protocols on follicular dynamics, plasma hormone concentrations and timing of ovulation in pasture-based, seasonal calving dairy cows in New Zealand

Abstract

This study compared the effects of GPG (GnRH, PGF_{2α} and GnRH) and GPG+Progesterone (P4) programmes on synchronisation of ovulation, and follicular and hormonal (P4 and oestradiol) profiles in postpartum anoestrous dairy cows. Friesian and Friesian x Jersey cows ($n = 22$) without a corpus luteum were randomly allocated to two oestrus synchronisation programmes. The first group (GPG+P4, $n = 11$) received 100 µg GnRH on Day 0, a progesterone releasing intravaginal device from Day 0 to Day 7, 500 µg PGF_{2α}, on Day 7 and 100 µg GnRH on Day 9, followed by fixed-time artificial insemination 16–20 h later. The programme for Group 2 (GPG, $n = 11$) was the same as Group 1, with the exclusion of the progesterone device. Daily transrectal ultrasonography was performed from Days 0 to 8 and then at 12 hourly intervals between Days 9 and 11. Blood samples were collected on Days 0, 1, 2, 3, 7, 8, 9, 12, 16 and 22 for determination of plasma oestradiol and progesterone concentrations.

There was no difference in the interval between the first GnRH administration and new follicular wave emergence between groups (GPG+P4: 3.3 ± 0.6 days, GPG: 2.4 ± 0.2 days; $P = 0.28$). The diameter of the ovulatory follicle after the first GnRH was significantly larger in the GPG group than in the GPG+P4 cows (16.6 ± 1.0 mm *versus* 14.0 ± 0.4 mm; $P = 0.04$), but there was no difference in the size of the preovulatory follicle after the second GnRH treatment (GPG: 17.1 ± 1.0 mm, GPG+P4: 15.2 ± 0.7 mm; $P = 0.14$). Whilst the GPG programme resulted in a relatively larger dominant follicle and higher peripheral oestradiol concentration than did the GPG+P4, there was no difference in synchronised ovulation within 48 h after Day 9 GnRH injection between the GPG+P4 and GPG groups ($P = 0.09$).

This study concluded that there was no difference in final synchronised ovulation between GPG+P4 and GPG treatment. However, the development of larger dominant follicle and presence of higher circulating oestradiol concentrations in the GPG compared to the GPG+P4

group may suggest that follicles in the GPG cows were physiologically better developed than were those in the GPG+P4 animals.

(**Key words:** anovulatory anoestrus, GPG, progesterone, synchronisation)

Introduction

Anovulatory anoestrus is a significant problem within the New Zealand dairy industry, as cows which are not observed in oestrus prior to the start of the breeding season (non-cyclers) have significantly lower conception and pregnancy rates than cows which are seen in oestrus (Xu and Burton, 2000; McDougall, 2001). Typically, approximately 20% of New Zealand dairy cows are classified as anoestrous, of which most have anovulatory anoestrus rather than representing a failure of oestrus detection (Rhodes *et al.*, 2003). This incidence of anoestrus has a major impact on the profitability of dairy farming in New Zealand, principally through delayed conception as well as failure to conceive by the end of the breeding season (McDougall and Rhodes, 1999; McDougall, 2010a).

In most anoestrous cows, there is a sequence of follicular waves prior to the first ovulation (McDougall *et al.*, 1995). A failure of the preovulatory surge of gonadotrophins, perhaps due to insufficient production of oestradiol by these follicles, may be the key mechanism preventing the resumption of cyclical activity (Chenault *et al.*, 1975; Beck and Convey, 1977; Kesler *et al.*, 1977). Hormonal intervention is the main treatment options for such cows, which aims to induce ovulation by stimulating maturation of ovarian follicles.

Before 2007, most programmes for the induction and synchronisation of oestrus in adult dairy cows in New Zealand were based on oestradiol benzoate and progesterone. However, in 2007, oestradiol treatments for dairy cows were prohibited in New Zealand following a policy change in Europe (European Union, 2003), leading to the loss of the most commonly used treatment programme (Lucy *et al.*, 2004; McDougall, 2010a). Treatments were revised, with GnRH being substituted for oestradiol, whilst retaining progesterone as the basis of the programme (McDougall, 2010a). These changes led to a new recommended treatment protocol for anoestrous dairy cows: a GPG/fixed-time artificial insemination (FTAI) + Progesterone programme (GPG+P4: two treatments of GnRH 9 days apart, a progesterone releasing intravaginal device from Day 0 to Day 7, PGF_{2α} injection on Day 7, followed by FTAI 16–20 h after the second GnRH injection; Laven, 2008) .

Whilst GPG programmes are well established as a means for synchronising oestrus in dairy cows, they have not proved particularly effective in treating anovulatory anoestrus (McDougall,

2010a). Progesterone has long been used as a means of synchronising oestrus, either by itself, or in combination with other hormones, particularly PGF_{2α} (Lammond, 1967a, b; Cooper, 1974; Macmillan, 1990; Darwash *et al.*, 1999). More recently, progesterone has been included in GPG programmes, typically between the first GnRH and the PGF_{2α} treatments, as a means of improving the effectiveness of synchronisation protocols. Such improvements are largely based upon the risk that a GPG programme (i.e. one that lacks exogenous progesterone) can lead to a short luteal phase after ovulation of the induced follicle (Sheffel *et al.*, 1982; Gümen *et al.*, 2003; McDougall, 2010a), leading to lower conception rates than in cycling cows (Moreira *et al.*, 2001). Indeed, several North American reports have shown that conception rates increased by 10 to 20% by using a combination of progesterone with a GPG programme in dairy (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b) and beef (Lamb *et al.*, 2001) cows. For example, El-Zarkouny *et al.* (2004) reported increased conception rates in cyclic lactating dairy cows treated with GPG+P4 compared with GPG alone (Day 29; 59.3% *versus* 36.3%; Day 57; 45.1% *versus* 19.8%). Conversely, other reports saw no improvement by the addition of progesterone: for example, Stevenson *et al.* (2006) found no improvement in 56-day conception rates in cycling (GPG = 39%, GPG+P4 = 43%) or non-cycling (GPG = 39%, GPG+P4 = 34%) lactating dairy cows.

Studies undertaken under New Zealand conditions have also corroborated North American evidence that a GPG+P4 programme results in higher conception rates than GPG alone ($46 \pm 3\%$ *versus* $34 \pm 2\%$; McDougall 2010a). However, the exact mechanism of action of the progesterone device in such programmes has yet to be elucidated. The improved conception rate may be due in part to either the premature oestrus that occurs in 5–11.8% of cows between the first GnRH and the PGF_{2α} injections (Roy and Twagiramungu, 1999; DeJarnette *et al.*, 2001); or the positive effects of progesterone on the endometrium (Clemente *et al.*, 2009) and conception rate (Fonseca *et al.*, 1983; Folman *et al.*, 1990); or the lower incidence of short luteal phases at the end of the synchronisation protocol.

Most of the studies comparing the efficacy of GPG and GPG+P4 programmes have been based on assessing conception and pregnancy rates rather than evaluating the effects of GPG and GPG+P4 treatment on ovarian follicle and luteal development. This is particularly the situation for cattle with postpartum anovulatory anoestrus, which is the most common reason for treatments of dairy cattle in New Zealand with synchronisation programmes (McDougall *et al.*, 2001; McDougall and Compton, 2008; McDougall, 2010b, a). It is important to understand the role of progesterone in a GPG programme, inasmuch as whilst it increases conception rate it also markedly increases the total cost of the programme. Identifying the mechanisms by which progesterone improves conception rate could provide valuable information on how to improve

the response of anoestrous cows to GPG-based programmes without the expense of an intravaginal progesterone-releasing device

The aim of this study was to therefore evaluate how the addition of a progesterone device to a GPG programme in dairy cows with postpartum anovulatory anoestrus affected ovarian follicular dynamics and the synchronisation of ovulation.

Materials and methods

All animal use was approved by Massey University Animal Ethics Committee, Palmerston North.

This study was conducted at Massey University's No. 4 Dairy Farm, a spring-calving pasture-based 460-cow dairy farm in Palmerston North (latitude 40°2'S, longitude 175°4'E), New Zealand, during spring (September to November) 2011.

Animals

Nine days before the planned start of the breeding season, all lactating cows which had been calved for >30 days, had not had an assisted calving, had not been diagnosed with endometritis, and had not been recorded in oestrus were examined. Oestrus detection had been undertaken by farm staff using a combination of tail paint and observation from 40 days prior to the planned start of mating. The reproductive tracts of all the selected animals ($n = 22$) were examined by trans-rectal ultrasonography and manual palpation *per rectum* to confirm the absence of utero-ovarian pathology as well as the presence or absence of a corpus luteum (CL). Cows with a corpus luteum in either of the ovaries, any abnormalities of the puerperium, or any evidence of uterine infections or adhesions or other pathology of the ovaries or uterus were excluded from the study. Body condition score (BCS, scale 1 to 10: Macdonald and Roche, 2004) of all the animals was also recorded at the time of enrolment.

Synchronisation protocols

Using a random allocation sheet (created using MS Excel, 2007), the cows were allocated to one of the two treatment groups, based on their order through the race on Day 0 (Figure 1.5). Treatments were: (1) Day 0: 100 µg i/m GnRH (Ovurelin, Bomac Laboratories Ltd, Auckland, New Zealand) and placement of a progesterone-releasing intravaginal device (1.56 g progesterone: Cue-Mate, Bomac Laboratories Ltd); Day 7: 500 µg cloprostol i/m, (Ovuprost, Bomac Laboratories Ltd) and removal of the progesterone device; Day 9: a second dose of GnRH. Fixed-time AI (FTAI) was performed on Day 10 (16–20 h after the second GnRH

treatment) (GPG+P4; n = 11); (2) As for Group 1, with the exclusion of the progesterone-releasing intravaginal device (GPG; n = 11).

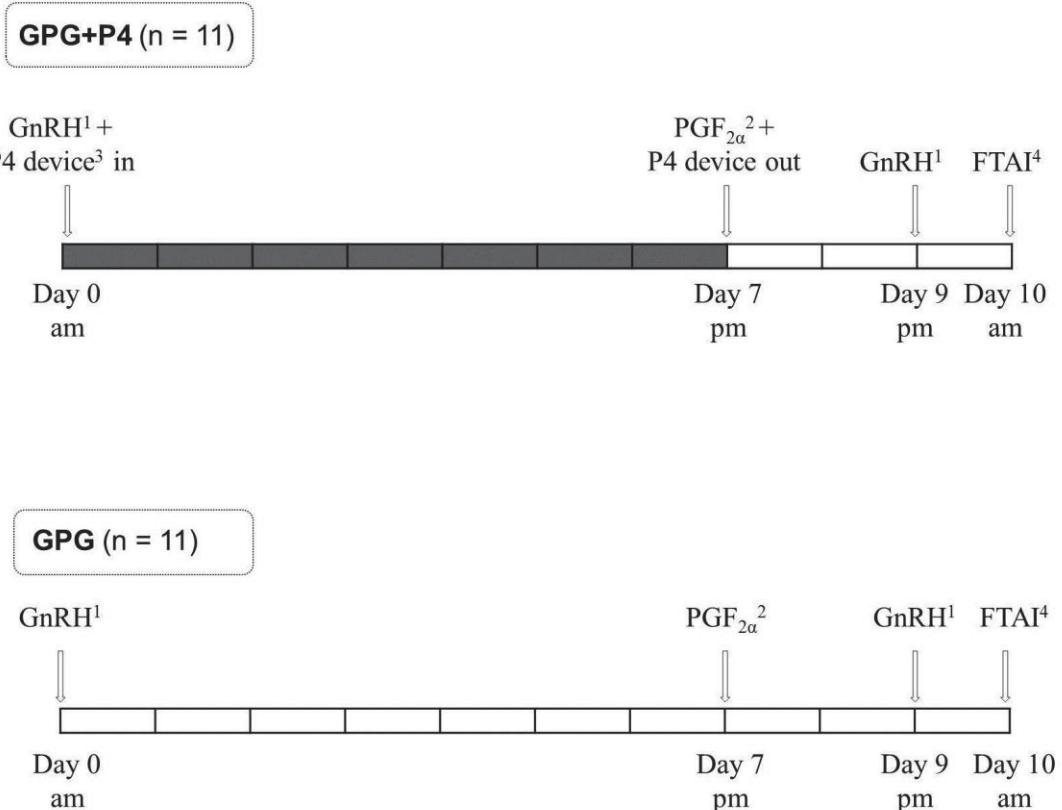


Figure 1.5 Synchronisation protocol (grey shading indicates progesterone releasing intravaginal device).

¹ 100 µg GnRH, i/m.

² 500 µg PGF_{2α} i/m.

³ Progesterone releasing intravaginal device containing 1.56 g of progesterone.

⁴ Fixed-time artificial insemination.

Ultrasonography

Ovarian structures of all the cows were monitored and studied as previously described (Pierson and Ginther, 1984) using a real time B-mode ultrasound scanner (DP-6600 Vet, Mindray, Szechuan, China), equipped with a variable linear transducer set at 7.5 MHz. Ultrasonography was performed on Days 0 to 9. On each occasion, digital ultrasound images of both ovaries were recorded and a corresponding ovarian map was also drawn manually on the recording sheet to locate and identify the structures on the ovary, particularly the presence or absence of a CL.

The impact of treatment and time on follicular dynamics was evaluated by measuring the size of the largest dominant follicle using ImageJ (v1.46d, National Institutes of Health, USA). The diameter of the dominant follicle was estimated by taking the average of two measurements: (i) the size at the widest point and (ii) the size at right angles to the first measurement.

Between Days 0 and 7, the response of the dominant follicle to treatment (persistence, ovulation or atresia) was studied. Ovulation was defined as the disappearance of a dominant follicle followed by the development of a CL (or accessory CL) and atresia was defined as the disappearance of a dominant follicle followed with no CL development. Persistence was defined as no disappearance of the dominant follicle.

The time to the emergence of a new follicular wave was identified as the day, within 7 days of the first treatment with GnRH, that the dominant follicle was retrospectively identified to have had a diameter of ≥ 4 mm. If the dominant follicle was not detected until it reached 6 or 7 mm, the previous day was taken as the first day (Ginther *et al.*, 1989b). The growth rate of the preovulatory follicle was established from the diameter reached on Day 9, minus the diameter on the day of its detection divided by the number of the days.

Timing of ovulation after Day 7 was determined by ultrasound examination of the ovaries every 12 h from the morning of Day 9 until the afternoon of Day 11; or ovulation, whichever was sooner. Ovulation was defined as the disappearance of a previously identified dominant follicle of ≥ 9 mm diameter.

Blood samples and hormone assays

Blood samples (10 mL) were collected via coccygeal venepuncture into heparinised vacutainers (Becton Dickinson New Jersey, USA) on Days 0, 1, 2, 3, 7, 8, 9, 12, 16 and 22 for determination of plasma oestradiol and progesterone concentrations. Plasma was separated by centrifugation at 1500 g for 20 min at 40°C within 2 h of collection. The plasma duplicates were taken and then stored at -20°C until assay.

Progesterone assay

Plasma progesterone concentrations were measured in duplicate 10 μ L aliquots by radioimmunoassay, using the ImmuChem Double Antibody Progesterone ^{125}I RIA kit for *in vitro* diagnostic use (MP Biomedicals, USA). The sensitivity of the assay was 0.14 ng progesterone/mL. The intra-assay coefficients of variation at 80, 50 and 20% binding on the standard curve were 16.1, 8.4 and 9.9% respectively; the inter-assay coefficients of variation were 19.1, 14.4 and 15.7% for low, medium and high solutions, respectively. The assay was

validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer (see Appendix 1 for a description of the method).

Oestradiol assay

Plasma oestradiol concentrations were measured in duplicate 40 µL aliquots by radioimmunoassay, using Ultra-Sensitive Estradiol ^{125}I RIA kit, (Immunotech, Czech Republic). The sensitivity of the assay was 3.3 pg oestradiol/mL. The intra-assay coefficients of variation at 60 and 20% binding on the standard curve were 10.8 and 7.1% respectively; the inter-assay coefficients of variation were 14.8 and 4.0% for low and high solutions, respectively (see Appendix 2).

Statistical analyses

Except where noted, all statistical analysis was performed using the Prism statistical package (v 5.03, GraphPad Software, Inc. La Jolla, USA). The key outcome variables were the diameter of the dominant follicle from Day 0 to 9, diameter of the preovulatory follicle, and growth rate of preovulatory follicle after Day 0. Secondary objectives were measurement of the proportion of cows ovulating in response to GnRH injection on Day 0 and Day 9; proportion with atresia of dominant follicle between Day 0 and Day 7; interval from Day 0 to the emergence of the follicular wave producing the dominant follicle on day 9; presence or absence of CL on Day 7 and 9, timing of ovulation after GnRH injection on Day 9; and plasma concentration of progesterone and oestradiol.

The balance of the treatment group for age and BCS was compared by one-way ANOVA to ensure that the groups were balanced for these factors which are known to have a key influence on fertility (McDougall, 2001; Roche *et al.*, 2007). The proportion of cows with atresia or ovulation and new follicular wave emergence within 7 days of treatment was compared by Fisher's exact test (two tailed). The interval to new wave emergence was analysed by Student's t-test. Age of the dominant follicle on Day 9 and the growth rate of the preovulatory follicles of new wave were analysed by Student's t-test. The proportion of cows which ovulated within 48 h after the Day 9 GnRH treatment, and the proportions of cows which ovulated at different time points (i.e. 12, 24, 36, 48 h), were compared using Fisher's exact test (two tailed). The areas under the curve (AUC) of oestradiol concentrations were calculated using the trapezoid rule and further comparison between the treatments was made by Student's t-test. Comparison of the progesterone concentrations on Day 16 and 22 for the cows that were recorded as having ovulated with those that did not was undertaken using Student's t-test.

Endocrine and follicular size data were subjected to repeated-measures analysis of variance with respect to treatment and time, using the MIXED procedure of SAS (v 9.3, Statistical analysis system, SAS Institute Inc., Cary, North Carolina, USA) with treatment as a fixed effect, day of measurement as a repeated fixed effect and cow as a random effect. Progesterone data from Days 0 to 7, and from Day 8 until the end of the experiment, were analysed separately. An unstructured covariance was used based on the lowest Akaike Information Criterion (Littell *et al.*, 1996).

The effects of treatment and of the sizes of the dominant follicle on Days 7 and 9 upon the ovulation response to the second GnRH injection were modelled by using binary logistic regression in Minitab (Logit Link function: Minitab v16, Minitab Inc., State College, PA, USA). The dependent variable was ovulation in response to the second GnRH injection, which was treated as a binomial variable with a logit transformation (1 = ovulated, 0 = not ovulated). The independent variables were treatment and dominant follicle size on Days 7 and 9 and all two-factor interactions. To avoid covariance due to two similar variables, a matrix plot of all the independent variable was first generated. Those with a P value of <0.2 were kept in the model, unless there was a significant correlation ($r>0.7$) between the predictor variables when the variable with the smallest P value was kept in the model. A backward elimination of nonsignificant factors were done one by one until all the variables were significant ($P\leq0.05$) in the model. Preliminary final model was checked by diagnostic plots involving event probability (delta chi square vs. probability) and leverage (delta chi-square vs. leverage) and goodness of fit tests and if there were no any unusual observations and P value of goodness of fit test was >0.05 then the model was declared final.

Results

One cow in the GPG+P4 group with a sustained large follicular diameter of 26.6 ± 0.7 mm from the beginning the experiment to the final days of observation was excluded from all the data analyses, as this animal was considered to have had a follicular cyst. Consequently, 21 animals were included in the final data analysis. There was no difference in age (GPG: 57.2 months, GPG+P4: 50.8 months; $P = 0.42$), BCS (GPG: 3.8, GPG+P4: 3.6; $P = 0.35$) and days post-partum at the start of the experiment (GPG: 32.6 days, GPG+P4: 32.2 days; $P = 0.90$) between the two treatment groups.

Ovarian dynamics from Day 0 to Day 9

Follicular development

The findings are summarised in Table 1.5 and Figure 2.5. The percentages of cows with follicular wave emergence within 7 days of treatment was not significantly different between the two treatment groups (GPG: 63.6%, GPG+P4: 70.0%; P>0.05), nor was the mean interval from treatment to follicular wave emergence (P = 0.28; Table 2.5). Similarly, there was no difference (P>0.05) in the proportion of cows which ovulated after the start of the treatment. The first ovulation percentage was 54.5 and 60% for the GPG and GPG+P4 groups, respectively.

The mean diameter of the ovulatory follicle after the first GnRH injection was significantly greater in the GPG than the GPG+P4 group (P = 0.04). The diameter of the dominant follicles of new wave changed markedly over time (P = 0.01; Figure 2.5). There was also an effect of treatment (P = 0.02), but there was no interaction between time and treatment (P = 0.93). The mean diameter of the dominant follicle was numerically greater in the GPG than the GPG+P4 group although it did not differ significantly between groups on any day (including Day 9, P = 0.59; Figure 3.5). However, on Day 9, there was more variation in the size of the dominant follicle in the GPG than the GPG+P4 group (Figure 3.5). The growth rate (mm per day) of the dominant follicle was also not different between the groups (GPG+P4: 1.0 ± 0.2 GPG: 0.9 ± 0.1; P = 0.68), nor was the age of the dominant follicle on Day 9 (P = 0.30).

Corpus luteum status

The proportions of cows with a CL on Days 0, 7 and 9 are shown in Table 2.5. There was no effect of treatment on CL status at any time point.

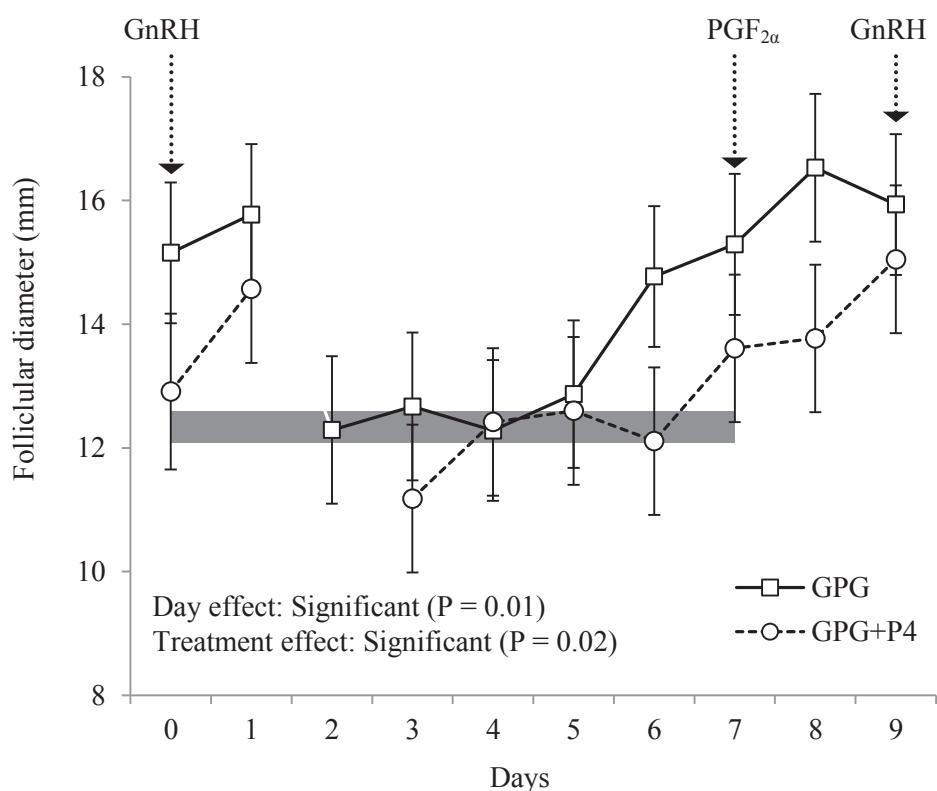


Figure 2.5 Follicular development in GPG and GPG+P4 groups from Days 0 to 9 (least square means \pm SEM). Grey shading represents the progesterone releasing intravaginal device between Days 0 and 7 in the GPG+P4 group. Time to emergence of the new follicular wave was 2.4 ± 0.2 days and 3.3 ± 0.6 days, respectively, for the GPG and GPG+P4 groups ($P = 0.28$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

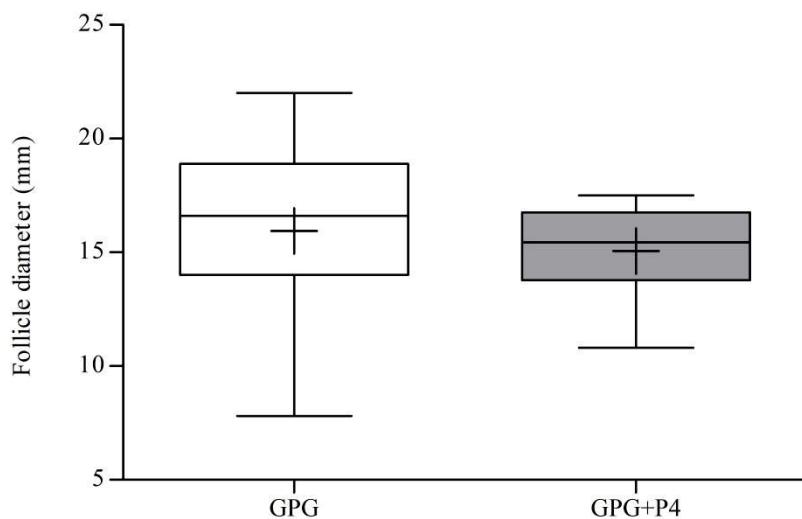


Figure 3.5 Comparison of Day 9 follicular diameter between GPG and GPG+P4 groups ($P = 0.59$). '+' shows the mean value in each group. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 1.5 Changes in follicular structures present in the ovary of cows between Days 0 and 9 of GPG and GPG+P4 synchronisation protocols.

	GPG (n= 11)	GPG+P4 (n = 10)
Atresia ¹	1 (9%)	1 (10%)
Ovulation ²	6 (55%)	6 (60%)
Cows with new follicular wave	7 (64%)	7 (70%)
Interval to follicular wave emergence (days)	2.4 ± 0.2	3.3 ± 0.6
Ovulatory follicle diameter after first GnRH (mm)	16.6 ± 1.0	14.0 ± 0.4
Age of the dominant follicle on Day 9 (days)	6.6 ± 0.2	5.8 ± 0.6

¹Disappearance of a previously identified dominant follicle followed by no development of corpus luteum.

²Disappearance of the dominant follicle followed by the development of corpus luteum or accessory corpus luteum.

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 2.5 Proportion of cows in each treatment group with a visible corpus luteum at different time point of the synchronisation protocol.

	GPG (n= 11)	GPG+P4 (n = 10)
CL Day 0	0	0
CL Day 7	9	7
CL Day 9	7	8

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Follicular dynamics – ovulatory follicle

Seventeen of the 21 cows in the study had a synchronised ovulation within 48 h after the final (Day 9) GnRH injection and four failed to respond. The mean diameter of the preovulatory follicle that ovulated within 48 h of Day 9 GnRH injection was not different between the treatments ($P = 0.14$). Table 3.5 shows the number of cows in each treatment group which ovulated in response to the injection of GnRH.

The difference in the overall proportion of cows with synchronised ovulation within 48 h after Day 9 GnRH injection approached, but did not reach, significance (GPG: 63.6 % [7/11], GPG+P4: 100 % [10/10]; P = 0.09, Figure 4.5). The median survival time of ovulation after the second GnRH injection was 24 h for both groups.

Table 3.5 Activity of the ovulatory follicle in cows undergoing synchronisation protocols with GPG and GPG+P4.

	GPG (n = 11)	GPG+P4 (n = 10)
Did not ovulate	4	0
Ovulated with 48 h of Day 9 GnRH (synchronised ovulation)	7	10
Mean ± SEM (range) maximal preovulatory follicle size of cows that ovulated within 48 h of Day 9 GnRH (mm)	17.1 ± 1.0 (12.8–20.6)	15.2 ± 0.7 (12.2–20.5)

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

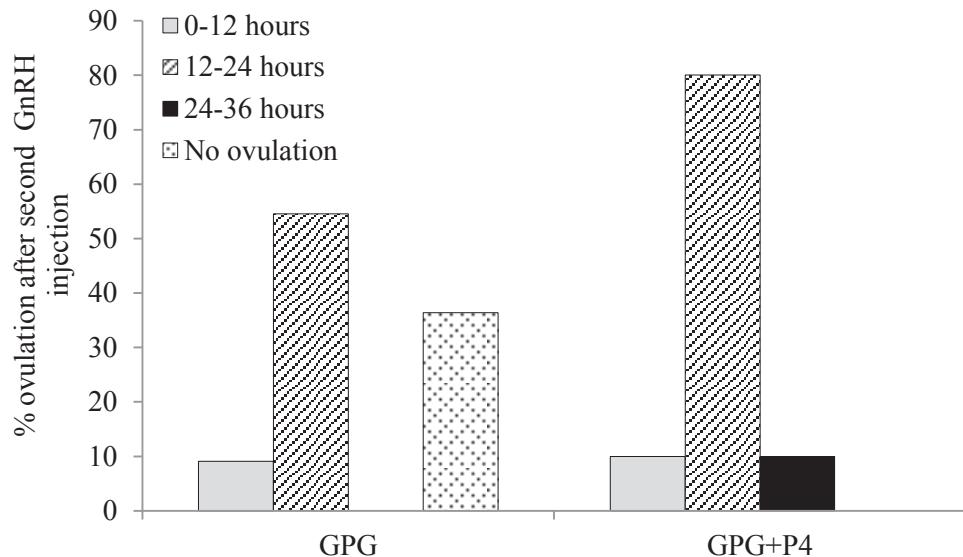


Figure 4.5 Distribution of proportion of ovulations at different time intervals after the administration of GnRH on Day 9 to GPG and GPG+P4 groups (P = 0.09). ‘No ovulation’ denotes the cows that failed to ovulate. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Relationship between follicle size and ovulation

There was no effect of treatment or the size of the dominant follicle on Day 7 on the ovulation response to Day 9 GnRH treatment ($P = 0.54$ and 0.31 respectively; 14.8 ± 0.7 mm for ovulated versus 13.8 ± 1.6 mm for not ovulated). However, effects of the Day 9 dominant follicle were closer to statistical significance ($P = 0.07$), such that the probability of ovulation was higher if the dominant follicle size on Day 9 was larger (16.6 ± 0.4 mm for ovulated *versus* 13.4 ± 1.7 mm for not ovulated).

Progesterone concentrations

Progesterone concentrations are shown in Figure 5.5. Between Days 0 and 7 there was a significant interaction between treatment and time (days) ($P < 0.001$ for all), such that the progesterone concentrations on Days 1, 2 and 3 were significantly higher in the GPG+P4 than the GPG group. In contrast, after Day 7, the only significant effect was that of time ($P < 0.001$).

Progesterone concentrations in cows that did, or did not, ovulate (regardless of treatment group) were similar between Days 8 and 12. Thereafter, progesterone concentrations diverged, such that concentrations were higher on Days 16 and 22 in cows that ovulated than in those that did not (Figure 6.5).

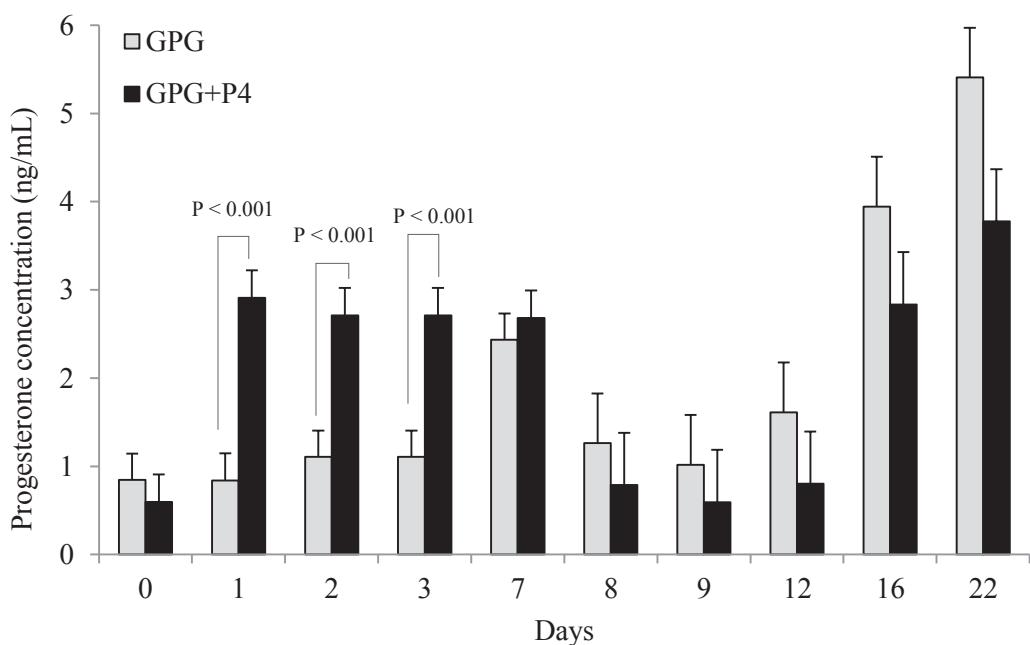


Figure 5.5 Comparison of progesterone concentrations between GPG and GPG+P4 groups. The values are presented as least square means \pm SEM. Between Day 0 and 7 there was an interaction between treatment and time ($P < 0.001$). From Day 8 onwards there was only a significant effect of time ($P < 0.001$). Abbreviations: G, GnRH; P, PGF 2α ; P4, progesterone.

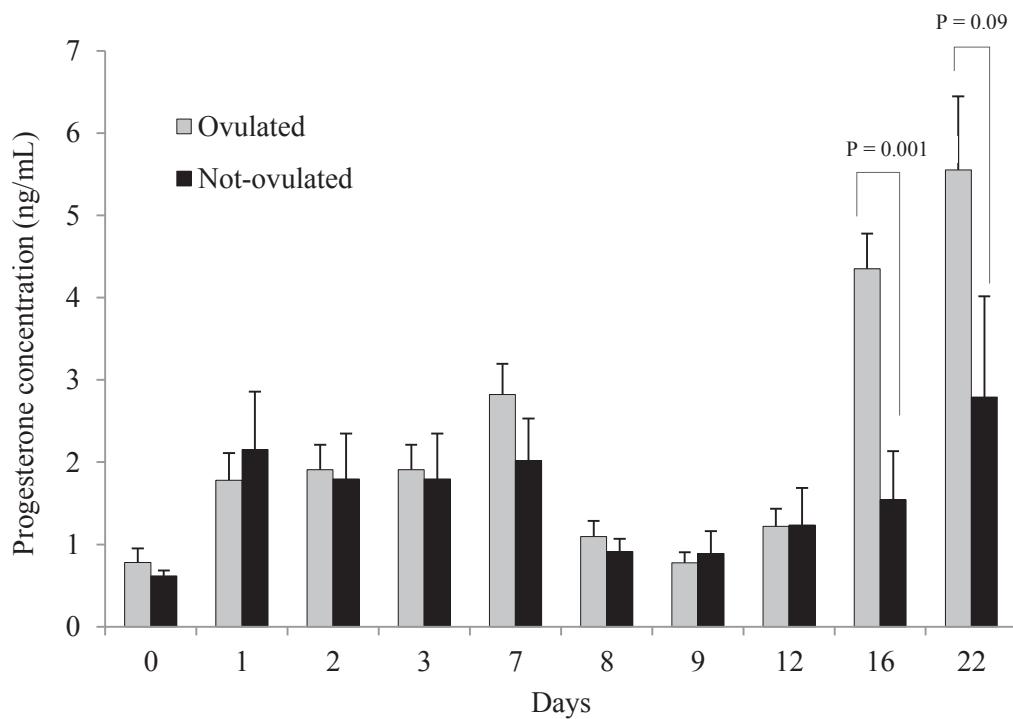


Figure 6.5 Comparison of progesterone concentrations between cows that ovulated ($n = 17$) or failed to ovulate ($n = 4$) for the combined GPG and GPG+P4 groups. Abbreviations: G, GnRH; P, PGF 2α ; P4, progesterone.

Oestradiol concentrations

Oestradiol concentrations are summarised in Figure 7.5. There was a significant effect of time ($P < 0.001$), but not of treatment ($P = 0.12$) or treatment by day interaction ($P = 0.64$). On the other hand, the AUC (ng/mL day) showed a significantly higher oestradiol concentrations in the GPG compared with the GPG+P4 group ($74.9 \text{ pg}^*\text{day}$ versus $60.6 \text{ pg}^*\text{day}$; $P = 0.002$).

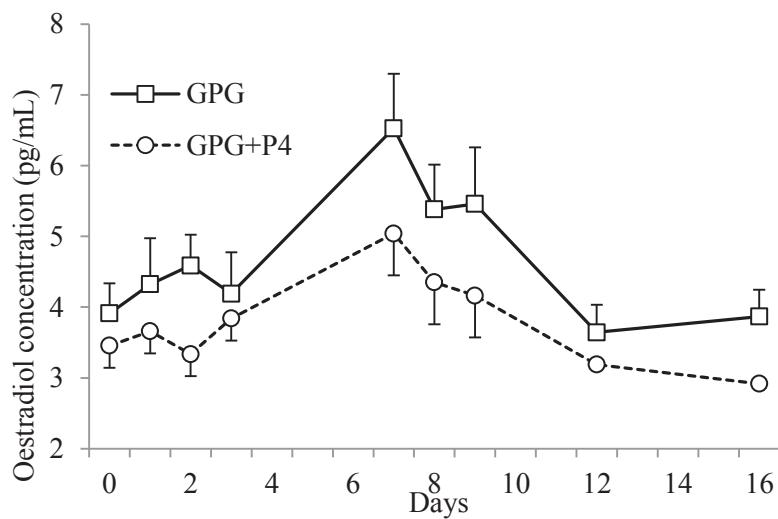


Figure 7.5 Comparison of oestradiol concentrations between GPG and GPG+P4 groups. Values are shown as least square means \pm SEM. Abbreviations: G, GnRH; P, PGF 2α ; P4, progesterone.

Discussion

This is the first study to directly compare follicular dynamics and hormonal profiles between the GPG and GPG+P4 synchronisation protocols in anoestrous cows managed under pastoral-based systems, in an attempt to explain the better conception rates that can occur with the progesterone-containing regimen than with GPG alone (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b; McDougall, 2010a). The results of this study have shown that the effects of a GPG+P4 programme and a GPG programme are similar in terms of synchronisation of ovulation, although the GPG programme led to the development of relatively larger dominant follicles and higher circulating concentrations of oestradiol than did GPG+P4.

The results of the present study do not appear to be related to the ovarian status of the animals at the start of the experiment. Progesterone concentration of < 1ng/mL are indicative of the absence of any luteal activity (Schams *et al.*, 1978), so Day 0 progesterone concentration of < 1ng/mL across both the GPG and the GPG+P4 groups confirmed the absence of luteal structures in the ovary and, hence, that the cows were anoestrous at the time of enrolment.

Several earlier reports have indicated a greater fertility in cycling cows that ovulate to the first GnRH of a GPG programme than in cows which do not (Gümen *et al.*, 2003; Bello *et al.*, 2006; Galvão and Santos, 2010). However, the responses to the initial steps (Day 0 GnRH, with or without intravaginal progesterone) of the synchronisation protocols in the present study were similar between the two groups, as shown by no difference in the number of days to the emergence of new follicular waves or the proportion of follicles that ovulated after first administration of GnRH on Day 0 (GPG: 55%, GPG+P4: 60%). Moreover, the ovulation rate after the first GnRH treatment in the present study was not dissimilar to that reported by Colazo *et al.* (2013) (55%), although somewhat lower than the 88% reported by Gümen *et al.* (2003). Whilst the size of the dominant follicle that ovulated after administration of GnRH on Day 0 was greater in the GPG than the GPG+P4 groups (16.7 ± 1.0 mm *versus* 14.0 ± 0.5 mm; $P = 0.04$; Table 1.5, Figure 2.5), it is difficult to attribute significance to this in terms of the behaviour of the (next) follicular wave that ovulated in response to the Day 9 GnRH.

The patterns of follicular growth after ovulation of the initial dominant follicle were quite dissimilar between the two treatment groups. Firstly, the follicular wave tended to emerge later in the GPG+P4 group than in the GPG group. Then, after a period of relatively slow growth, rapid follicular development started a day earlier in the GPG group (Day 6) than in the GPG+P4 group (Day 7). Thereafter, growth rates were similar between the groups, thereby maintaining a size differential between GPG and GPG+P4 cows for the remainder of the experiment. Interestingly, the follicle in the GPG group was reduced in size, albeit not significantly, on Day

9, whereas that in the GPG+P4 group continued to increase. Also, oestradiol concentrations did not mirror follicular size particularly well, which was surprising inasmuch as larger sized follicles are generally considered to secrete more oestradiol than smaller ones (Sartori *et al.*, 2004; Atkins *et al.*, 2008). Whether these differences can be attributed to the inclusion of a progesterone insert in the treatment protocol is unclear. On one hand, it is known that supplementation of exogenous progesterone in cattle suppresses the follicle development in a dose dependent manner (Adams *et al.*, 1992; Savio *et al.*, 1993b; Burke *et al.*, 1994). The exogenous progesterone supplementation in the GPG+P4 group might have therefore suppressed follicular growth in this group. Evidence to support this notion is that progesterone concentrations in the GPG+P4 group were significantly higher than in the GPG group on Days 1 to 3 of the treatment which could be attributed to the progesterone device in this group increasing overall concentrations of progesterone concentration (Lima *et al.*, 2009). Similar findings were also reported by Herlihy *et al.* (2012) in cyclic postpartum dairy cows at 24 h after insertion of progesterone device (GPG+P4, 5.0 ng/mL; GPG, 1.8 g/mL). On the other hand, concentrations of progesterone of ~3 ng/mL (i.e. those which occurred in the GPG+P4 group between Days 1–3) are not atypical of the early luteal phase when follicular recruitment is occurring in a normal oestrous cycle, whereas the concentrations in the GPG group (~1.5 ng/mL) were very much lower and more characteristic of the early/late follicular phase. In other words, rather than arguing that the exogenous progesterone in the GPG+P4 protocol delayed follicular growth, it might be more appropriate to argue that its absence in the GPG group allowed premature follicular recrudescence.

Evidence for the foregoing is also apparent in the pattern of ovulations after the Day 9 administration of GnRH, inasmuch as the overall proportion of cows with synchronised ovulation (within 48 h after the second GnRH injection) for the GPG+P4 group was 100%, whereas that for the GPG group was only 63.6% ($P = 0.09$, Figure 4.5). Moreover, this difference in synchronised ovulations between the GPG and the GPG+P4 groups was accentuated by the fact that there was a great deal of variation in the size of the dominant follicle on Day 9 in the GPG group, but that the variation was very much less in the GPG+P4 group, supporting the notion that the follicle growth in the GPG group was not well synchronised. This difference in variation of Day 9 dominant follicle size seems to be one of the reasons for the spread in final ovulations at different times and relatively more non-responders in the GPG than the GPG+P4 group although this conclusion would require further investigation with greater numbers of cows to be made definitively.

Curiously however, the majority of ovulations in the present experiment took place at 12–24 h after the second GnRH treatment in both GPG and GPG+P4 groups (Figure 4.5 and Table 4.5);

a figure that is at variance not only with the data of Pursley *et al.* (1995), who reported that all cows (20/20) ovulated between 24–32 h after the second GnRH injection, and with the data reported in Chapter 4 in which the peak of ovulations occurred at 24–36 h after the Day 9 administration of GnRH. Although some other studies (e.g. Peters *et al.*, 1999) have reported that the highest proportion of ovulations (10/11) occurred within 24 h of the second GnRH treatment in GPG cows (or 72–96 h after PGF_{2α} injection) in cyclic cows, it is unclear why there was a difference of 12 h in the peak period of ovulations in the present groups of experiments.

An appropriate response to PGF_{2α} on Day 7 is critical for the successful synchronisation of ovulation in a GPG programme. A higher concentration of progesterone in the GPG than the GPG+P4 treated cows (>1 ng/mL) on Day 8 (after PGF_{2α} injection on Day 7) and on Day 9, and near the time of AI (Day 12) indicated that luteolysis was delayed in the GPG group after the administration of PGF_{2α} on Day 7. It is well recognised that cows with incomplete or delayed luteolysis have a much lower fertility after spontaneous (Burke *et al.*, 1996) and induced ovulation (Moreira *et al.*, 2001; Souza *et al.*, 2008; Galvão and Santos, 2010). Moreover, three cows in the GPG group which did not ovulate to the final GnRH injection had progesterone concentrations <1 ng/mL on Day 7. In other words, it appears that the GPG group had poorer regulation of luteal development on Day 7 than did the GPG+P4 cows. Some appear to have had unresponsive corpora lutea that underwent delayed or partial luteolysis in response to PGF_{2α} on Day 7, whereas others had corpora lutea that were inadequately developed so had (presumably) failed to provide adequate progesterone priming of the developing dominant follicle. Taken together, it seems that there was a benefit of inclusion of a progesterone device in the GPG+P4 group which facilitated the development of a more responsive CL on Day 7 in this group, which thereafter led to a more synchronous response to the administration of GnRH on Day 9. The difficulty with this idea is that the progesterone concentrations in the present study are poorly correlated with the numbers of animals with observable CL on Days 7 and 9 (Table 2.5 and Figure 6.5). However, many authors have shown that the ability of ultrasonography to detect early, late or small corpora lutea is much poorer (i.e. as their echogenicity is less different to ovarian stroma than is that of the mature CL; Pieterse *et al.*, 1990; Hanzen *et al.*, 2000; Siqueira *et al.*, 2009) than its ability to detect mid-luteal phase or large structures, so perhaps greater weight should be given to the blood progesterone data than to the visualisation of the ovarian structures.

The results of this study therefore present a number of difficulties in reconciling them with the higher conception rates and pregnancy outcomes after GPG+P4 rather than GPG synchronisation protocols in a number of previous reports (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b; McDougall, 2010a). Firstly, the size of the dominant

follicle on Day 9 in the GPG+P4 group was slightly, albeit not significantly, smaller than that of the GPG group; secondly, the post-ovulation progesterone concentrations were also numerically (but not statistically significantly) higher in the GPG than the GPG+P4 group; and, thirdly, oestradiol concentrations were higher during the follicular growth phase in GPG than GPG+P4 cows. The smaller follicle that was present on Day 9 in the GPG+P4 group would be expected to have led to the formation of a relatively smaller CL, since ovulation of smaller follicles leads to the development of a smaller CL and lower progesterone concentrations (Vasconcelos *et al.*, 2001; Santos *et al.*, 2004; Perry *et al.*, 2007). Likewise, recent reports have shown that GnRH-induced ovulation of a small dominant follicle leads to reduced circulating oestradiol concentrations, decreased oocyte competence (Arlotto *et al.*, 1996), a lower fertilization rate and reduced chances of successful establishment of pregnancy (Jinks *et al.*, 2013). Taken together, these data would not lead one to expect better reproductive outcomes in the GPG+P4 group.

On the other hand, the ovulation rate in the first 48 h after administration of GnRH on Day 9 was relatively lower in the GPG than the GPG+P4 group (63.6% *versus* 100%). Given that field use of both GPG and GPG+P4 protocols requires FTAI rather than insemination to observed oestrus, the difference in synchrony of ovulation therefore appears to be the most probable explanation from the present results of the reported improvement in fertility in GPG+P4 compared to GPG treated cows. Nonetheless, the role of progesterone on Day 7 is also intriguing. Clearly, cows that failed to respond to PGF_{2α} treatment with either complete or timely luteolysis would contribute to the poorer synchronisation of ovulation, since an LH surge cannot be developed in the face of elevated progesterone concentrations. The cows that had low progesterone on Day 7 were perhaps prone to develop follicles that produced a short luteal phase, which is indeed a well-recognised shortcoming of GPG programmes in anoestrous cows. Numbers of cows in the present study were too small to be certain about any such effect, but, nevertheless, it would be compatible with data in the literature.

Conclusions

This study endeavoured to understand the physiological basis of improved conception rates in field studies of GPG+P4 compared to GPG oestrus synchronisation and induction programmes in anoestrous, pastoral dairy cattle. There were few differences in follicular dynamics during the nine days of the treatment process between the two protocols that would readily explain this difference in conception rate. However, ovulation was better synchronised in the GPG+P4 than the GPG cows, probably based upon a more synchronous luteolysis after administration of PGF_{2α} on Day 7. Whether this difference is sufficient to outweigh the larger dominant follicle on Day 9 in GPG than GPG+P4 cows, or the trend towards high post-ovulation progesterone

concentrations in the GPG cows is not clear, and would have to be studied further with much larger groups of animals.

Acknowledgements

This research was supported by a New Zealand International Doctoral Research scholarship and a Massey University Doctoral Research Scholarship. The product provided by Bomac Laboratories is gratefully acknowledged. The contributions of the farm manager of Massey University No.4 Dairy Farm and undergraduate veterinary students of Massey University are acknowledged.

CHAPTER 6

Conception rate to GPG + Progesterone and Progesterone + Prostaglandin oestrus synchronisation and fixed-time artificial insemination in pasture based dairy heifers

Abstract

This study evaluated the conception rate (CR) to fixed time artificial insemination (FTAI) of two oestrus synchronisation programmes in dairy heifers in New Zealand across eight locations during two different periods. In Year 1 and Year 2, postpubertal nulliparous Friesian and Friesian x Jersey heifers (13–15 months of age) were randomly allocated to two oestrus synchronisation programmes. Group 1 (GPG+P4; n = 330) received 100 µg of GnRH i/m on Day 0 (morning), a progesterone releasing intravaginal device from Day 0 to Day 7, 500 µg of PGF_{2α}; i/m on Day 7 (morning) and a second dose of GnRH at the time of FTAI on Day 9 (afternoon). The second group (P4+PG; n = 343), received a progesterone releasing intravaginal device from Day 0 to Day 7, 500 µg PGF_{2α} on Day 6 (morning) and FTAI on Day 9 (afternoon). Pregnancy was diagnosed on Day 42–52 by transrectal ultrasonography. There was no difference in CR between the two treatment groups (GPG+P4: 52.4%, P4+PG: 54.8%; P = 0.60), nor were there any differences between groups in different years (Year 1 (GPG+P4: 56.1%; P4+PG: 57.5%; P = 0.82); Year 2 (GPG+P4, 49.1%; P4+PG 52.3%; P = 0.58)). Interactions between the effects of farm and treatment were small and inconsistent. This study indicated that synchronising heifers with P4+PG resulted in CR equivalent to those resulting from GPG+P4 treatment, but with reduced drug costs. However, because heifers in the GPG+P4 group received the second GnRH injection at the time of AI, they needed only three yardings as opposed to the four required for the heifers treated with P4+PG. Thus, the choice of programme for an individual farm will depend on that farm's circumstances, in particular the cost of yarding the heifers.

(Key words: oestrus synchronisation, heifer, conception rate, GPG)

Introduction

Maiden heifers are the largest parity group in most dairy herds in New Zealand. They have the greatest genetic potential and, before their first calving, have incurred significant costs to their owners without any return (Parker *et al.*, 2007). In pasture-based dairy production systems, active reproductive management of these heifers can be difficult, particularly as they are often

grazed on separate properties away from the main farm (Xu and Burton, 1999b). Hence, unlike lactating cows, which can be relatively easily checked and regularly observed at milking, special efforts have to be made to detect oestrus in heifers. Difficulties associated with the detection of oestrus have limited the widespread use of artificial insemination (AI) in maiden heifers in New Zealand. However, there are several good reasons to use AI in yearling heifers (Dairy NZ, 2007), principally because using AI allows the farmers to rear increased numbers of replacement heifers with high breeding value. In turn, this allows for either a more rapid increase in herd size without buying in additional stock, or, if herd size remains constant, farmers can get the same number of high BV replacements from a short AI period in the milking herd and, as a bonus, increase the rate of genetic gain of the herd (Macmillan, 1998; Dairy NZ, 2007; Pickering, 2008).

Whilst synchronising oestrus in dairy heifers under New Zealand conditions will facilitate the use of AI (Xu and Burton, 1999a), programmes which do not require oestrus observation have significant advantages (Laven, 2008). However the use of such programmes, in either heifers or older animals, has been less common in New Zealand than elsewhere, probably because lactating dairy cows in New Zealand express heat better than such cows do in North America and Europe (Macmillan *et al.*, 1996; Harris and Kolver, 2001). Consequently, the focus of synchronisation in New Zealand has, historically, been on inducing oestrus behaviour rather than on synchronising ovulation. This is in contrast to the situation in North America and Europe, where weaker expression of oestrus behaviour and poorer detection of oestrus has highlighted the advantages of synchronising ovulation and using fixed time AI in lactating dairy cows (Christian, 1948; Roche, 1976; Butler, 2000b).

Until the European Union banned the use of oestradiol in 2007 there had been well established heifer synchrony programmes that were based upon the use of a progesterone releasing intravaginal device, oestradiol benzoate and PGF_{2α} (Pickering, 2002). These programmes were described as satisfactory for the heifers because they resulted in an average CR of approximately 60% after a single fixed-time AI (FTAI) at 48–52 h after progesterone insert removal (Pickering, 2002).

After the prohibition of the use of oestradiol esters in food producing animals by the European Union, alternative, cost-effective synchronisation programmes were needed for dairy heifers. This led to the introduction of synchronisation programmes based upon GnRH, progesterone and PGF_{2α}, which only require a single FTAI to achieve acceptable pregnancy rates. In this regard, McDougall *et al.* (2013) compared the three oestrus synchrony programmes in heifers a large-scale study: 1) Double prostaglandin (two injections of PGF_{2α} 11 days apart with AI to

detected oestrus); 2) ‘GPG + Progesterone + FTAI’ (100 µg GnRH and insertion of an intravaginal progesterone releasing device on Day 0, 500 µg cloprostenol on Day 7, 100 µg GnRH on Day 9, with FTAI 16 h after final GnRH injection); and 3) ‘GPG + Progesterone-Cosynch + FTAI (same as Treatment 2 but FTAI at the time of final GnRH injection). They reported that GPG + Progesterone-Cosynch + FTAI resulted in a higher first service CR and a higher 21 day pregnancy rate (57% and 76%) than in the other groups (Group 1: 48 % and 63%; Group 2: 47% and 72%). Pickering (2008) reported that when used in the field (1061 heifers in 10 different herds) the programme recommended by McDougall *et al.* (2013) resulted in an average CR of 53% (with individual herd averages ranging from 40–70%).

Progesterone is an important part of FTAI synchronisation programmes in heifers, because in nulliparous dairy heifers the CR to GPG+FTAI programmes (i.e. that omit progesterone) are relatively low (~40%); a figure that is similar to conception rates achieved in dairy cows despite the higher inherent fertility of heifers compared to cows (Schmitt *et al.*, 1996b; Pursley *et al.*, 1997; Tenhagen *et al.*, 2005). A higher proportion of heifers having premature oestrus (18% in heifers, Rivera *et al.* 2004 *versus* 6–9% in lactating cows, Roy and Twagiramungu 1996) before the FTAI in GPG+FTAI protocols has been suggested as one of the causes of their poor CR. Progesterone has therefore been added to GPG protocols, in an effort to reduce the proportion of premature ovulations, in a number of studies (Peeler *et al.*, 2004; Ambrose *et al.*, 2005; Cavalieri *et al.*, 2007). However, although this is effective in improving CR, the inclusion of progesterone in a synchronisation programme markedly increases its cost (Laven, 2008). Treatment costs are important as oestrus synchronisation is primarily used as a management tool for economic reasons; its use has to be justified based on improvements in submission and pregnancy rates and the time between calving and conception (Laven *et al.*, 2006). Increasing costs may improve outcomes, but can still result in reduced economic benefit. Thus, it is important to properly and fully evaluate the reproductive outcomes and costs of all synchronisation programmes to ensure that they produce the optimal benefits.

An alternative synchronisation programme, which was not evaluated by either Pickering (2008) or McDougall *et al.* (2013), is a combined therapy of progesterone and PGF_{2a} without the GnRH (P4+PG). Prostaglandin F_{2a} treatment at the end of a 7 day progesterone treatment was first proposed for synchronising oestrus by Deletang (1975). Later, Roche (1976) used this programme in beef heifers and cows, but pregnancy rates were low in both treated and control animals. However modification to the protocol by which PGF_{2a} injection was given 24 h before progesterone device removal (Hansel and Beal, 1979) resulted in better synchrony of oestrus and high fertility in dairy heifers. A more detailed study of pregnancy rates achieved using this programme in dairy heifers was undertaken by Smith (1984). In that study, a single

FTAI was performed 2 days after PGF_{2α} injection, which was preceded by 7 days of progesterone treatment, and reported CR to single FTAI was 66% (compared to 52% in heifers treated with a double PGF_{2α} injection 11 days apart).

The precise synchronisation resulting from the combined use of intra-vaginal progesterone-releasing devices and PGF_{2α} has meant that this combination has been widely used elsewhere in the world. Indeed, combined programmes involving a 7 day progesterone treatment with PGF_{2α} injection on either Day 6 or 7 are currently the only authorised methods of using intravaginal-progesterone devices in the UK, Canada and US (Canadian Animal Health Institute, 2005; National Office of Animal Health, 2012). In contrast, in New Zealand and Australia, intravaginal progesterone devices are licensed for use in combination with PGF_{2α} alone and with GPG programmes (Company data Zoetis Animal Health, Bioniche Animal Health). However, there are no peer reviewed published studies of the use, under New Zealand conditions, of combined progesterone and PGF_{2α} programmes in dairy heifers.

The aim of this study was to compare the CR to FTAI of post-pubertal nulliparous dairy heifers under New Zealand managemental conditions treated with either GPG+P4 (Cosynch) or P4+PG programmes.

Materials and methods

All animal use was approved by Massey University Animal Ethics Committee, Palmerston North.

The study was conducted at multiple locations across two years using eight farms in the vicinity of Palmerston North (latitude 40°2'S, longitude 175°4'E), in the lower North Island of New Zealand.

Animals

In Year 1 (2008) and Year 2 (2010), postpubertal nulliparous Friesian and Friesian x Jersey heifers that were 13–15 months of age were selected for this study. The numbers of heifers enrolled for the study from different farms is summarised in Table 1.6.

Power analysis

The number of animals used in the study was based on detecting an increase of more than 10% in conception rate after GPG+P4 compared to P4+PG. With $\alpha = 0.05$, using 330 animals in each group, resulted in $\beta = 0.83$.

Synchronisation protocols

All heifers were randomly allocated to one of two treatment groups by the use of a random allocation table generated using Microsoft Excel, 2007. Group 1 (see Figure 1.6, GPG+P4; n = 330) received 100 µg of gonadorelin i/m (Ovurelin, Bomac Laboratories Ltd, Auckland, New Zealand) on Day 0 (morning) along with the insertion of a progesterone releasing intravaginal device containing 1.56 g of progesterone (Cue-Mate, Bomac Laboratories Ltd) between Day 0 and Day 7. At the time of progesterone removal on Day 7 (morning), heifers were given 500 µg cloprostenol i/m (Ovuprost, Bomac Laboratories Ltd). A second dose of gonadorelin (100 µg i/m) was given on Day 9 at the time of FTAI. In Year 1, all inseminations used semen from Angus sires, whilst in Year 2, Jersey or Friesian semen was used depending on the farm.

Heifers in Group 2 (P4+PG; n = 343) received a progesterone releasing intravaginal device (Cue-Mate, Bomac Laboratories Ltd) from Day 0 to Day 7, and 500 µg cloprostenol, i/m (Ovuprost, Bomac Laboratories Ltd) on Day 6 (morning). On Day 9 (morning) FTAI was undertaken as described for Group 1. After the completion of FTAI, groups of heifers were combined. Bulls were introduced to the heifers seven days after FTAI, and remained with them until after the pregnancy diagnosis was undertaken.

Pregnancy diagnosis

Pregnancy diagnosis was undertaken on Day 42–52 after FTAI, using a real time B-mode ultrasound scanner (DP-6600 Vet, Mindray, Szechuan, China), equipped with a 7.5 MHz linear transducer. Conception date was confirmed by measuring crown rump length to determine the age of the conceptus. The CR per AI was defined as the percentage of heifers that were confirmed to be pregnant to the FTAI.

Statistical analyses

The data were initially subjected to the frequency procedure of SAS and a table of exposure (treatment groups) and outcome (pregnant or not pregnant) groups was created for each year and for both years together. Pearson Chi-square test as well as estimation of the odds ratio were undertaken for each year and for both years together.

The number of pregnant heifers on Day 42–52, which was treated as a binomial trait (0 = nonpregnant, 1 = pregnant), was analysed with a logistic regression model after a logit transformation using the generalised linear mixed-model (GLIMMIX) procedure of SAS (v9.3, SAS Institute Inc., Cary, North Carolina, USA). As farms were not repeated between years, two models were created: the first model included the fixed effects of the treatment, farm and the

interaction between treatment and farm. The second model included the fixed effects of the treatment, year and the interaction between treatment and year. Back-transformed marginal means and standard errors for each year or farm and treatment were obtained and used for the multiple comparisons, and differences were considered to be significant at $P \leq 0.05$.

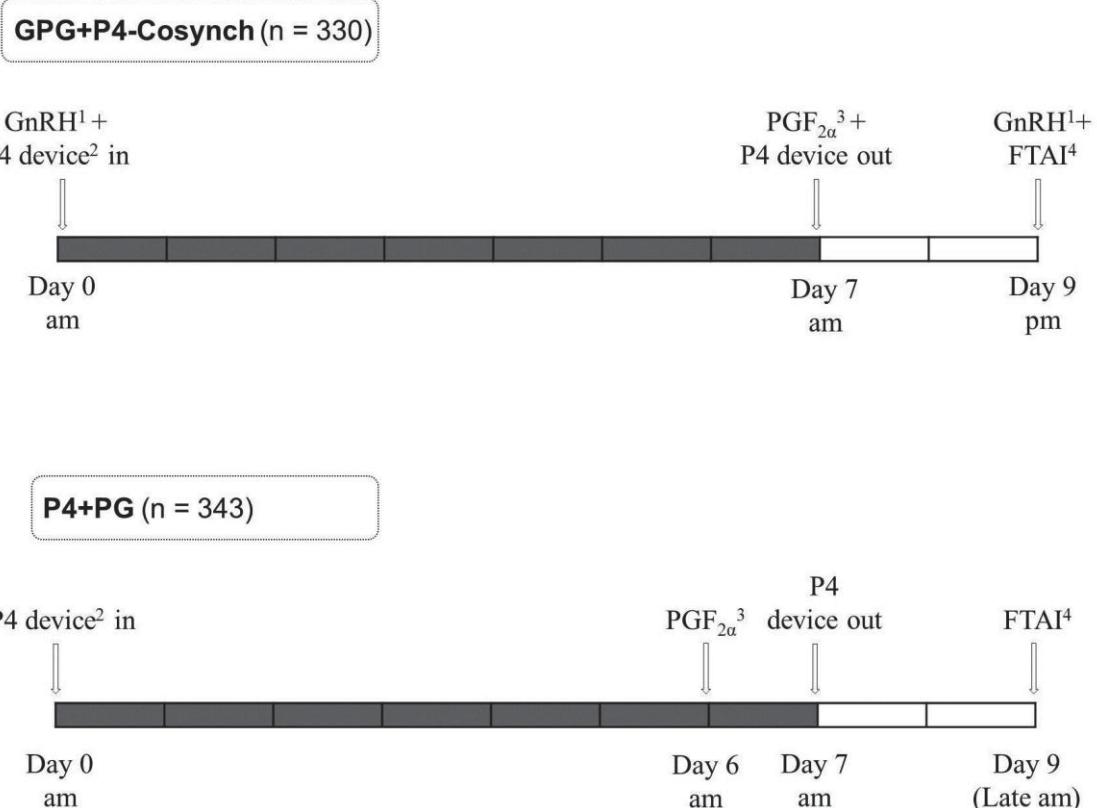


Figure 1.6 Synchronisation protocol for two groups of heifers (grey shading indicates the duration of progesterone releasing intravaginal device).

¹ 100 µg of GnRH i/m.

² Progesterone releasing intravaginal device containing 1.56 g of progesterone.

³ 500 µg cloprostenol i/m.

⁴ Fixed-time artificial insemination.

Results

The overall conception rates to FTAI were 52.4% and 54.8% for the GPG+P4 and P4+PG groups, respectively (Table 1.6). The odds of pregnancy between the two treatment were not significant ($OR = 0.90$; CI: 0.67–1.23). Results from Model 1 showed that there was no effect of treatment ($P = 0.54$), year ($P = 0.12$) and treatment by year interaction ($P = 0.79$) upon CR. There was no difference in CR between the GPG+P4 and P4+PG groups in different years [Year 1 [GPG+P4: 56.1%; P4 + PG: 57.5%; $P = 0.82$]; Year 2 [GPG+P4, 49.1%; P4 + PG 52.3%; $P = 0.58$]]. On the other hand, analysis by Model 2 showed that there was a significant ($P = 0.002$) farm effect (Figure 2.6) on overall CR, but no effect of treatment ($P = 0.67$) or treatment by farm interaction ($P = 0.92$).

Table 1.6 Conception rates to FTAI (as determined by transrectal ultrasonography on Days 42–52), across different farms and years, of heifers that had been synchronised with GPG+P4 or P4 + PG programmes.

Year	Farm	Conception rate (% and actual numbers)	
		GPG+P4	P4+PG
Year 1	1	58.6 (17/29)	61.3 (19/31)
	2	70.6 (12/17)	76.2 (16/21)
	3	66.2 (43/65)	60.9 (42/69)
	4	34.1 (15/44)	41.3 (19/46)
Total for Year 1		56.1 (87/155)	57.5 (96/167)
Year 2	5	50.0 (15/30)	40.0 (8/20)
	6	46.2 (24/52)	56.4 (31/55)
	7	52.2 (12/23)	52.2 (12/23)
	8	50.0 (35/70)	52.6 (41/78)
Total for Year 2		49.1 (86/175)	52.3 (92/176)
Total conception rate (Years 1 and 2)		52.4 (173/330)	54.8 (188/343)

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone; PG, PGF_{2α}.

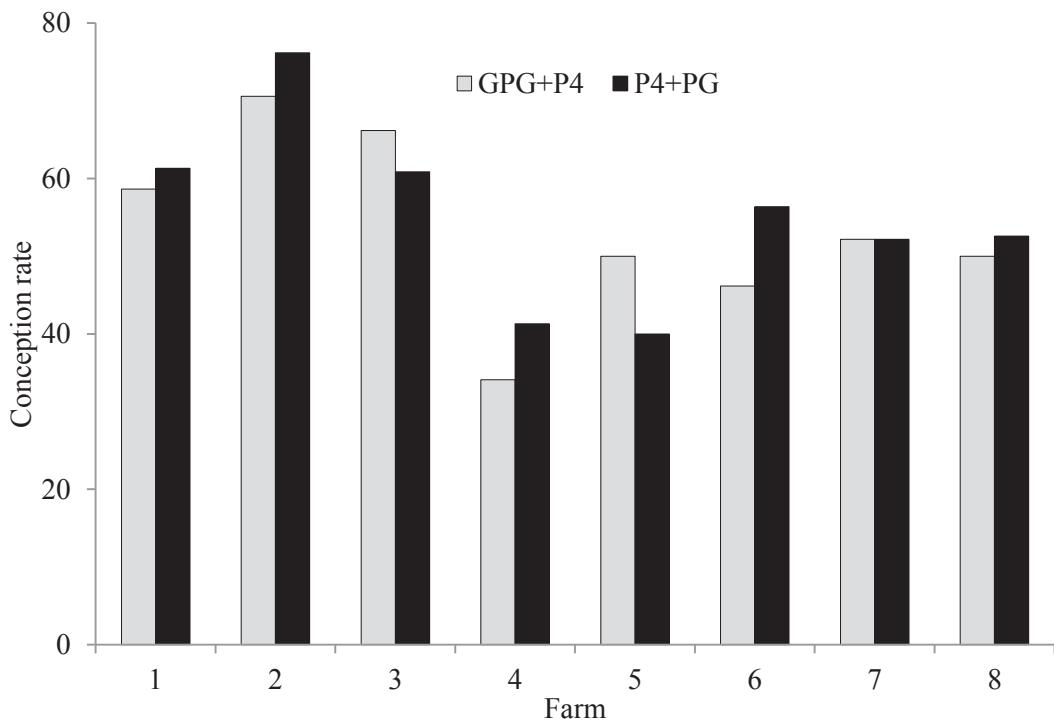


Figure 2.6 Differences in conception rates to FTAI between different farms (1 to 8) and treatments in heifers treated either with GPG+P4 or P4+PG programmes. There was a significant farm effect ($P = 0.002$) but there was no effect of treatment, year, treatment by farm interaction and treatment by year interaction ($P > 0.05$) on overall conception between groups. Abbreviations: G, GnRH; P, PGF_{2a}; P4, progesterone; PG, PGF_{2a}.

Discussion

This is the first New Zealand-based study undertaken comparing the efficacy in heifers of a GPG plus progesterone (GPG+P4) protocol for oestrus synchronisation (McDougall *et al.*, 2013) with the cheaper synchronisation programme using just progesterone and PGF_{2a} (P4+PG) that is commonly used outside of Australasia. In the present study, synchronising heifers with the P4+PG protocol resulted in similar CR to synchronising with GPG+P4 (54.8% *versus* 52.4%, respectively).

The CR reported in this study for both synchronisation programmes are similar to previous results of these programmes in dairy heifers. For GPG+P4, the results in this study tended to be marginally lower than previous reports from other countries. For example, Stevenson *et al.* (2008a) recorded a CR of 55.3% (21/38) (US study); Colazo and Ambrose (2011) (Canada) reported a CR of 58.1% (18/31) in dairy heifers. Conception rates were very similar to the 53% reported by Pickering (2008) in dairy heifers across 10 commercial dairy herds in New Zealand,

but were marginally lower than the 57% CR (213/374) reported by McDougall et al (2013), in New Zealand dairy heifers.

The response to synchronisation using progesterone and PGF_{2α} only, the CR of 54.8% in present study is similar to the 55.6% reported by Ambrose *et al.* (2008) in Canada using FTAI, and 54% reported by Lucy *et al.* (2001b) in the USA involving AI after detected oestrus. However, a higher CR (66%) was reported in a single herd of dairy heifers using the P4+PG programme by Smith *et al.* (1984) in which FTAI was done 84 h after PGF_{2α} injection. Nevertheless it was within the range of the herd CR seen in the present study, and the study was undertaken in a herd in which the 25 day pregnancy rate was also very high (73%) in untreated control heifers. Notwithstanding, the results of Smith *et al.* (1984), the commensurate results between the heifers in the present study and those of Ambrose *et al.* (2008) and Lucy *et al.* (2001b) suggests that P4+PG plus a single FTAI would be an acceptable programme for synchronising oestrus in dairy heifers.

Although it resulted in similar conception as the GPG+P4 programme, the P4+PG regime was significantly less expensive. As of July 2013, the costs of the products used in these programmes were: Cuemate (NZ\$20.02/unit), Ovuprost (NZ\$2.04/mL) and Ovurelin (NZ\$5.60/mL). Therefore the cost of synchronisation using GPG+P4 was NZ\$35.30, whereas the cost for P4+PG was just NZ\$24.10 per animal. Thus, assuming each programme resulted in a CR of 53.6%, (the overall average seen in this study), the cost per pregnancy for GPG+P4 was NZ\$65.85, whereas the cost for the P4+PG treated animals was NZ\$44.96, a difference of more than NZ\$20. However, the GPG+P4 programme required only three days of yardings and manipulations, whereas the P4+PG programme required four, which would clearly add to costs. Nonetheless, for a group of 84 heifers (the average number of heifers treated per farm in this study), the difference in cost between synchronising the heifers using a GPG+P4 programme rather than a P4+PG programme would be in excess of NZ\$940, a difference that is likely to be significantly more than the cost of an extra yarding.

The financial advantage of using P4+PG rather than GPG+P4 could still remain even if the odds of pregnancy after treatment with GPG+P4 were at the top end of the 95% CI (0.67 to 1.23) found in this study; i.e. if the odds of pregnancy after GPG+P4 were 1.23 times higher than those after treatment with P4+PG. Assuming a 53.6% CR after P4+PG, an OR of 1.23 for GPG+P4 would indicate a true CR of 58.1; the cost per pregnancy for the two programmes would be NZ\$45.6 and NZ\$60.75, a NZ\$15 advantage for the P4+PG programme. Furthermore, based on the calculations by McDougall *et al.* (2013), the advantage of a 5% increase in CR in synchronised heifers would be ~NZ\$13/treated animal, only marginally more than the increased

cost of the GPG+P4 programme compared to the P4+PG programme. Consequently, it seems reasonable to infer that the P4+PG programme is a more cost effective means of synchronising heifers under New Zealand conditions than GPG+P4. Nevertheless, further research with a specific economic focus is required to more accurately establish the veracity of this conclusion in terms of financial benefit.

Nevertheless, cost is not the only important criterion, as the convenience of the three yardings required for the GPG+P4 programme may outweigh the cost benefit of the P4+PG programme, but the simplicity of the latter has other advantages - there were double treatments on all treatment days during the GPG+P4 programme, but only single treatments with the P4+PG programme. Under commercial conditions, multiple treatments at a single yarding may lead to a higher proportion of incorrect treatments, again favouring the use of the P4+PG programme rather than GPG+P4.

Conclusions

In conclusion, the results of this study suggest that inseminating heifers at a single fixed time after synchronisation of ovulation using a P4+PG programme resulted in a CR which was as high as was achieved in those heifers synchronised using a GPG+P4 programme, even though the programme cost significantly less. Further research is required to better establish the economic benefit of using the P4+PG programme, and to identify whether other programmes that are cheaper than a GPG+P4 programme, such as a GPG+P4 without the first GnRH treatment, can result in similar CR to the two programmes tested in this study whilst retaining the major benefit of the GPG+P4 programme (i.e. only three yardings).

Acknowledgements

This research was supported by a New Zealand International Doctoral Research scholarship and a Massey University Doctoral Research Scholarship. The product provided by Bomac Laboratories is gratefully acknowledged. The contributions of the dairy farmers and farm managers of the Manawatu region are acknowledged.

CHAPTER 7

The effects of exclusion of progesterone or Day 0 GnRH from a GPG+Progesterone programme on synchronisation of ovulation in pasture-based dairy heifers

Abstract

This study evaluated the effect of removing the GnRH injection on Day 0 or the progesterone (P4) device from a GnRH, PGF_{2α}, GnRH (GPG) + P4 programme on follicular dynamics and synchronisation of ovulation in dairy heifers. In Year 1 ($n = 35$) and Year 2 ($n = 38$) Friesian and Friesian x Jersey heifers were randomly allocated to one of three oestrus synchronisation programmes. The first group (GPG+P4) received 100 µg GnRH on Day 0, a progesterone releasing intravaginal device from Day 0 to Day 7, 500 µg PGF_{2α}, on Day 7 and 100 µg GnRH on Day 9, followed by fixed-time artificial insemination 16–20 h later. The programme for Group 2 (GPG) was the same as Group 1 with the exclusion of the progesterone device. Group 3 (P+G+P4) was treated the same as Group 1, except for the absence of the GnRH treatment on Day 0.

Ultrasonography was performed on Days 0, 1, 2 3 and 7 and then at 12 hourly intervals on Days 9 to 11. Plasma concentration of progesterone on Days 2, 7, 13, 15, 18 and 27 and oestradiol on Days 2 and 13 were measured. Dominant follicle size was affected by both treatment and time and there was also a significant interaction between treatment and time ($P < 0.02$). Mean dominant follicle size was larger in the heifers treated with P+G+P4 on Days 1 to 3 than those treated with GPG+P4 ($P < 0.02$) and, larger on Day 2, than those treated with GPG ($P = 0.005$). However, on Day 7, mean dominant follicle size was larger in heifers treated with GPG than heifers treated with P+G+P4 ($P = 0.03$). There was a significant effect of treatment on progesterone concentration on Days 18 and 27. On Day 18, heifers treated with GPG had higher progesterone concentrations than heifers treated with GPG+P4 whereas on Day 27 higher progesterone concentrations were recorded in both GPG and P+G+P4 groups than in GPG group ($P \leq 0.05$). There was no effect of treatment, time, or their interaction on mean oestradiol concentration ($P > 0.05$ for all).

The emergence of a new follicular wave was later in heifers treated with P+G+P4 than heifers which received a GnRH injection on Day 0 (4.3 ± 0.7 days compared with 3.0 ± 0.3 days in combined GPG+P4 and GPG; $P = 0.03$). The proportion of heifers which ovulated within the

first 48 h after the Day 9 injection of GnRH was not affected by treatment (GPG: 81%, GPG+P4: 84% and P+G+P4: 100% (including early ovulation); $P = 0.11$). The timing of the ovulation was not different between treatments ($P = 0.97$).

(**Key words:** heifer, GPG, synchronisation, ovulation)

Introduction

The primary reason for synchronising oestrus in dairy heifers is to facilitate use of artificial insemination (AI) and, in such animals, synchronisation programmes which do not require oestrus observation have significant advantages (Laven, 2008). Nevertheless, the use of synchronisation to avoid the need for heat detection has not been very widespread in New Zealand, probably because lactating dairy cows in New Zealand express heat far better than in the US and Europe (Macmillan *et al.*, 1996), so farm staff are more used to successful heat detection.

In 2003, the European Union prohibited the use of oestradiol and its esters in food producing animals (European Union, 2003) which has led to the loss of oestradiol benzoate (ODB) based synchronisation programmes. Some alternative options for New Zealand dairy heifers were studied in a large field study by McDougall *et al.* (2013), who compared the efficacy of three programmes: (1) Double PGF_{2α} (two injections of PGF_{2α} 11 days apart, with AI following detection of oestrus within 4 days after the second PGF_{2α}; $n = 380$); (2) GPG+P4 (two treatments of GnRH (given 9 days apart) with a PGF_{2α} injection on Day 7, combined with intravaginal progesterone from Day 0 to 7, with fixed time artificial insemination (FTAI) 16–20 h after the Day 9 GnRH injection; $n = 383$); and (3) Cosynch (the same sequence of treatments as for GPG+P4 but with the FTAI coincident with the final GnRH; $n = 374$). They reported that Cosynch was the best of the three programmes, as it was associated with the highest first service conception rate (57% *versus* 48% and 47% for GPG+P4-Cosynch *versus* double PGF_{2α} and GPG+P4, respectively) and highest 21 day pregnancy rate (76% *versus* 63% and 72% for Treatment 3 *versus* Treatments 1 and 2, respectively).

Other alternative synchronisation programmes that have been used in heifers include (i) a GPG programme without intravaginal progesterone, or (ii) a combined progesterone+PGF_{2α} programme with a 7-day intravaginal progesterone from Day 0 to 7, PGF_{2α} injection on Day 6 and single FTAI at 56 h after progesterone device removal (Laven, 2008). The former programme is rarely used in heifers because the reduction in fertility resulting from the use of GPG protocols is greater in heifers than in dairy cattle (Twagiramungu *et al.*, 1995; Pursley *et al.*, 1997), probably because of the higher incidence (18%) of oestrus before FTAI in dairy

heifers (Rivera *et al.*, 2004) than in cows (6–9%; Roy and Twagiramungu, 1996). For this reason, intravaginal progesterone is commonly used in GPG programmes in heifers to delay ovulation (Peeler *et al.*, 2004; Ambrose *et al.*, 2005; Cavalieri *et al.*, 2007).

However, although the inclusion of a progesterone releasing device in a GPG programme has led to improved pregnancy rates (Martinez *et al.*, 2002; Ambrose *et al.*, 2008; McDougall *et al.*, 2013), it greatly adds to the costs of such programmes. Thus the identification of cheaper, but equally effective, alternatives to progesterone could markedly reduce costs and improve the economic benefits of synchronisation programmes (Laven *et al.*, 2006). To identify alternatives it is necessary to identify the mechanism by which progesterone improves the response to synchronisation. However, there is only limited evidence of its mechanism of action, and other information particularly whether it improves the synchronisation of ovulation.

Additionally, although the first GnRH treatment has been shown to be an important part of a GPG+P4 programme in lactating dairy cattle since it leads to synchronous follicular wave emergence, larger preovulatory follicles, synchronous ovulation and improved pregnancy rates (65% in GPG+P4 *versus* 30% in P+G+P4; Kim *et al.*, 2005), its importance in the GPG+P4 protocol in heifers is less certain inasmuch as the pattern of follicular development of dairy heifers is different from that of lactating dairy cows (Sartori *et al.*, 2004). Heifer follicles thus seem to be responsive to GnRH for a lower proportion of their time, resulting in a lower success rate in inducing follicle turnover and failure to induce a new follicular wave (Haughian and Wiltbank, 2002). This is consistent with the findings of Colazo & Ambrose (2011) that removal of a Day 0 GnRH injection did not affect the recruitment of a new follicular wave or conception rate in dairy heifers given a GPG+P4-Cosynch programme. Therefore, the benefit of using GnRH at the start of such programmes needs to be further evaluated in heifers.

The present study was therefore undertaken to evaluate the effect of removing either progesterone or the Day 0 GnRH from a GPG+P4 programme on the follicular dynamics, hormonal profiles and synchronisation of ovulation of nulliparous dairy heifers.

Materials and methods

All animal use was approved by the Massey University Animal Ethics Committee, Palmerston North.

This study was conducted at Haurongo farm, a research farm of Massey University, Palmerston North (latitude 40°2'S, longitude 175°4'E), New Zealand, during autumn (May to June) 2009 ($n = 35$) and spring (September to November) 2010 ($n = 38$).

Animals

Nulliparous dairy heifers (Friesian and Friesian x Jersey) of approximately 13–15 months of age with a body weight range from 350–420 kg were used for this study. The reproductive tract of all the selected heifers were palpated and examined by ultrasonography to confirm the absence of utero-ovarian pathology and the presence or absence of a corpus luteum (CL). Body condition score (BCS, Scale 1 to 10: Macdonald and Roche, 2004) was also recorded.

Synchronisation protocols

All 73 heifers were randomly allocated to one of three treatments using a random allocation sheet based on their order in the race on Day 0 (Figure 1.7): (1) 100 µg i/m of GnRH (Ovurelin Bomac Laboratories Ltd, Auckland, New Zealand) on Day 0 (morning), a progesterone-releasing intravaginal device containing 1.56 g of progesterone (Cue-Mate, Bomac Laboratories Ltd) from Day 0 to Day 7, 500 µg cloprostenol i/m, (Ovuprost, Bomac Laboratories Ltd) on Day 7 (late afternoon) and a second dose of GnRH on Day 9 (late afternoon), followed by FTAI on Day 10 (16–20 h after Day 9 GnRH treatment; GPG+P4; n = 25); (2) as for Group 1, with the exclusion of a progesterone-releasing intravaginal device (GPG; n = 27); and (3) as for Group 1, but omitting the GnRH treatment on Day 0 (P+G+P4; n = 21).

Ultrasonography

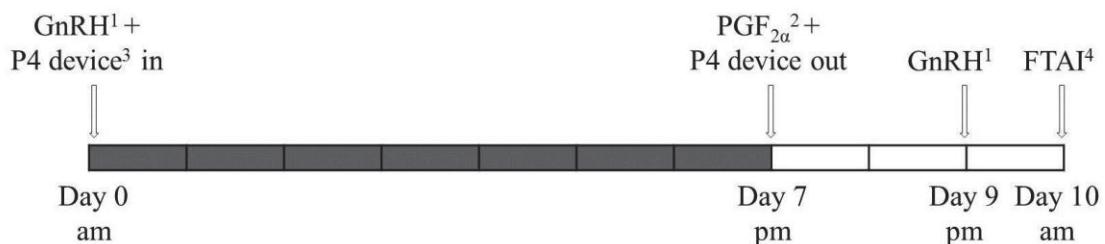
Ovarian structures of all the heifers were monitored and studied as previously described (Pierson and Ginther, 1984) using a real time B-mode ultrasound scanner (DP-6600 Vet, Mindray, Szechuan, China), equipped with a variable linear transducer set at 7.5 MHz. Ultrasonography was performed on Days 0, 1, 2, 3, 7 and 9. On each occasion, digital ultrasound images of both ovaries were recorded (Figure 2.7) and a corresponding ovarian map was also drawn manually on the recording sheet to locate and identify the structures on the ovary, particularly the presence or absence of a CL.

The impact of treatment and time on follicular dynamics was evaluated by measuring the size of the largest dominant follicle using ImageJ (v1.46d, National Institutes of Health, USA). The diameter of the dominant follicle was estimated by taking the average of two measurements: size at the widest point and size at right angles to the first measurement.

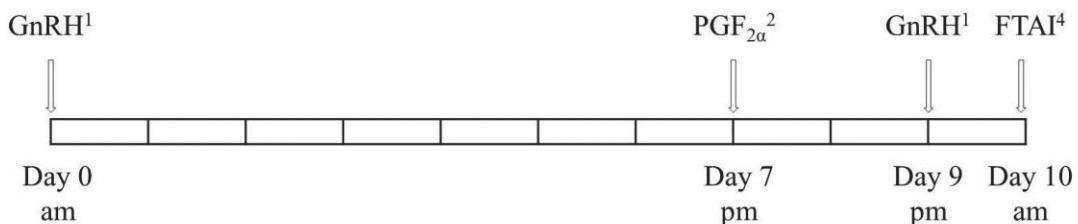
Between Days 0 and 7, the response of the dominant follicle to treatment, in terms of its persistence, ovulation or atresia, was recorded. Ovulation was defined as the disappearance of a dominant follicle followed by the development of a CL (or accessory CL), whereas atresia was

defined as the disappearance of a dominant follicle followed with no CL development. Persistence was defined as no disappearance of the dominant follicle.

GPG+P4 (n = 25)



GPG (n = 27)



P+G+P4 (n = 21)

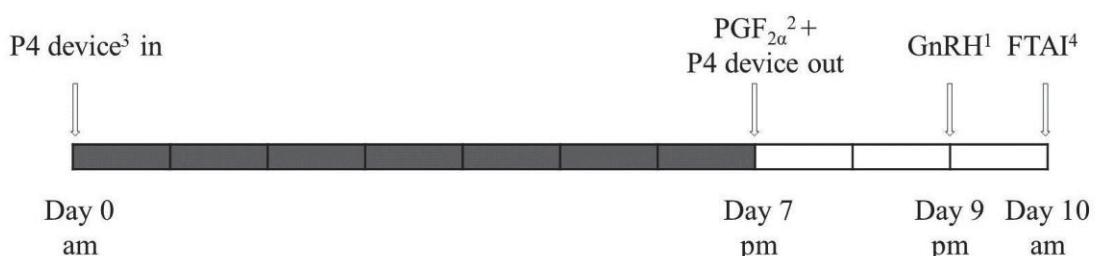


Figure 1.7 Synchronisation protocol for three groups of heifers (grey shading indicates progesterone releasing intravaginal device).

¹100 µg gonadorelin i/m.

²500 µg cloprostenol i/m.

³intravaginal progesterone device with 1.56g of progesterone.

⁴fixed-time artificial insemination.

The days to the emergence of a new follicular wave, within 7 days of the treatment, was identified as the day that the dominant follicle was retrospectively identified to have had a diameter of ≥ 4 mm. If the dominant follicle was not detected until it reached 6 or 7 mm, the previous day was taken as the first day (Ginther *et al.*, 1989b).

The growth rate of the preovulatory follicle was established from the diameter reached on Day 9, minus the diameter the day of its detection, divided by the number of the days.

Timing of ovulation after Day 7 was determined by ultrasound examination of the ovaries every 12 h from the morning of Day 9 until the afternoon of Day 11, or ovulation, whichever was sooner. Ovulation was defined as the disappearance of a previously identified dominant follicle ≥ 9 mm.

Pregnancy diagnosis to confirm the success of the FTAI was undertaken on Day 42 using the same ultrasound equipment.

Blood samples and hormone assay

Blood samples (10 mL) were collected via coccygeal venepuncture into heparinised Vacutainers (Becton Dickinson, New Jersey, USA) on Days 2, 7, 13, 15, 18 and 27 of the study. The blood samples were centrifuged for separation of plasma at 1500 g for 20 min at 4°C within 2 h of collection. The plasma was aspirated using disposable Pasteur pipettes and was stored at -20°C until assay.

Progesterone assay

Blood samples taken on Days 2, 7, 13, 15, 18 and 27 were assayed for plasma progesterone concentrations. Plasma progesterone concentrations were measured in duplicate 10 µL aliquots by radioimmunoassay, using the ImmuChem Double Antibody Progesterone ^{125}I RIA kit for *in vitro* diagnostic use (MP Biomedicals, USA). The sensitivity of the assay was 0.14 ng progesterone/mL. The intra-assay coefficients of variation at 80, 50 and 20% binding on the standard curve were 16.1%, 8.4% and 9.9% respectively; the inter-assay coefficients of variation were 19.1%, 14.4% and 15.7% for low, medium and high solutions, respectively. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer (see Appendix 1 for a description of the method).

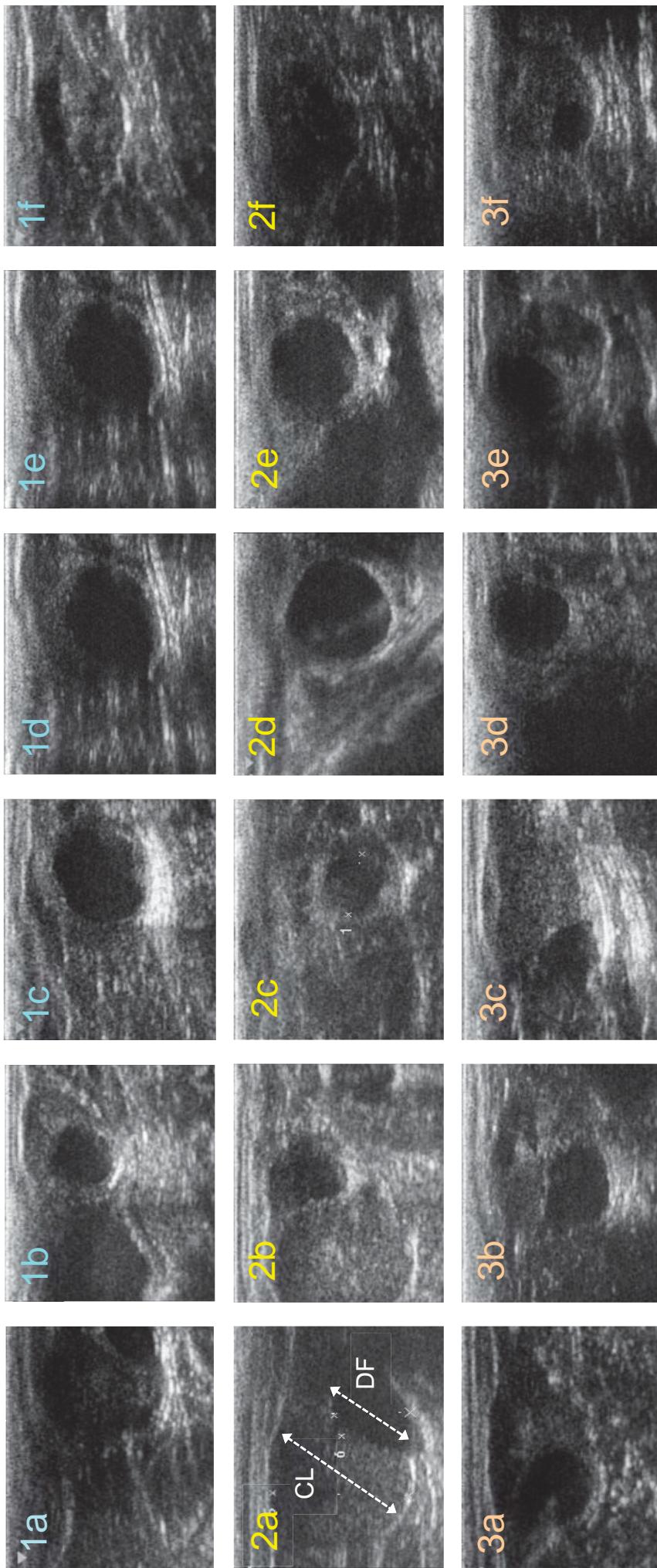


Figure 2.7 Growth of dominant follicle in three groups of heifers in response to synchronisation treatment. 1. GPG (Heifer No. 12), 1a. Day 0 (left ovary), 1b. Day 3 (left ovary), 1c. Day 7(right ovary), 1d. Day 9 (right ovary), 1e. Preovulatory follicle (right ovary), 1f. Confirmation of ovulation (right ovary). (Heifer No 25), 2a. Day 0 (corpus luteum and dominant follicle in the right ovary), 2b. Day 3 (left ovary), 2c. Day 7 (left ovary), 2d. Day 9 (left ovary), 2e. Preovulatory follicle (left ovary), 2f. Confirmation of ovulation (left ovary). 3. P+G+P4 (Heifer No 61), 3a. Day 0 (corpus luteum and dominant follicle in right ovary), 3b. Day 3 (right ovary), 3c. Day 7(right ovary), 3d. Day 9 (right ovary), 3e. Preovulatory follicle (right ovary), 3f. Confirmation of ovulation (right ovary). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Oestradiol assay

Blood samples taken on Days 2 and 13 were assayed for plasma oestradiol concentration. Plasma oestradiol concentrations were measured in duplicate 40 µL aliquots by radioimmunoassay, using Ultra-Sensitive Estradiol ^{125}I RIA kit, (Immunotech, Czech Republic). The sensitivity of the assay was 3.3 pg oestradiol/mL. The intra-assay coefficients of variation at 60 and 20% binding on the standard curve were 10.8 and 7.1% respectively; the inter-assay coefficients of variation were 14.8 and 4.0% for low and high solutions, respectively (see Appendix 2).

Statistical analyses

The key outcome variables were the diameter of the dominant follicle from Day 0 to 9, diameter of the preovulatory follicle, and growth rate of preovulatory follicle after Day 0. Secondary objectives were measurement of the proportion of heifers ovulating in response to GnRH injection on Day 0 and Day 9; proportion with atresia of dominant follicle between Day 0 and Day 7; interval from Day 0 to the emergence of the follicular wave producing the dominant follicle on day 9; presence or absence of CL on Day 7 and 9, timing of ovulation after GnRH injection on Day 9; and plasma concentration of progesterone and oestradiol. The balance of the treatment group for age and BCS was compared by one-way analysis of variance (ANOVA) to ensure that the groups were balanced for these factors which are known to have a key influence on fertility (McDougall, 2001; Roche *et al.*, 2007).

Except where noted, all statistical analyses were performed using Prism (v 5.03, GraphPad Software, Inc. La Jolla, USA). The proportion of heifers with atresia or ovulation and new follicular wave emergence within 7 days of treatment was compared by chi-squared test. The interval to new wave emergence was subjected to one way ANOVA and Student's t-test. Age of the dominant follicle on Day 9 and the growth rate of the preovulatory follicles of new follicle waves were analysed by ANOVA with Tukey's multiple-comparison test post-hoc where the ANOVA was significant. The proportion of heifers with synchronised ovulation within 48 h after the Day 9 GnRH treatment and the proportion of heifers with an ovulation (before the Day 9 GnRH injection or within 12, 24, 36, 48 h) or no ovulation after the Day 9 GnRH injection, were compared using chi-squared analysis and simple correspondence analysis (SAS v 9.3). Simple correspondence analysis of the frequency table of the ovulation at different time interval was done using the CORRESP procedure. A low-dimensional graphical representation of the rows and columns of the frequency table was generated in which each row and column was represented by a point in the plot determined from the cell frequencies. Correspondence analysis plot locates all the categories in a Euclidean space and is like two different overlaid plots, one for each categorical variable.

The influence of progesterone concentration on the response to Day 9 GnRH was analysed by Student's t-test by comparing the area under the curve (AUC) of the progesterone concentration (calculated using the trapezoid rule) for the heifers that did ovulate with that of the heifers that did not ovulate.

The changes in the dominant follicle size from Day 0 to Day 3, Day 7 and Day 9, preovulatory follicle, plasma oestradiol concentrations on Days 2 and 13 and progesterone concentrations on Days 2, 7, 13, 15, 18 and 27 were subjected to a repeated measures analysis of variance with respect to treatment and day, using the MIXED procedure of SAS (v 9.3, Statistical analysis system, SAS Institute Inc., Cary, North Carolina, USA), in which treatment was a fixed effect, day of measurement a repeated fixed effect and cow a random effect. An unstructured covariance was used based on the lowest Akaike Information Criterion (Littell *et al.*, 1996).

The effects of treatment, and Day 7 and Day 9 dominant follicle size on the ovulation response to second GnRH injection was modelled by using a generalised linear mixed-model following a logit transformation (GLIMMIX procedure, SAS v 9.3). The dependent variable was ovulation in response to the second GnRH injection, which was treated as a binomial variable with a logit transformation (1 = ovulated, 0 = not ovulated). The independent variables were treatment and dominant follicle size on Days 7 and 9 and all two-factor interactions. To avoid covariance due to two similar variables, a matrix plot of all the independent variable was first generated. Those with a P value of <0.2 were kept in the model, unless there was a significant correlation ($r>0.7$) between the predictor variables when the variable with the smallest P value was kept in the model. A backward elimination of nonsignificant factors was done one by one until all the variables were significant ($P\leq0.05$) in the model. Preliminary final model was checked by diagnostic plots involving event probability (delta chi square vs. probability) and leverage (delta chi-square vs. leverage) and goodness of fit tests and if there were no unusual observations and P value of goodness of fit test was >0.05 then the model was declared final.

Results

The number, age and BCS of the heifers allocated to each treatment groups are shown in Table 1.7. One heifer allocated to the GPG treatment group in 2010 was excluded from the analysis as she developed an ovarian cyst by Day 3, which left a total of 72 heifers. There was no difference between treatment groups in age ($P = 0.10$) or BCS ($P = 0.77$). The heifers in Year 2 tended to have a lower BCS than those in Year 1 (4.7 ± 0.10 versus 5.0 ± 0.09 , respectively, $P = 0.06$).

Ovarian dynamics from Day 0 to Day 9

The findings are summarised in Table 2.7 and Figure 2.7. The percentage of heifers with follicular wave emergence within 7 days of treatment was not significantly different between the three treatment groups (GPG: 69.2, GPG+P4: 80.0, P+G+P4: 57.1; $P = 0.25$), nor was there a significant difference in mean interval from treatment to follicular wave emergence ($P = 0.07$; Table 2.7). However, when the data from the GPG and GPG+P4 groups (the groups which received a GnRH treatment on Day 0) were combined, the mean interval from the start of treatment to follicular wave emergence was shorter in these groups compared to the groups treated with P+G+P4 (3.0 *versus* 4.3 days; $P = 0.03$). There was a significant difference ($P = 0.003$) in the proportion of heifers which ovulated after the start of the treatment. The first ovulation percentage was 38.5, 40 and 0% for the GPG, GPG+P4 and P+G+P4 groups, respectively.

Table 1.7 Number, age and BCS¹ of the heifers in the GPG, GPG+P4 and P+G+P4 groups.

	Treatment		
	GPG	GPG+P4	P+G+P4
No. of heifers			
Year 1	13	12	10
Year 2	13	13	11
Total	26	25	21
Age (months) (range)	14 (13.1–14.4)	13.8 (13.1–14.6)	14.3 (12.8–20.6)
BCS (1–10) (range)	4.9 (3.5–6)	4.8 (3.5–6)	4.8 (3.5–6.5)

¹Body condition score (Scale 1–10, Macdonald and Roche, 2004).

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 2.7 Ovarian dynamics in three groups of heifers after the start of the treatment (Day 0–9)

	GPG (n = 26)	GPG+P4 (n = 25)	P+G+P4 (n = 21)
Atresia ¹	8 (31%)	10 (40%)	12 (57%)
Ovulation ²	10 (38.5)	10 (40%)	0
Heifers with new follicular wave	18 (69%)	20 (80%)	12 (57%)
Interval to follicular wave emergence (days)	2.7 ± 0.3	3.2 ± 0.4	4.3 ± 0.7
Age of the dominant follicle on Day 9 (days)	6.3 ± 0.3	5.8 ± 0.4	4.7 ± 0.57

¹ Disappearance of a previously identified dominant follicle followed by no development of corpus luteum.

² Disappearance of the dominant follicle followed by the development of corpus luteum or accessory corpus luteum.

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone

Mean dominant follicle size changed markedly over time ($P < 0.001$; Figure 3.7). There was also an effect of treatment ($P = 0.005$) and an interaction between time and treatment ($P = 0.02$). Heifers treated with P+G+P4 had larger dominant follicles than heifers treated with GPG+P4 on Days 1, 2 and 3 ($P < 0.02$) and heifers treated with GPG on Day 2 ($P = 0.005$), but smaller dominant follicles compared with the GPG group on Day 7 ($P = 0.03$). No difference in dominant follicle diameter was seen at any stage between heifers treated with GPG or GPG+P4 ($P = 0.88$). On Day 9 there was no significant difference between the groups in mean size of the dominant follicle ($P = 0.70$). On Day 9, dominant follicle size ranged from 5.0 to 20.7 mm, 9.0 to 19.0 mm and 4.0 to 20.2 mm for the GPG, GPG+P4 and P+G+P4 groups, respectively. Although the age of the dominant follicle on Day 9 was not different between the groups ($P = 0.07$, Table 2.7), it was significantly lower for the P+G+P4 group compared with the combined GPG and GPG+P4 ($P = 0.03$; 4.7 *versus* 6 days) groups.

The growth rate (mm per day) of the dominant follicle was not different either between the groups (GPG+P4: 1.2 ± 0.1 GPG: 1.0 ± 0.1 P+G+P4: 1.0 ± 0.1 ; $P = 0.21$) or between P+G+P4 and the combined GPG and GPG+P4 groups (P+G+P4: 1.1 ± 0.1 *versus* combined GPG and GPG+P4: 0.09 ± 0.1 ; $P = 0.14$).

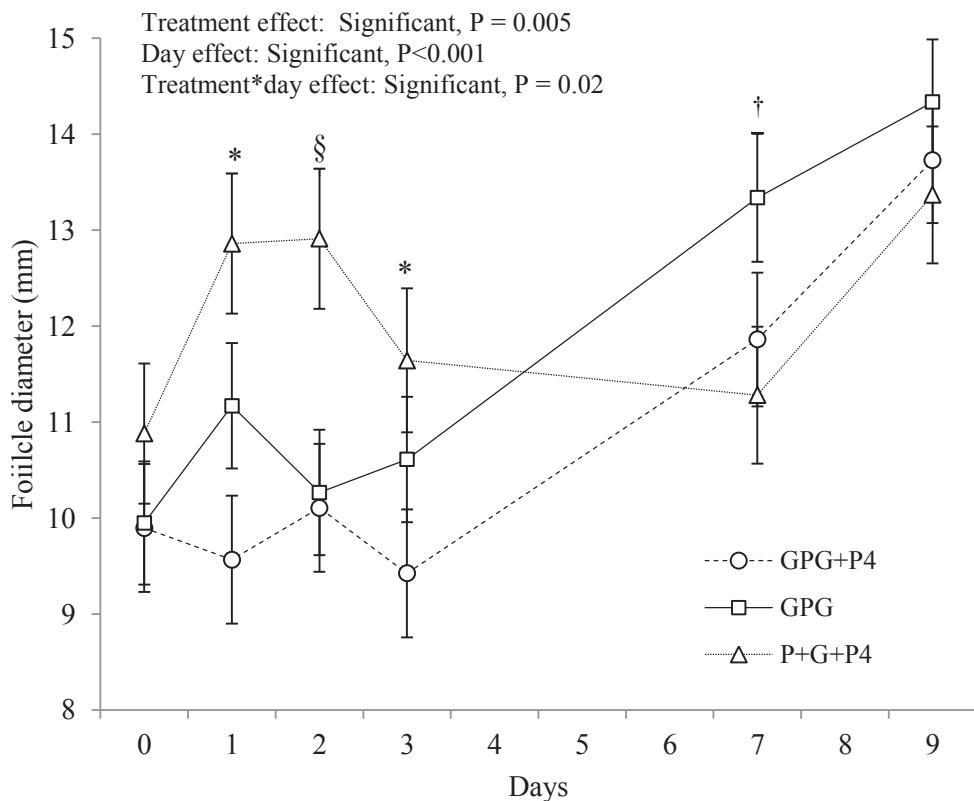


Figure 3.7 Least square mean \pm SEM of changes in dominant follicle diameter from Day 0 to Day 9 in GPG, GPG+P4 and P+G+P4 groups. *P+G+P4 significantly different from GPG+P4 ($P<0.02$); §, P+G+P4 significantly different from GPG+P4 and GPG ($P<0.004$); †GPG significantly different from P+G+P4 ($P = 0.03$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Corpus luteum status

The proportions of heifers with a CL on Days 0, 7 and 9 are shown in Table 3.7. There was no effect of treatment group on CL status at any time point.

Table 3.7 Number (% in parentheses) of heifers in each treatment group with a visible CL at different time point of the treatment.

	GPG (n = 26)	GPG+P4 (n = 25)	P+G+P4 (n = 21)
CL Day 0	24 (92%)	23 (92%)	16 (76%)
CL Day 7	26 (100%)	23 (92%)	17 (81%)
CL Day 9	11 (42%)	10 (40%)	6 (29%)

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone

Follicular dynamics – ovulatory follicle

Of the 72 heifers, four ovulated prior to the GnRH injection on Day 9, 59 had a synchronised ovulation within 48 h after GnRH injection and 9 failed to respond in that time period. Table 4.7 shows the number of heifers in each treatment group which ovulated in response to the injection of GnRH. The distribution of ovulation timing (very early, early, mid, late and no ovulation) in each group is summarised in Table 5.7 and Figure 4.7. The mid category (24–36 h) was the most common category (GPG: 11/26, GPG+P4: 10/25, P+G+P4: 10/21).

Table 4.7 Follicular dynamics of the ovulatory follicle in GPG, GPG+P4 and P+G+P4 groups of heifers.

	GPG (n = 26)	GPG+P4 (n = 25)	P+G+P4 (n = 21)
Ovulated between Day 7 and 11 (n)	22 (85%)	23 (92%)	18 (86%)
Ovulated prior to GnRH (n)	1 (4%)	2 (8%)	1 (5%)
Did not ovulate (n)	4 (15%)	2 (8%)	3 (14%)
Synchronised ovulation (n)	21 (81%)	21 (84%)	17 (81%)
Mean preovulatory follicle size (mm)	15.4 ± 0.6 (8.3–19.6)	15.3 ± 0.4 (10.1–18.0)	15.3 ± 0.4 (13.2–19.1)

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone

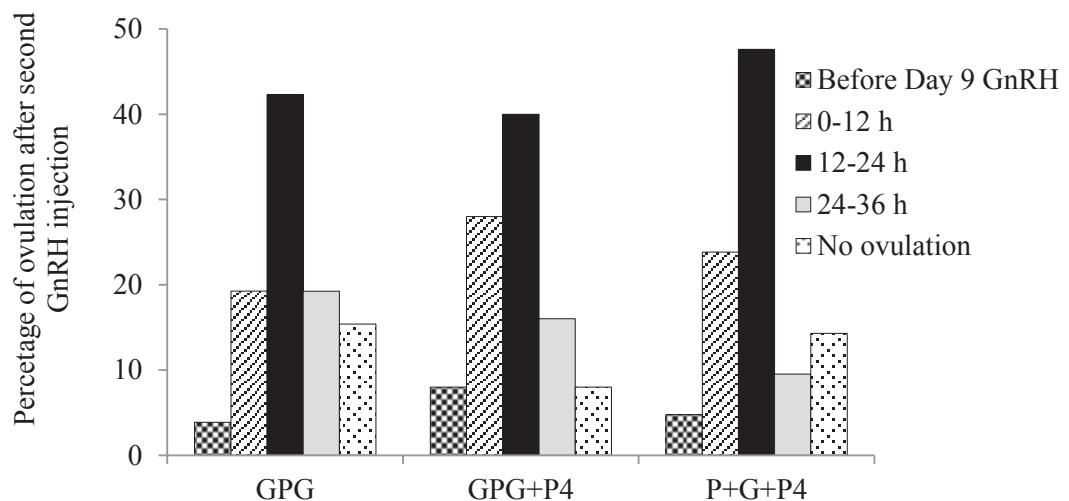


Figure 4.7 Distribution of final ovulation percentage at different time interval in GPG, GPG+P4 and P+G+P4 groups of heifers. The differences in the proportions that ovulated at different time intervals was not significant ($P = 0.97$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Table 5.7 Distribution of number of ovulation (% in parentheses) at different time interval in three treatment groups

Treatment	Very Early ¹	Early ²	Mid ³	Late ⁴	No ovulation ⁵	Total
PGP	1(3.8%)	5(19.2%)	11(42.3%)	5(19.2%)	4(15.4%)	26
PGP+ P4	2(8.0%)	7(28.0%)	10(40.0%)	4(16.0%)	2(8.0%)	25
P+G+P4	1(4.8%)	5(23.8%)	10(47.6%)	2(9.5%)	3(14.3%)	21
Total	4	17	31	11	9	72

¹ ovulation before Day 9 GnRH injection

²ovulation from 0–12 h after Day 9 GnRH injection

³ ovulation from 12–24 h after Day 9 GnRH injection

⁴ovulation from 24–36 h after Day 9 GnRH injection

⁵ no ovulation within 48 h after Day 9 GnRH injection

Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone

Figure 5.7 shows the results of the correspondence analyses. The first dimension of the analysis accounted for a large proportion of the inertia or variation in the data present in the original correspondence table (63.1%; Table 5.7). The correspondence analysis can be read at three levels. Reading from left to right along the x-axis, the first dimension strongly differentiates PGP+P4 from the other two treatments, whilst the second dimension separates PGP and P+G+P4. A similar examination of the five outcome variables (ovulation timing) shows that the first dimension differentiates between very early and early ovulation and the three other categories, while the second dimension separates mid and late ovulation. Finally, the association between the predictor variables and the outcome variables can be assessed; treatment with PGP+P4 is associated with the early and very early categories while P+G+P4 is associated with mid timing of ovulation. PGP treatment is in the same quadrant as the late category but the association is not close as there is a large difference along the second dimension.

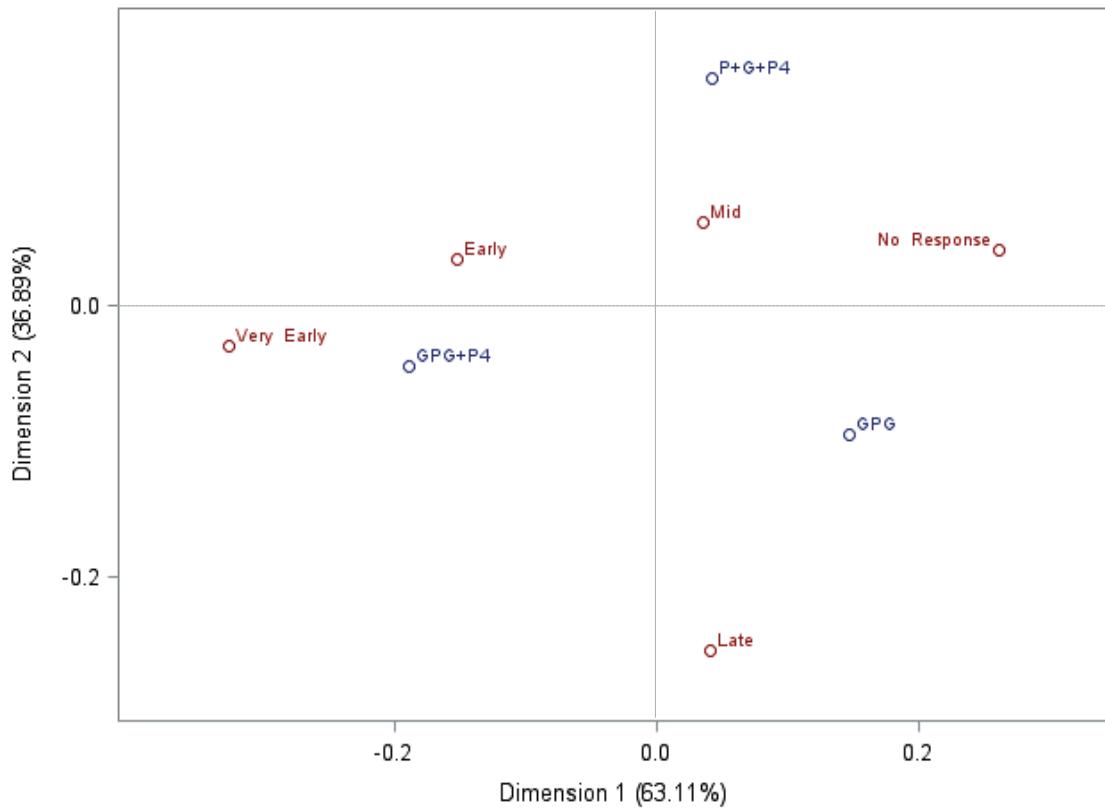


Figure 5.7 Symmetric plot of predictor variable (treatment) and outcome variable (timing of ovulation; Very early: before Day 9 GnRH injection; Early: 0–12 h; mid: 12–24 h; late: 24–36 h and no response (no ovulation during observation) after Day 9 GnRH injection. The figure in parentheses for each of the dimensions is the proportion of the variance explained by the original correspondence table accounted for by each dimension. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Impact of follicle size on ovulation

There was a significant effect of dominant follicle size on Day 7 and Day 9, and also a significant interaction between results on the two days on the ovulation response to GnRH treatment on Day 9 ($P = 0.02, 0.01$ and 0.02 respectively), such that the probability of ovulation was higher if the dominant follicle size on these days was larger.

Progesterone concentration

Mean progesterone concentrations for all three groups showed an increase between Days 0 and 7, followed by a sharp decline to Day 13 and then an increase in concentration to Day 27 ($P<0.05$; Figure 6.7). There was a significant effect of treatment on progesterone concentration ($P = 0.01$) which was only seen on Days 18 and 27. On Day 18, heifers treated with GPG had higher progesterone concentrations than heifers treated with GPG+P4. However, on Day 27, higher progesterone concentrations were recorded in both the GPG and the P+G+P4 groups than in the GPG group ($P\leq0.05$).

Although progesterone concentration in cows that ovulated prior to Day 12 tended to be higher throughout the study period, these differences were not significant at any time point, nor was there any effect on AUC ($P = 0.68$, see Figure 7.7).

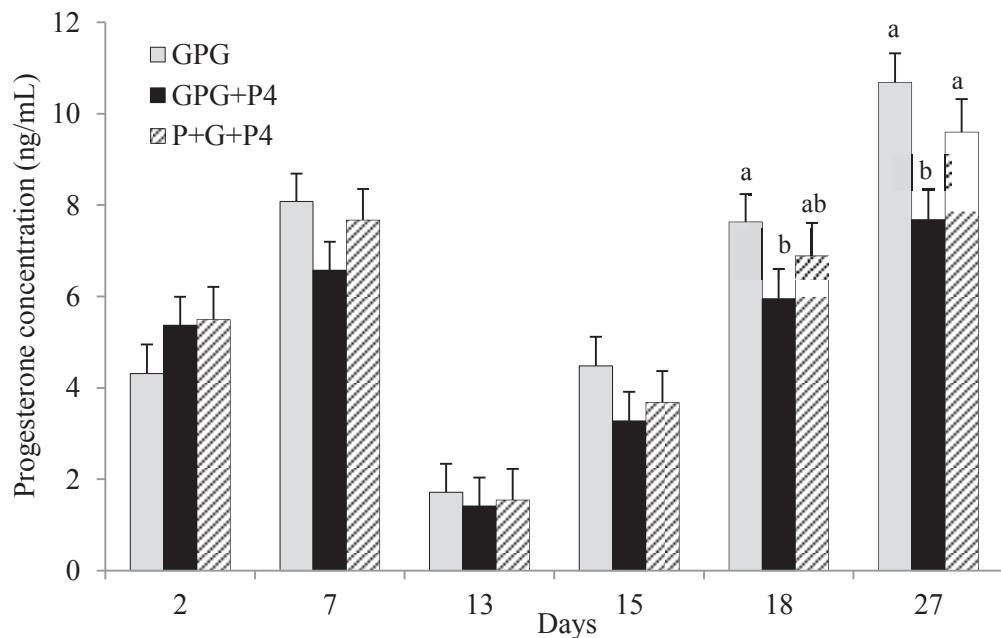


Figure 6.7 Effect of synchronisation treatment on progesterone concentrations (least square mean \pm SEM) in GPG, GPG+ P4 and P+G+P4 group of heifers. Bars labelled within the same day with different letters are significantly different (Day 27, $P\leq0.05$) or tended to be different (Day 18, $P = 0.06$) from each other. Abbreviations: G, GnRH; P, PGF 2α ; P4, progesterone.

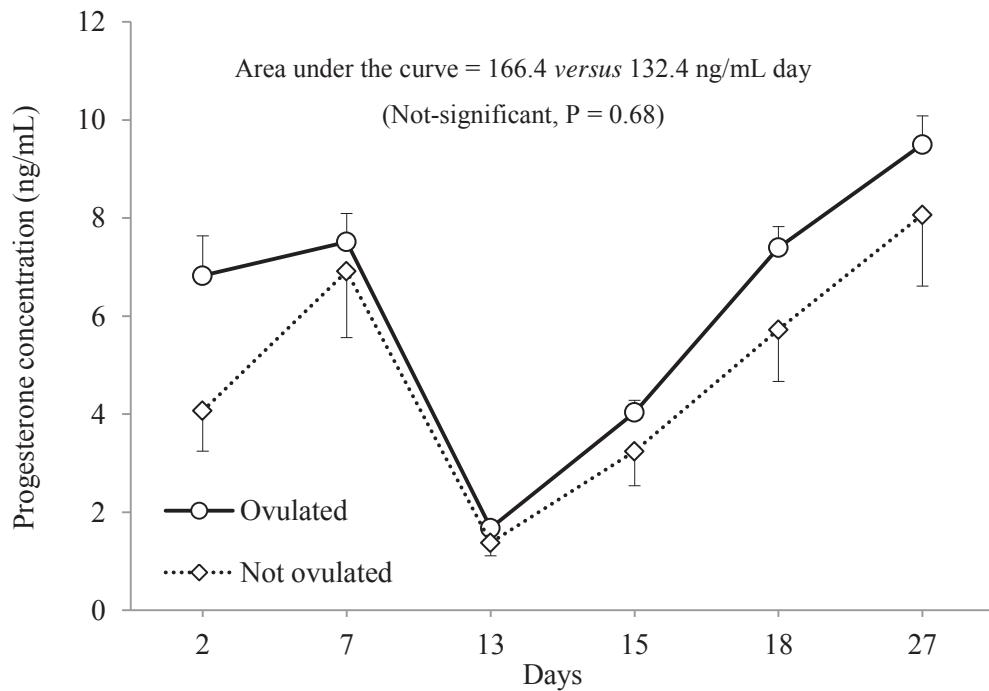


Figure 7.7 Comparison of progesterone concentration between ovulated ($n = 63$) and non-ovulated ($n = 9$) heifers of combined GPG, GPG+ P4 and P+G+P4 groups. Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Oestradiol concentration

There was no effect of treatment or day, or their interaction on mean oestradiol concentration ($P>0.05$ for all; Figure 8.7).

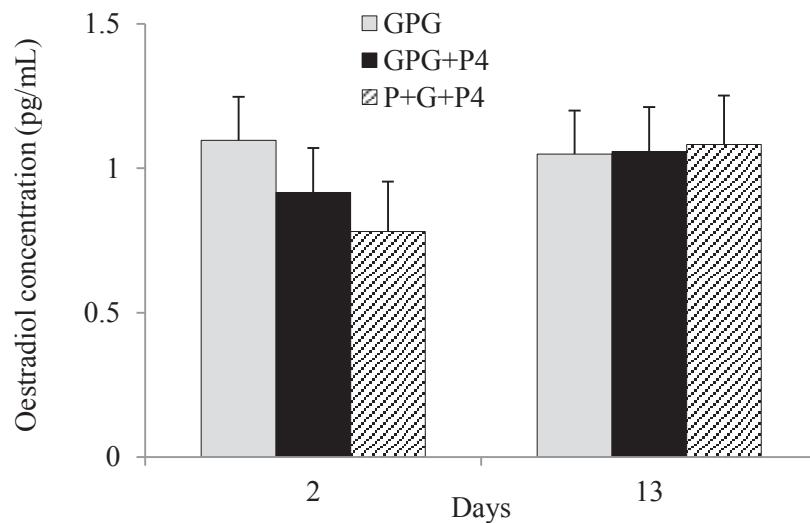


Figure 8.7 Oestradiol concentrations (least square mean \pm SEM) in GPG, GPG+ P4 and P+G+P4 groups of heifers. Treatment, day and treatment by day interaction effect were not significant ($P>0.05$). Abbreviations: G, GnRH; P, PGF_{2α}; P4, progesterone.

Discussion

The present study was undertaken to evaluate the effect of alterations to the GPG+P4 synchronisation programme on follicular dynamics, hormonal concentrations and synchronisation of ovulation in nulliparous dairy heifers. Two changes to the standard synchronisation programme were tested: The removal of the progesterone device and the removal of the Day 0 GnRH injection.

The first change, the removal of the progesterone-releasing device (comparing GPG+P4 with GPG alone), had no effect on follicular dynamics or on the proportion of heifers which ovulated after the GnRH injection on either Day 0 or Day 9. This is the first study that has directly compared the follicular dynamics in heifers, after treatment with GPG versus treatment with GPG+P4, so direct comparisons within the literature are not available. However, at least some previous data from studies in dairy heifers which have ovulated after treatment with either GPG or GPG+P4 appear to be at variance with the results of this study.

Firstly, previous studies in dairy heifers have reported ovulation rates of over 56% after the administration of GnRH on Day 0 in a GPG program (Pursley *et al.*, 1995; Moreira *et al.*, 2000a) rather than the 38.5% seen in this study. However, the small number of heifers in this ($n = 26$) and the previously published studies ($n = 24$) mean that the difference is not statistically significant even when the data from the two studies is combined ($P = 0.14$, $\chi^2 = 2.2$).

In contrast, the proportion of heifers ovulating after the administration of GnRH on Day 0 in the GPG+P4 group (40%) tended to be higher than reported in previous studies of dairy heifers treated with GPG+P4, with reported rates ranging from 25 to 31% (Hittinger *et al.*, 2004; Stevenson *et al.*, 2008a; Stevenson, 2008; Colazo and Ambrose, 2011). As for GPG-treated heifers, the small size of this study means that the difference in ovulation rate between this and other studies such as that by Stevenson 2008 (25% in 141 heifers) is not significant ($P = 0.15$, $\chi^2 = 2.1$). Further research is required to establish whether the absence of an effect of removing the progesterone device seen in this study is correct or whether the differences in the published literature better reflect the situation.

The interval to new follicular wave emergence was not different between the GPG+P4 and GPG groups in the present study (3.2 ± 0.4 *versus* 2.7 ± 0.3 days, respectively); these results were consistent with the data from Moreira *et al.* (2000) who found a similar range after treatment of dairy heifers with GPG. No previous data on time to new wave emergence in heifers treated with GPG+P4 was found.

This lack of impact of the use of progesterone on follicular dynamics continued after the removal of the progesterone device on Day 7. The diameter of the largest follicle on Day 9 ranged from 9.0 to 19.0 mm for the GPG+P4 group and 5 to 20.7 mm for the GPG group, out of which 24.0% (6/25) and 11.5% (3/26), respectively were small dominant follicles (≤ 11 mm) in the GPG+P4 and GPG groups. Stevenson *et al.* (2008) reported that, in heifers treated using a GPG+P4 protocol, the ovulation of a dominant follicle ≤ 11 mm in diameter resulted in a reduced chance of pregnancy. The presence of a relatively high proportion of small dominant follicles at the time of the second GnRH treatment is probably caused by the failure of the GnRH injection on Day 0 to initiate a new follicular wave (Atkins *et al.*, 2008). Results from the present study seem to support this hypothesis, as five out of six heifers in the GPG+P4 group and one out of three heifers in the GPG group with a small dominant follicle on Day 9 had not ovulated after the GnRH injection on Day 0 and, thus, had failed to initiate a new follicular wave. The importance of these observations is that induced ovulation of smaller follicles is associated with lower fertility, possibly because it leads to the development of a smaller CL and decreased circulating progesterone concentrations (Vasconcelos *et al.*, 2001; Santos *et al.*, 2004; Perry *et al.*, 2007). In contrast to this conclusion, Colazo and Ambrose (2011) reported that lack of response to the first GnRH was not a problem, as the majority of heifers in their study (87.5%) started a new follicular wave prior to Day 7 even though they did not ovulate in response to the Day 0 GnRH. Furthermore, heifers which did not respond to the first GnRH treatment had a higher pregnancy rate than those which did. However, the present study does not seem to agree with Colazo and Ambrose (2011), as in the present study, the heifers which did ovulate in response to Day 0 GnRH had a higher conception rate (66.7%) than those did not (41.4%). Further research is required to better establish the importance of response to first GnRH on subsequent fertility of dairy heifers.

In contrast to the removal of progesterone, the second change in the protocol, omitting the GnRH injection on Day 0, had the expected significant impact on follicular dynamics between Day 0 and Day 7 as, in the P+G+P4 group, there were no ovulations before Day 7 (*versus* 40% in the GPG+P4 group) and new follicular waves started later than in the heifers treated with GnRH on Day 0 (3.0 *versus* 4.3 days). However, the proportion of heifers which started a new follicular wave was not markedly less in the P+G+P4 group than in the GPG group (57 *versus* 69%, respectively), although it was lower than in the GPG+P4 group (80%), a finding that is consistent with the data from Colazo and Ambrose (2011) who reported a new follicular wave in 87.5% of dairy heifers treated with a GPG+P4 programme.

The lack of ovulations in the early days of the treatment protocol in the P+G+P4 group meant that treatment initially led to the development of larger dominant follicle compared with the

GPG+P4 group (Day 1, 12.9 ± 0.7 mm *versus* 9.6 ± 0.6 mm; Day 2, 12.9 ± 0.7 mm *versus* 10.1 ± 0.6 ; Day 3, 11.6 ± 0.7 mm *versus* 9.4 ± 0.6 mm; P<0.05). This was due to exclusion of GnRH injection on Day 0 in the P+G+P4 group. However, subsequent dominant follicle size in the P+G+P4 group was no different than the GPG+P4 group. This may have been mediated by the progesterone device as it results in suppression of the dominant follicle during its growing phase and also decreases in oestradiol production (Adams *et al.*, 1992; Burke *et al.*, 1994).

Interestingly, in the 57% of the heifers treated with P+G+P4 that had later emergence of a new follicular wave, the preovulatory follicle was of similar size to those in the two groups which received GnRH on Day 0. In the remaining 43% of heifers in the P+G+P4 group in which no new follicle wave emerged, the aged dominant follicle of the previous wave was still present on Day 9 and, again, was of similar size to those of the groups that received GnRH on Day 0. Therefore, the dominant follicle on Day 9 in all the three groups of the present study had a similar potential of ovulating in response to a GnRH injection. On the other hand, the age of the dominant follicle could potentially affect fertility after ovulation, inasmuch as increasing the duration of dominance of the preovulatory follicle has been shown to reduce fertility in cattle (Stock and Fortune, 1993; Mihm *et al.*, 1994; Austin *et al.*, 1999). It needs further exploration as to how 57% of younger and 43% of older dominant follicles could have affected the subsequent conception rate in P+G+P4 treated heifers.

Although mean follicle size on Day 9 was not affected by the absence of GnRH on Day 0, only one heifer (1/21 [5%]) in the P+G+P4 group had a small dominant follicle (≤ 11 mm) on Day 9, a figure which was much lower than the 24% in the GPG+P4 group and 11% in the GPG group. That heifer was one of the three heifers which did not ovulate in response to the Day 9 GnRH. If this lack of small follicles is a consistent finding it might be indicative of an advantage of omitting the GnRH on Day 0, but this finding needs further confirmation in a larger group of heifers.

Although there was no difference in ovulation rate after giving GnRH on Day 9, the correspondence analysis clearly discriminated between treatments in terms of timing of ovulation, with each of the three treatments in a different quadrant and associated with different ovulation times. It appears that the present study is the first that has used correspondence analysis to analyse ovulation time, and its results show the added value of using such an analysis compared to a simple chi-square analysis which is much less powerful. It is less clear what the significance of these differences is - Colazo and Ambrose (2011) reported that heifers which ovulated prior to being given GnRH on Day 9 still had high pregnancy rates even though ovulation prior to insemination is usually associated with low pregnancy rates (Trimberger,

1944; Barrett and Casida, 1946). Nevertheless, the correspondence analysis clearly shows that removal of the first GnRH did not result in significantly delayed ovulation at the end of the programme, inasmuch as treatment with P+G+P4 was associated with earlier ovulation than GPG.

Conclusions

Neither removing the first GnRH nor the progesterone device from a GPG+P4 programme had a significant effect on follicular dynamics of the preovulatory follicle in this study. These results suggest that the benefit of progesterone treatment in synchronisation programmes in heifers is not related to an effect mediated via follicular dynamics. Further research, focussing on other areas such as frequency of short cycles after synchronisation, is required to better establish how the effect of progesterone is mediated if cheaper alternatives are to be developed. In contrast, the lack of effect on follicular dynamics of removing the first GnRH treatment supports a lack of benefit of the first GnRH in heifers treated with a GPG+P4 programme. Colazo and Ambrose (2011) suggested that this lack of benefit may be principally seen in programmes based on 5 days of progesterone, but the present data suggest that there may be limited benefit in a 7-day programme too. Large-scale studies under New Zealand conditions (including both 5- and 7-day programmes) are required to confirm this suggestion.

Acknowledgements

This research was supported by a New Zealand International Doctoral Research scholarship and a Massey University Doctoral Research Scholarship. The product provided by Bomac Laboratories is gratefully acknowledged. The contributions of the farm manager of Haurongo Farm and undergraduate veterinary students of Massey University are acknowledged.

CHAPTER 8

General discussion

Under the pastoral systems which predominate New Zealand dairy farms, synchronisation of ovulation is not only an important heifer management tool but is also the foremost treatment option for dairy cows with postpartum anoestrus. Although the underlying physiological actions of the Day 0 GnRH injection and progesterone in the GPG+P4 programme are not fully understood, the GPG+P4 programme is currently the recommended synchronisation treatment for both heifers and cows, mainly based on acceptable conception rates in field studies. However, the physiological actions of at least two of the components (Day 0 GnRH injection and progesterone device) of this programme is not addressed very well in the literature, so the objectives of this thesis were to better understand the role and importance of both the Day 0 GnRH injection and the progesterone device in a GPG+P4 programme in nulliparous heifers and anoestrous dairy cows. In addition, the impact of negative energy balance on the response to synchronisation in postpartum anoestrous dairy cattle was also studied.

Most of the studies reported in this thesis focused on the physiological response to synchronisation, primarily the changes in ovarian follicular dynamics, steroid hormone concentrations (oestradiol and progesterone) and the timing of the final synchronised ovulation. One study, the heifer field study (Chapter 6), differed from this model as, whilst a field study on anoestrous dairy cows (McDougall, 2010a) had shown that the currently recommended GPG+P4 synchronisation programme was the best of the feasible alternatives under New Zealand conditions, no such information existed for either GPG, progesterone PGF_{2α} synchrony of heifers in New Zealand farming systems.

Effects of removing Day 0 GnRH injection

The first study in this thesis, undertaken in postpartum anoestrous cows (Chapter 3), focussed on the size of the dominant follicle and the CL on Day 7 (i.e. at the time of PGF_{2α} treatment) as these appear to be of importance in influencing the synchronisation of ovulation (Savio *et al.*, 1993b; Diskin *et al.*, 2002). In this study, CL volume was used as an outcome variable rather than CL diameter. This was chosen on the basis of the assumption that many corpora lutea contains a fluid filled cavity and, therefore, by calculating the CL volume, the inclusion of the volume of the vacuole is avoided and will lead to a more accurate estimation of the functional size of the organ. The size of Day 7 dominant follicle was not affected when Day 0 GnRH treatment of GPG+P4 was excluded, nor was response affected by Day 0 CL status or interaction between treatment and CL status. However, the result showed a variation in Day 7 CL volume in cows based on the presence of absence of CL on Day 0 of the treatment. In CL

negative cows, removal of Day 0 GnRH led to reduced CL development whereas in CL positive cows no such effect was seen. Another variable tested was the presence or absence of CL by Day 7 for the combined data (which indeed made the power of study acceptable for the given sample size). When Day 0 GnRH was excluded from the GPG+P4 treatment there was a significant reduction in the proportion of cows with an observable CL on Day 7 (P+G+P4: 42% [10/24] *versus* GPG+P4: 69% [20/29]). This finding is of significance in showing the role of Day 0 GnRH for treating anoestrous dairy cows, which leads to a conclusion that when Day 0 GnRH is excluded from a GPG+P4 programme there is less precise synchronisation of ovulation which may in part be due to the absence of a CL or poorly developed CL on Day 7. This, in turn, may cause incomplete luteal regression or a CL that is unresponsive to PGF_{2α} on Day 7 and further may increase the risk of conception failure after FTAI (Burke *et al.*, 1996; Peters *et al.*, 1999b; Moreira *et al.*, 2000b; Kim *et al.*, 2003). Complete luteolysis of a responsive CL may later lead to more synchronised ovulations and elevated circulating progesterone concentrations during the post-insemination period, resulting in an increased pregnancy rate. This notion is supported by a positive correlation between serum progesterone concentration before AI and conception rate (Fonseca *et al.*, 1983) and decreased conception rates in dairy cows without a CL at the end of progesterone treatment (Smith and Stevenson, 1995) as compared to those having a CL. The size of the dominant follicle on Day 7 is likely to be another important factor affecting the synchronisation of ovulation after treatment with GnRH on Day 9. However it was not affected by the exclusion of Day 0 GnRH treatment, perhaps as the study needed more power to detect such difference.

In Chapter 4, follicular dynamics and ovulation synchronisation was studied in postpartum cows which were given the similar hormonal treatment as in Chapter 3. There was no difference between treatment groups in the percentages of CL negative cows which underwent either ovulation or atresia of the original dominant follicle after the start of the synchronisation protocol (Chapter 4, Table 4.4). However, emergence of the new follicular wave tended to be later (0.7 days) in cows treated with the P+G+P4 protocol than in those that had received GnRH on Day 0 ($P=0.09$). The persistence of the dominant follicle, together with a largely unregulated emergence of a new follicular wave in the middle of the synchronisation protocol, led to a greater rate of growth in the dominant follicle in P+G+P4 group compared to the GPG or GPG+P4 groups. Regardless of this, the size of the dominant follicle on Day 9 did not affect its ability to ovulate in response to the final GnRH treatment. Whether the fertility of the larger follicles in the P+G+P4 group would have been affected is unclear as some studies have shown that larger ovulatory follicles result in higher conception rates than do smaller ones (Perry *et al.*, 2005; Bello *et al.*, 2006; Lopes *et al.*, 2007), whilst some other studies have emphasized the age

of the dominant follicle to be of greater significance than size *per se* (Mihm *et al.*, 1994; Ahmad *et al.*, 1997; Austin *et al.*, 1999; Townson *et al.*, 2002).

In contrast, omitting the Day 0 GnRH injection in nulliparous heifers had a significant impact on follicular dynamics between Days 0 and 7 as, in the P+G+P4 group, there were no ovulations before Day 7 (*versus* 40% in GPG+P4 group) and new follicular waves started later than in the heifers treated with GnRH on Day 0 (3.0 *versus* 4.3 days). Although there was no difference in ovulation rate after giving GnRH on Day 9 in heifers, the correspondence analysis clearly showed that the removal of the Day 0 GnRH did not result in significantly delayed ovulation at the end of the programme: indeed treatment with P+G+P4 was associated with earlier ovulation than GPG. Furthermore, in the P+G+P4 group, only one heifer (5%) had a small dominant follicle (≤ 11 mm) on Day 9, a figure which was much lower than the 24% in the GPG+P4 group and 11% in the GPG group. That heifer was one of the three heifers which did not ovulate in response to the Day 9 GnRH. If this lack of small follicles is a consistent finding it might be indicative of an advantage of omitting the GnRH on Day 0 in heifers, but this finding needs further confirmation in a larger group of heifers.

Effects of removing progesterone device

Removal of progesterone device had no effect on dominant follicle size on Day 7 or on the proportion of cows with observable CL on Day 7 (combined data; GPG: 78% [22/28] vs. GPG+P4: 69% [20/29], Chapter 3). The situation in regard to CL volume was more complex and inconclusive due to the lack of power in the study reported in Chapter 3. This study suggested that, when a CL is present, its support by GnRH may not be necessary when progesterone is also supplied exogenously. Such a conclusion is in contrast to previous studies where GnRH was used and was shown to improve the duration of luteal activity (MacMillan *et al.*, 1985; Thatcher *et al.*, 1989; Peters *et al.*, 1999). Indeed the present results suggest that exogenous progesterone may directly support CL size. In contrast, when there is no CL present, omission of GnRH on Day 0 reduces the chance of a CL being present on Day 7, and, perhaps more importantly, if a CL is present it may be younger, smaller CL and, hence, less responsive to PGF_{2α}. Further data is required to confirm these suggestions, as at the univariable level the study lacked power, across the combined data a difference of 764 mm³ in CL volume could have been detected with 80% power ($\alpha = 0.05$).

Further study in Chapter 4 comparing the follicular dynamics of GPG and GPG+P4 programmes inferred that cows in both the groups were similarly competent at developing an LH surge in response to exogenous GnRH. Likewise, the similar, and normal, interval to emergence of a new follicular wave also indicated that the FSH-based stimulation of the

follicular wave was unaffected by the presence or absence of progesterone. Furthermore, the final ovulation response, at least in terms of the proportions of cows that ovulated, was largely unaffected by either the presence of a CL on Day 7 or the circulating concentrations of progesterone during the synchronisation protocol. Thus, whilst the GPG treatment resulted in a slightly greater probability (76%) of a CL being present on Day 7 than did GPG+P4 (61%), there was no difference in the proportion of cows that ovulated in response to GnRH administration on Day 9 (85.7% *versus* 88.9%). This effect was also independent of the CL status of the cows at the start of the study.

In the CL negative cows, progesterone concentrations on Day 7 did not differ significantly between groups, which suggests that the presence of a progesterone insert (GPG+P4 and P+G+P4 groups) did not elevate progesterone concentrations above those produced by an induced endogenous CL alone (i.e. in the GPG group). Although this result is difficult to explain in terms of combinations of pre-existing and induced corpora lutea, even if there was a small augmentation of progesterone concentrations due to the insert, this finding matches that of the preliminary cow study (Chapter 3) in which the addition of a progesterone device had a beneficial effect in CL-positive cows, in both the GPG+P4 and P+G+P4 groups (i.e. as it resulted in a relatively higher Day 7 CL volume). This finding is further supported by the literature reporting that progesterone secretion is directly proportional to CL size (Stabenfeldt *et al.*, 1969; Erb *et al.*, 1971; Wise *et al.*, 1982; Ott *et al.*, 1986; Sprecher *et al.*, 1989; Kastelic *et al.*, 1990). In contrast to the limited differences in progesterone concentration, there were significant differences in the distribution of ovulation times between the groups. Overall, cows treated with GPG, regardless of their CL status at the start of the experiment, tended ($P = 0.06$) to have a higher proportion of ovulations between 24 and 36 h after GnRH administration (66.7%) than did the other groups (GPG+P4: 50%, P+G+P4: 38.9%). These data closely accord with the findings of Pursley *et al.* (1995), who reported that the highest proportion of ovulations (60%) occurred 26–28 h after final GnRH administration.

As the first two synchronisation studies (Chapter 3 and 4) in cows had both shown the Day 0 GnRH to be an important part of regimen for the treatment of anoestrous cows, a further direct comparison of the GPG and the GPG+P4 treatments (Chapter 5) was studied only in a small group of CL negative cows. This involved daily ultrasonography and blood sampling to study progesterone and oestradiol concentrations. The effects of GPG+P4 and GPG programmes were remarkably similar in terms of the synchronisation of ovulation, although the GPG programme led to the development of relatively larger dominant follicles and higher circulating concentrations of oestradiol than did the GPG+P4 programme. The responses to the Day 0 GnRH (with or without intravaginal progesterone) of the synchronisation protocols in the

present study were similar between the two groups, as shown by similar rates of atresia of the original dominant follicles, no difference in the number of days to the emergence of new follicular waves, and no difference in the proportion of follicles that ovulated after first administration of GnRH on Day 0. The findings of this study (Chapter 5) were therefore similar to those of the previous study in anoestrous cows (Chapter 4).

However, the overall proportion of cows with synchronised ovulation (within 48 h after the second GnRH injection) for the GPG+P4 group was 100%, whereas that for the GPG group was only 63.6% ($P=0.09$; Chapter 5, Figure 4.5). Moreover, this difference in synchronised ovulations between the GPG and the GPG+P4 groups was accentuated by the fact that there was a great deal of variation (very small to very large) in the size of the dominant follicle on Day 9 in the GPG group. The variation was very much less in the GPG+P4 group, supporting the notion that the follicle growth in the GPG group was not well synchronised. This difference in variation of Day 9 dominant follicle size seems to be one of the reasons for the spread in the timing of final ovulation and relatively more non-responders in the GPG than the GPG+P4 group. This conclusion would require further investigation with greater numbers of cows to be made definitively.

An appropriate response to PGF_{2α} on Day 7 is critical for the successful synchronisation of ovulation in a GPG programme. The data from Chapter 5 suggest that there was no difference in response to PGF_{2α} between cows treated with GPG and those treated with GPG+P4. The progesterone concentrations on Day 9 were not significantly different between treatments (GPG: 1.02 ± 0.57 ng/mL; GPG+P4: 0.59 ± 0.59 ng/mL; $P=0.61$). The proportion of cows with progesterone <1 ng/mL was also similar (9/11 and 10/10, respectively).

The results of Chapter 5 provide a little evidence as to why GPG+P4 synchronisation protocols produce better conception rates and pregnancy outcomes than those based on GPG alone (El-Zarkouny *et al.*, 2004; Melendez *et al.*, 2006; Stevenson *et al.*, 2008b; McDougall, 2010a). Firstly, the mean size of the dominant follicle on Day 9 in the GPG+P4 group was slightly, albeit non-significantly, smaller than that of the GPG group. Secondly, the post-ovulation progesterone concentrations were also numerically (but not statistically significantly) higher in the GPG than the GPG+P4 group; and thirdly, oestradiol concentrations were higher during the follicular growth phase in GPG cows than GPG+P4 cows. The smaller follicle that was present on Day 9 in the GPG+P4 group would be expected to have led to the formation of a relatively smaller CL, since ovulation of smaller follicles leads to the development of a smaller CL and lower progesterone concentrations (Vasconcelos *et al.*, 2001; Santos *et al.*, 2004; Perry *et al.*, 2007). Likewise, reports have shown that GnRH-induced ovulation of a small dominant follicle

leads to reduced circulating oestradiol concentrations, decreased oocyte competence (Arlotto *et al.*, 1996), a lower fertilization rate and reduced chances of successful establishment of pregnancy (Jinks *et al.*, 2013). Taken together, these data would not lead one to expect better reproductive outcomes in the GPG+P4 group. On the other hand, the ovulation rate in the first 48 h after administration of GnRH on Day 9 was relatively lower in the GPG than the GPG+P4 group (63.6% *versus* 100%). Given that field use of both GPG and GPG+P4 protocols requires FTAI rather than insemination to observed oestrus, it could be concluded from Chapters 4 and 5 that there may be some difference in synchrony of ovulation but such differences are not major and therefore not likely to be the principal factor determining the improved fertility in GPG+P4 compared to GPG treated cows. In conclusion, there was no definitive evidence in these studies in cows of how a GPG+P4 programme is better than a GPG programme, supporting the conclusion that it is probably due to progesterone priming with longer inter-oestrus intervals after treatment as suggested by McDougall (2010).

In nulliparous dairy heifers (Chapter 7), the removal of the progesterone-releasing device from a GPG+P4 programme had no effect on follicular dynamics or on the proportion of heifers which ovulated after either the GnRH injection on Day 0 or Day 9. The lack of impact of the use of progesterone on follicular dynamics continued even after the removal of the progesterone device on Day 7. A key finding of the comparison between GPG and GPG+P4 programme in heifer was the relationship between ovulation response to Day 0 GnRH and conception rate. Although there was not enough statistical power, the heifers which did ovulate in response to Day 0 GnRH had a higher conception rate (66.7%) than those did not (41.4%). Further research is required to better establish the importance of response to first GnRH on subsequent fertility of dairy heifers.

Effect of energy balance on response to oestrus synchronisation

The results of Chapter 3 showed that nutritional status exerted an influence upon treatment response, as significant effects of Day 0 insulin, Day 0 IGF-I and Day 7 NEFA concentrations on Day 7 CL volume were recorded. These findings are in accord with the literature inasmuch as insulin and IGF-I have direct effects on ovarian cells *in vitro*, including stimulation of proliferation of granulosa cells and progesterone production by bovine granulosa and luteal cells (Poretsky and Kalin, 1987; Spicer and Echternkamp, 1995); whilst *in vivo*, their concentrations have been related to postpartum ovarian follicular development (Adashi *et al.*, 1985; Hammond *et al.*, 1988). There was also a significant interaction between treatment and Day 7 NEFA concentrations. Elevated NEFA concentrations have been shown to have detrimental effects on *in vitro* follicle cell viability and function (Leroy *et al.*, 2005; Vanholder *et al.*, 2006) and to retard luteal growth (Zulu *et al.*, 2002b; Jorritsma *et al.*, 2004). However, in the present study,

increased Day 7 NEFA concentrations had a different effect depending on treatment, with higher Day 7 NEFA concentrations being associated with a greater CL volume on Day 7 in cattle treated with either GPG or P+G+P4 but a smaller CL volume in the GPG+P4 group.

The studies in this thesis have also shown that the interval between calving and the initiation of hormonal treatment affected treatment response. A significant negative effect of a short postpartum interval has been previously reported in treating anoestrous dairy cows in New Zealand (Nation *et al.*, 1997). However, in contrast to that study, the effect seen in this study depended on the treatment, with a longer interval being associated with a greater CL volume in both GPG+P4 and P+G+P4 treated cattle, whereas in the GPG group it was associated with a smaller CL volume.

To further understand the underlying mechanism of the effects of energy balance on response to hormonal treatment (follicular dynamics and synchronisation of ovulation) in cows with postpartum anoestrus more intensive ultrasonography and blood sampling regimens were used (Chapter 4). The interval between calving and the start of the synchronisation protocols (9–10 weeks), together with the small overall change in weight before, during and after the experiment (Chapter 4, Table 1.4 and 2.4), indicated that most cows used in this experiment had probably passed through the nadir of negative energy balance. McDougall *et al.* (1993) showed that the period of negative energy balance typically lasts for 4 to 14 weeks postpartum, and that anoestrous cows tend to have a longer, and more significant negative energy balance than do those cows that resume oestrous cycles by the start of mating. Hence, it was notable that NEFA concentrations were significantly higher on Days 0, 7 and 9, and insulin concentrations were significantly lower on Day 0, in cows that failed to ovulate in response to the synchronisation protocol compared with cows that did ovulate. These findings were supported by previous *in vitro* studies in which the elevated NEFAs had a detrimental effects on follicle cell viability and function (Leroy *et al.*, 2005; Vanholder *et al.*, 2006) and the retardation of luteal growth due to elevated NEFA (Zulu *et al.*, 2002b; Jorritsma *et al.*, 2004). Similarly, the areas under the curve of insulin and IGF-I concentrations were numerically lower in cows that did not ovulate than in those that did. Nonetheless, the clear and significant relationship between NEFA concentrations and ovulation in response to all synchronisation protocols was a key finding of Chapter 4, which showed that, regardless of the regimen that is used to treat anoestrous cows, the response will be moderated and limited by the degree of negative energy balance. Furthermore, BCS of all the treated cows was significantly less than the industry standard (≥ 4 at the time mating) therefore studying the effect of BCS on the response to treatment would have added more value to the present findings. Future research on the effects of BCS on response to hormonal treatment may be a useful study for the benefit of the New Zealand dairy industry as

it might suggest an optimum BCS to be maintained to get the best possible response to the treatment.

One overall conclusion that can be drawn from Chapters 3, 4 and 5 is that anoestrous New Zealand dairy cows respond to hormonal synchronisation as well as, if not better than, high-producing cyclic North American dairy cows. The lack of response at the time of Day 0 GnRH treatment (Moreira *et al.*, 2001; Gümen *et al.*, 2003; Lopez *et al.*, 2004) in a significant proportion of synchronised North American dairy cows (20–30%) led to the addition of treatment pre-synchronisation in order to ensure that when cows are treated with the main synchronisation programme they are in Days 5 to 12 of the oestrous cycle (Moreira *et al.*, 2001; El-Zarkouny *et al.*, 2004; Navanukraw *et al.*, 2004; Chebel *et al.*, 2006; Bicalho *et al.*, 2007; Souza *et al.*, 2008). However, although the conception rate to synchronisation treatment was not studied in the present study, the CL development and synchrony of ovulation in response to treatment has shown that anovulatory anoestrous New Zealand dairy cows could be effectively treated by the GPG-based programmes without the need of any presynchronisation.

Comparison of conception rates to two synchronisation programmes

The heifer field study (Chapter 6) compared the pregnancy rates of a GPG+P4 programme recommended by McDougall *et al.* (2013) with that of a rather different oestrus synchronisation programme (P4+PG) which had never been evaluated under New Zealand conditions. In this study, synchronising heifers with the P4 + PG protocol resulted in similar conception rates to synchronising with GPG+P4 (54.8% *versus* 52.4%, respectively) which was in turn similar to the previous reports using such programmes in dairy heifers in New Zealand (Pickering, 2008; McDougall *et al.*, 2013), Canada (Ambrose *et al.*, 2008) and USA (Lucy *et al.*, 2001b). This study provides further evidence that Day 0 GnRH is not required in heifer synchrony. The P4+PG programme produced similar conception rates to the GPG + P4 programme but it was significantly less expensive per animal (NZ\$24.10 *versus* 35.30) and significantly less costly per pregnancy (NZ\$44.96 *versus* \$65.85). Although this study had sufficient statistical power, further such experiments to study the effects of location and season would be useful in order to understand the extent to which the results of this thesis can be generalised.

Conclusions

The novel outcomes of this thesis have shown that the physiological effects of the GPG and the GPG+P4 programmes were similar when used to treat dairy cows not detected in oestrus. The higher conception rate in cows treated with a GPG+P4 programme rather than a GPG programme does not seem to be modulated by the actions on follicular dynamics and improved synchronised ovulation in dairy cattle with postpartum anoestrous (or in nulliparous heifers).

However, inclusion of the Day 0 GnRH still seems reasonable in a GPG programme for cows with postpartum anoestrus as it leads to a higher probability of a CL on Day 7. Additionally, negative energy balance can significantly affect the treatment response in postpartum cows not detected in oestrus, as evidenced by a significant negative effect of Day 7 NEFA concentration on the probability of ovulation after the Day 9 GnRH injection.

In nulliparous dairy heifers, neither removing the Day 0 GnRH nor the progesterone device from a GPG+P4 programme had a significant effect on follicular dynamics of the preovulatory follicle, suggesting that the known benefits of progesterone treatment in synchronisation programmes in heifers is not related to an effect mediated via follicular dynamics. The lack of an effect of Day 0 GnRH is consistent with the finding in the field study that the potentially cheaper P4+PG programme was as effective at heifer synchronisation as the GPG+P4 programme.

Limitations of the present research

There were several limitations of the present research. The sample size used for some of the experiments were the biggest limiting factor for confirming the validity of the results and conclusions. Furthermore, the maximum number of animals used in each experiments was also based upon (i) the maximum number of animals that could be scanned and blood sampled in a day and (ii) the total number of animals available in the research farm during a breeding season. The frequency of ultrasonography using hand-held probe was another issue in primiparous heifers, as the rectum of some of the relatively younger heifers was very narrow. This difficulty in scanning was a leading factor to exclude several heifers from further scanning causing a significant data loss and which further compromised the power of the study. Therefore, to increase the power of the study, the experiment in heifers was repeated for another year. Moreover, because all the experiments documented in this thesis were carried out at Massey-owned research farms (except one farm in Chapter 6) it was not possible to examine whether there was any variability in results due to farm and locations effects. The anoestrous cows allocated for this research were also in the poor body condition (<4) compared to the recommended industry standard of ≥ 4 at the time of breeding. Although the animals were balanced for BCS at the beginning of the study, the lower BCS reflects a selection bias. If such experiments are repeated in future then it would be interesting to see the effects of BCS on the outcome variables.

Suggestions for future research

Future research is recommended to resolve the limitations and selection and measurement biases discussed in this thesis. Future work may be conducted to address the following points:

1. Carrying out similar experiments with larger sample size and across various locations and seasons.
2. Study of more precise timing of ovulation by increasing the frequency of ultrasound scanning and more frequent blood sampling to study the concentration of LH hormone.
3. Study of effects of BCS on response to synchronisation of ovulation in both nulliparous heifers and anoestrous cows.
4. Study of the effects of inclusion of progesterone device in GPG programmes on the subsequent growth, development and vitality of the embryo.
5. A cost-benefit study of the programmes for heifers and cows under commercial settings.

References

- Adams, G. P.; Matteri, R. L.; Ginther, O. J. 1992: Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *Journal of Reproduction and Fertility* **96**: 627-640.
- Adams, G. P.; Jaiswal, R.; Singh, J.; Malhi, P. 2008: Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology* **69**: 72-80.
- Adashi, E. Y.; Resnick, C. E.; D'ercole, A. J.; Svoboda, M. E.; Wyk, J. J. V. 1985: Insulin-Like Growth Factors as Intraovarian Regulators of Granulosa Cell Growth and Function. *Endocrine Reviews* **6**: 400-420.
- Adewuyi, A. A.; Gruys, E.; van Eerdenburg, F. J. C. M. 2005: Non esterified fatty acids (NEFA) in dairy cattle. A review. *Veterinary Quarterly* **27**: 117-126.
- Ahmad, N.; Townsend, E. C.; Dailey, R. A.; Inskeep, E. K. 1997: Relationships of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. *Animal Reproduction Science* **49**: 13-28.
- Alila, H. W.; Hansel, W. 1984: Origin of different cell types in the bovine corpus luteum as characterized by specific monoclonal antibodies. *Biology of Reproduction* **31**: 1015-1025.
- Ambrose, D. J.; Emmanuel, D. G. V.; Colazo, M. G.; Kastelic, J. P. 2008: Short communication: Pregnancy rates to timed artificial insemination in Holstein heifers given prostaglandin F_{2α} twenty-four hours before or concurrent with removal of an intravaginal progesterone-releasing insert. *Journal of Dairy Science* **91**: 2678-2683.
- Ambrose, J. D.; Kastelic, J. P.; Rajamahendran, R.; Aali, M.; Dinn, N. 2005: Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. *Theriogenology* **64**: 1457-1474.
- Amstalden, M.; Harms, P. G.; Welsh Jr, T. H.; Randel, R. D.; Williams, G. L. 2005: Effects of leptin on gonadotropin-releasing hormone release from hypothalamic-infundibular explants and gonadotropin release from adenohypophyseal primary cell cultures: further evidence that fully nourished cattle are resistant to leptin. *Animal Reproduction Science* **85**: 41-52.
- Arlotto, T.; Schwartz, J.; First, N.; Leibfried-Rutledge, M. 1996: Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes. *Theriogenology* **45**: 943-956.
- Armstrong, D. G.; Baxter, G.; Hogg, C. O.; Woad, K. J. 2002: Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles. *Reproduction* **123**: 789-797.
- Atkins, J. A.; Busch, D. C.; Bader, J. F.; Keisler, D. H.; Patterson, D. J.; Lucy, M. C.; Smith, M. F. 2008: Gonadotropin-releasing hormone-induced ovulation and luteinizing hormone release in beef heifers: Effect of day of the cycle. *Journal of Animal Science* **86**: 83-93.

- Austin, E. J.; Mihm, M.; Ryan, M. P.; Williams, D. H.; Roche, J. F. 1999: Effect of duration of dominance of the ovulatory follicle on onset of estrus and fertility in heifers. *Journal of Animal Science* **77**: 2219-2226.
- Bao, B.; Garverick, H. A. 1998: Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of Animal Science* **76**: 1903-1921.
- Baratta, M.; Grasselli, F.; Tamanini, C. 1994: Effects of gonadal steroids on tonic luteinizing hormone (LH) release and luteinizing hormone-releasing hormone-induced LH release from bovine pituitary cells cultured in vitro. *Biology of Reproduction* **50**: 1320-1327.
- Barrett, G. R.; Casida, L. E. 1946: Time of insemination and conception rates in artificial breeding. *Journal of Dairy Science* **29**: 556.
- Bauman, D. E.; Currie, W. B. 1980: Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. *Journal of Dairy Science* **63**: 1514-1529.
- Beam, S. W.; Butler, W. R. 1997: Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. *Biology of Reproduction* **56**: 133-142.
- Beam, S. W.; Butler, W. R. 1998: Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. *Journal of Dairy Science* **81**: 121-131.
- Beck, T. W.; Convey, E. M. 1977: Estradiol control of serum luteinizing hormone concentrations in the bovine. *Journal of Animal Science* **45**: 1096-1101.
- Bello, N. M.; Steibel, J. P.; Pursley, J. R. 2006: Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of ovsynch in lactating dairy cows. *Journal of Dairy Science* **89**: 3413-3424.
- Bicalho, R.; Cheong, S.; Warnick, L.; Guard, C. 2007: Evaluation of progesterone supplementation in a prostaglandin F_{2α} - based presynchronization protocol before timed insemination. *Journal of Dairy Science* **90**: 1193-1200.
- Bilby, T. R.; Sozzi, A.; Lopez, M. M.; Silvestre, F. T.; Ealy, A. D.; Staples, C. R.; Thatcher, W. W. 2006: Pregnancy, bovine somatotropin, and dietary n-3 fatty acids in lactating dairy cows: I. ovarian, conceptus, and growth hormone - insulin -like growth factor system responses. *Journal of Dairy Science* **89**: 3360-3374.
- Blache, D.; Chagas, L. M.; Martin, G. B. 2007: Nutritional inputs into the reproductive neuroendocrine control system a multidimensional perspective. *Reproduction in Domestic Ruminants* **6**: 123-139.
- Bo, G. A.; Cutaia, L. E.; Souza, A. H.; Baruselli, P. S. 2007: Systematic reproductive management in dairy herds. *Proceedings of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 155-168.
- Boulware, S. D.; Tamborlane, W. V.; Rennert, N. J. 1994: Comparison of the metabolic effects of recombinant human insulin-like growth factor-I and insulin. *Journal of Clinical Investigation* **93**: 1131-1139.

- Boulware, S. D.; Tamborlane, W. V.; Matthews, L. S.; Sherwin, R. S. 1992: Diverse effects of insulin-like growth factor I on glucose, lipid, and amino acid metabolism. *American Journal of Physiology - Endocrinology and Metabolism* **262**: E130-E133.
- Britt, J. H. 1992: Impacts of early postpartum metabolism on follicular development and fertility. *Proceedings of Annual Convention American Association of Bovine Practitioner* Pp. 39-43.
- Brockman, R. P. 1979: Roles for insulin and glucagon in the development of ruminant ketosis - a review. *Canadian Veterinary Journal* **20**: 121-126.
- Brown, J.; Reeves, J. 1983: Absence of specific luteinizing hormone releasing hormone receptors in ovine, bovine and porcine ovaries. *Biology of Reproduction* **29**: 1179-1182.
- Brown, J. G.; Peterson, D. W.; Foote, W. D. 1972: Reproductive response of beef cows to exogenous progestogen, estrogen and gonadotropins at various stages postpartum. *Journal of Animal Science* **35**: 362-369.
- Brownlie, T. S.; Morton, J. M.; Heuer, C.; Hunnam, J.; McDougall, S. 2013: Reproductive performance of seasonal-calving, pasture-based dairy herds in four regions of New Zealand. *New Zealand Veterinary Journal* **62**: 77-86.
- Brownlie, T. S.; Weir, A. M.; Tarbotton, I.; Morton, J. M.; Heuer, C.; McDougall, S. 2011: Reproductive management of dairy herds in New Zealand: Attitudes, priorities and constraints perceived by farmers managing seasonal-calving, pasture-based herds in four regions. *New Zealand Veterinary Journal* **59**: 28-39.
- Burke, C. R.; Mihm, M.; Macmillan, K. L.; Roche, J. F. 1994: Some effects of prematurely elevated concentrations of progesterone on luteal and follicular characteristics during the oestrous cycle in heifers. *Animal Reproduction Science* **35**: 27-39.
- Burke, J. M.; De La Sota, R. L.; Risco, C. A.; Staples, C. R.; Schmitt, É. J. P.; Thatcher, W. W. 1996: Evaluation of timed insemination using a gonadotropin-releasing hormone agonist in lactating dairy cows. *Journal of Dairy Science* **79**: 1385-1393.
- Butler, S. T.; Pelton, S. H.; Butler, W. R. 2004: Insulin increases 17β -estradiol production by the dominant follicle of the first postpartum follicle wave in dairy cows. *Reproduction* **127**: 537-545.
- Butler, W. R. 2000a: Nutritional interactions with reproductive performance in dairy cattle. *Animal Reproduction Science* **60-61**: 449 - 457.
- Butler, W. R. 2000b: Nutritional interactions with reproductive performance in dairy cattle. *Animal Reproduction Science* **60-61**: 449-457.
- Butler, W. R.; Smith, R. D. 1989: Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *Journal of Dairy Science* **72**: 767 - 783.
- Butler, W. R.; Everett, R. W.; Coppock, C. E. 1981: The relationships between energy balance, milk production and ovulation in postpartum Holstein cows. *Journal of Animal Science* **53**: 742-8.
- Canadian Animal Health Institute 2005: Compendium of veterinary products 9th Edition. Canadian Animal Health Institute Guelph, Ontario, Canada.

- Canfield, R. W.; Butler, W. R. 1990: Energy balance and pulsatile LH secretion in early postpartum dairy cattle. *Domestic Animal Endocrinology* **7**: 323-330.
- Cartmill, J. A.; El-Zarkouny, S. Z.; Hensley, B. A.; Lamb, G. C.; Stevenson, J. S. 2001: Stage of cycle, incidence, and timing of ovulation, and pregnancy rates in dairy cattle after three timed breeding protocols. *Journal of Dairy Science* **84**: 1051-1059.
- Cavalieri, J.; Hepworth, G.; Smart, V. M.; Ryan, M.; Macmillan, K. L. 2007: Reproductive performance of lactating dairy cows and heifers resynchronized for a second insemination with an intravaginal progesterone-releasing device for 7 or 8 d with estradiol benzoate injected at the time of device insertion and 24 h after removal. *Theriogenology* **67**: 824-834.
- Celik, H. A.; Aydm, I.; Sendag, S.; Dinc, D. A. 2005: Number of follicular waves and their time effect on pregnancy rate in the cow *Reproduction in Domestic Animals* 87-92.
- Cerri, R. L. A.; Santos, J. E. P.; Juchem, S. O.; Galvao, K. N.; Chebel, R. C. 2004: Timed artificial insemination with estradiol cypionate or insemination at estrus in high-producing dairy cows. *Journal of Dairy Science* **87**: 3704-3715.
- Cerri, R. L. A.; Chebel, R. C.; Rivera, F.; Narciso, C. D.; Oliveira, R. A.; Thatcher, W. W.; Santos, J. E. P. 2011: Concentration of progesterone during the development of the ovulatory follicle: I. Ovarian and embryonic responses. *Journal of Dairy Science* **94**: 3342-3351.
- Chagas, L. M.; Bass, J. J.; Blache, D.; Burke, C. R.; Kay, J. K.; Lindsay, D. R.; Lucy, M. C.; Martin, G. B.; Meier, S.; Rhodes, F. M. and others 2007: Invited review: new perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. *Journal of Dairy Science* **90**: 4022-4032.
- Channing, C. P.; Tsai, V.; Sachs, D. 1976: Role of insulin, thyroxin and cortisol in luteinization of porcine granulosa cells grown in chemically defined media. *Biology of Reproduction* **15**: 235-247.
- Chebel, R. C.; Santos, J. E. P.; Cerri, R. L. A.; Rutigliano, H. M.; Bruno, R. G. S. 2006: Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. *Journal of Dairy Science* **89**: 4205-4219.
- Chenault, J.; Thatcher, W.; Kalra, P.; Abrams, R.; Wilcox, C. 1975: Transitory changes in plasma progestins, estradiol, and luteinizing hormone approaching ovulation in the bovine. *Journal of Dairy Science* **58**: 709-717.
- Chenault, J. R.; Kratzer, D. D.; Rzepkowski, R. A.; Goodwin, M. C. 1990: LH and FSH response of Holstein heifers to ferteirelin acetate, gonadorelin and buserelin. *Theriogenology* **34**: 81-98.
- Christian, R. E. 1948: The effects of progesterone in altering the estrous cycle of the cow. *Journal of Animal Science* **7**: 540.
- Clark, B. A.; Chagas, L. M.; Gore, P. M.; Dow, B.; Verkerk, G. A. 2000: Prediction of post-partum anovulatory interval in dairy cows. *Proceedings of the New Zealand Society of Animal Production*. Pp. 15-18.
- Clark, D. A.; Caradus, J. R.; Monaghan, R. M.; Sharp, P.; Thorrold, B. S. 2007: Issues and options for future dairy farming in New Zealand. *New Zealand Journal of Agricultural Research* **50**: 203-221.

- Clemente, M.; de La Fuente, J.; Fair, T.; Al Naib, A.; Gutierrez-Adan, A.; Roche, J. F.; Rizos, D.; Lonergan, P. 2009: Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138**: 507-517.
- Colazo, M. G.; Ambrose, D. J. 2011: Neither duration of progesterone insert nor initial GnRH treatment affected pregnancy per timed-insemination in dairy heifers subjected to a Co-synch protocol. *Theriogenology* **76**: 578-588.
- Colazo, M. G.; Dourey, A.; Rajamahendran, R.; Ambrose, D. J. 2013: Progesterone supplementation before timed AI increased ovulation synchrony and pregnancy per AI, and supplementation after timed AI reduced pregnancy losses in lactating dairy cows. *Theriogenology* **79**: 833-841.
- Cooper, M. J. 1974: Control of oestrous cycles of heifers with a synthetic prostaglandin analogue. *Veterinary Record* **95**: 200-203.
- Crowe, M.; Padmanabhan, V.; Mihm, M.; Beitins, I.; Roche, J. 1998: Resumption of follicular waves in beef cows is not associated with periparturient changes in follicle-stimulating hormone heterogeneity despite major changes in steroid and luteinizing hormone concentrations. *Biology of Reproduction* **58**: 1445-1450.
- Cunningham, J. G. 2002: Textbook of veterinary physiology, Third Edition. Philadelphia, Pennsylvania, W.B. Saunders Company.
- Dailey, R. A.; Inskeep, E. K.; Washburn, S. P.; Price, J. C. 1983: Use of prostaglandin F_{2α} or gonadotropin releasing hormone in treating problem breeding cows. *Journal of Dairy Science* **66**: 1721-1727.
- Dairy NZ 2007: The InCalf book for New Zealand dairy farmers. Dairy NZ, Private Bag 3221 Hamilton New Zealand.
- Dairy NZ 2009: Strategy for New Zealand dairy farming 2009/2020. Dairy NZ, Private Bag 3221 Hamilton New Zealand. Pp. 8.
- Darwash, A.; Lamming, G.; Woolliams, J. 1997: The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. *Animal Science* **65**: 9-16.
- Darwash, A. O.; Lamming, G. E.; Woolliams, J. A. 1999: The potential for identifying heritable endocrine parameters associated with fertility in post-partum dairy cows. *Journal of Animal Science* **68**: 333 - 347.
- De Rensis, F.; Peters, A. R. 1999: The control of follicular dynamics by PGF2α, gnrh, hCG and oestrus synchronization in cattle. *Reproduction in Domestic Animals* **34**: 49-59.
- de Vries, M. J.; Veerkamp, R. F. 2000: Energy balance of dairy cattle in relation to milk production variables and fertility. *Journal of Dairy Science* **83**: 62-69.
- DeJarnette, J. M. Ovsynch, co-synch, presynch and kitchensynch: how did breeding cows get so complicated? At: http://www.selectsires.com/programs/docs/ovsynch_cosynch_presync_h.pdf (no date). Retrieved 01/02/2014.
- DeJarnette, J. M.; Salverson, R. R.; Marshall, C. E. 2001: Incidence of premature estrus in lactating dairy cows and conception rates to standing estrus or fixed-time inseminations after synchronization using GnRH and PGF_{2α}. *Animal Reproduction Science* **67**: 27-35.

- Deletang, F. 1975: Synchronisation of oestrus in cattle using a progestagen (SC 21,009) and a synthetic analogue of prostaglandin F2alpha (cloprostenol). *Veterinary Record* **97**: 453-454.
- Diskin, M. G.; Austin, E. J.; Roche, J. F. 2002: Exogenous hormonal manipulation of ovarian activity in cattle. *Domestic Animal Endocrinology* **23**: 211-228.
- Diskin, M. G.; Mackey, D. R.; Roche, J. F.; Sreenan, J. M. 2003: Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Animal Reproduction Science* **78**: 345-370.
- Donaldson, L.; Hansel, W. 1968: Cystic corpora lutea and normal and cystic Graafian follicles in the cow. *Australian Veterinary Journal* **44**: 304-308.
- Donaldson, L. E.; Bassett, J. M.; Thorburn, G. D. 1970: Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation, and the effects of under-nutrition or exogenous oxytocin on progesterone concentration. *Journal of Endocrinology* **48**: 599-614.
- Doraiswamy, V.; Knutson, D. L.; Grazul-Bilska, A. T.; Redmer, D. A.; Reynolds, L. P. 1998: Fibroblast growth factor receptor (FGFR)-1 and -2 in the ovine corpus luteum throughout the estrous cycle. *Growth Factors* **16**.
- Driancourt, M. A. 2001: Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology* **55**: 1211-1239.
- Echternkamp, S.; Howard, H.; Roberts, A.; Grizzle, J.; Wise, T. 1994: Relationships among concentrations of steroids, insulin-like growth factor-I, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. *Biology of Reproduction* **51**: 971-981.
- El-Zarkouny, S. Z.; Cartmill, J. A.; Hensley, B. A.; Stevenson, J. S. 2004: Pregnancy in dairy cows after synchronized ovulation regimens with or without presynchronization and progesterone. *Journal of Dairy Science* **87**: 1024-1037.
- Erb, R. E.; Randel, R. D.; Callahan, C. J. 1971: Female sex steroid changes during the reproductive cycle. *Journal of Animal Science* **32**: 80-106.
- Erb, R. E.; Garverick, H. A.; Randel, R. D.; Brown, B. L.; Callahan, C. J. 1976: Profiles of reproductive hormones associated with fertile and nonfertile inseminations of dairy cows. *Theriogenology* **5**: 227-242.
- European Union 2003: Directive 2003/74/ec of the European Parliament and of the Council of 22 September 2003 amending Council Directive 96/22/EC concerning the prohibition on the use of stock farming of certain substances having a hormonal or thyrostatic action and of beta agonists. *Official Journal of the European Union L262 of 14.10.2003*: 17-21.
- Fahey, J.; Morgan, S. R.; Napper, A. R.; Roche, J. R.; Kolver, E. S.; Verkerk, G. A. 2003: Comparative study of ovarian follicle dynamics and IGF-I concentration during an oestrus cycle in two genotypes of lactating Holstein-Friesian cows offered pasture or a total mixed ration. *Proceedings of the fifteenth conference of the association for the advancement of the animal breeding and genetics*. Pp. 227-231.
- Fielden, E. D.; Macmillan, K. L.; D., W. J. 1973: The anoestrous syndrome in New Zealand dairy cattle. 1. A preliminary investigation. *New Zealand Veterinary Journal* **21**: 77-81.

- Figueiredo, R. A.; Barros, C. M.; Pinheiro, O. L.; Soler, J. M. P. 1997: Ovarian follicular dynamics in nelore breed (*Bos indicus*) cattle. *Theriogenology* **47**: 1489-1505.
- Fike, K. E.; Day, M. L.; Inskeep, E. K.; Kinder, J. E.; Lewis, P. E.; Short, R. E.; Hafs, H. D. 1997: Estrus and luteal function in suckled beef cows that were anestrous when treated with an intravaginal device containing progesterone with or without a subsequent injection of estradiol benzoate. *Journal of Animal Science* **75**: 2009-2015.
- Flint, A. P. F.; Sheldrick, E. L. 1982: Ovarian secretion of oxytocin is stimulated by prostaglandin. *Nature* **297**: 587-588.
- Foley, R. C.; Greenstein, J. S. 1958: Cytological changes in the bovine corpus luteum during early pregnancy. *Reproduction and Infertility*, Pergamon Press, New York, USA. Pp. 88-96.
- Folman, Y.; Rosenberg, M.; Herz, Z.; Davidson, M. 1973: The relationship between plasma progesterone concentration and conception in post-partum dairy cows maintained on two levels of nutrition. *Journal of Reproduction and Fertility* **34**: 267-278.
- Folman, Y.; Kaim, M.; Herz, Z.; Rosenberg, M. 1990: Comparison of Methods for the Synchronization of Estrous Cycles in Dairy Cows. 2. Effects of Progesterone and Parity on Conception. *Journal of Dairy Science* **73**: 2817-2825.
- Fonseca, F. A.; Britt, J. H.; McDaniel, B. T.; Wilk, J. C.; Rakes, A. H. 1983: Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open1. *Journal of Dairy Science* **66**: 1128-1147.
- Forde, N.; Beltman, M. E.; Lonergan, P.; Diskin, M.; Roche, J. F.; Crowe, M. A. 2011: Oestrous cycles in *Bos taurus* cattle. *Animal Reproduction Science* **124**: 163-169.
- Fortune, J. E.; Sirois, J. 1989: The use of ultrasonography to study the regulation of follicular development in cattle and horses. In: Tsafirri, A.; Dekel, N. ed. *Follicular Development and the Ovulatory Response*, Ares-Serono Symposia, Rome, Serono Symp. Rev. Pp. 11-20.
- Fortune, J. E.; Sirois, J.; Quirk, S. M. 1988: The growth and differentiation of ovarian follicles during the bovine estrous cycle. *Theriogenology* **29**.
- Fricke, P. M.; Wiltbank, M. C. 1999: Effect of milk production on the incidence of double ovulation in dairy cows. *Theriogenology* **52**: 1133-1143.
- Galvão, K. N.; Santos, J. E. P. 2010: Factors affecting synchronization and conception rate after the ovsynch protocol in lactating Holstein cows. *Reproduction in Domestic Animals* **45**: 439-446.
- Garverick, H.; Zollers Jr, W.; Smith, M. 1992: Mechanisms associated with corpus luteum lifespan in animals having normal or subnormal luteal function. *Animal Reproduction Science* **28**: 111-124.
- Geary, T. W.; Whittier, J. C. 1998: Effects of a timed insemination following synchronization of ovulation using the ovsynch or co-synch protocol in beef cows. *The Professional Animal Scientist* **14**: 217-220.

- Geary, T. W.; Downing, E. R.; Bruemmer, J. E.; Whittier, J. C. 2000: Ovarian and estrous response of suckled beef cows to the select synch estrous synchronization protocol. *The Professional Animal Scientist* **16**: 1-5.
- Ghanem, M. E.; Nakao, T.; Nakatani, K.; Akita, M.; Suzuki, T. 2006: Milk progesterone profile at and after artificial insemination in repeat-breeding cows: effects on conception rate and embryonic death. *Reproduction in Domestic Animals* **41**: 180-183.
- Gibbons, J. R.; Wiltbank, M. C.; Ginther, O. J. 1999: Relationship between follicular development and the decline in the follicle-stimulating hormone surge in heifers. *Biology of Reproduction* **60**: 72-77.
- Ginther, O. J.; Kastelic, J. P.; Knopf, L. 1989a: Composition and characteristics of follicular waves during the bovine estrous cycle. *Animal Reproduction Science* **20**: 187-200.
- Ginther, O. J.; Knopf, L.; Kastelic, J. P. 1989b: Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. *Journal of Reproduction and Fertility* **87**: 223-230.
- Ginther, O. J.; Kot, K.; Kulick, L. J.; Wiltbank, M. C. 1997: Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology* **48**: 75-87.
- Ginther, O. J.; Wiltbank, M. C.; Fricke, P. M.; Gibbons, J. R.; Kot, K. 1996: Selection of the dominant follicle in cattle. *Biology of Reproduction* **55**: 1187-94.
- Giuliodori, M. J.; Delavaud, C.; Chilliard, Y.; Becú-Villalobos, D.; Lacau-Mengido, I.; de la Sota, R. L. 2011: High NEFA concentrations around parturition are associated with delayed ovulations in grazing dairy cows. *Livestock Science* **141**: 123-128.
- Goff, A. K. 2004: Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. *Biology of Reproduction* **71**: 11-16.
- Gong, J. G. 2002: Influence of metabolic hormones and nutrition on ovarian follicle development in cattle: practical implications. *Domestic Animal Endocrinology* **23**: 229-241.
- Gong, J. G.; Lee, W. J.; Garnsworthy, P. C.; Webb, R. 2002: Effect of dietary-induced increases in circulating insulin concentrations during the early postpartum period on reproductive function in dairy cows. *Reproduction* **123**: 419-427.
- Greenfield, R. B.; Cecava, M. J.; Donkin, S. S. 2000: Changes in mRNA expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation. *Journal of Dairy Science* **83**: 1228-1236.
- Guilbault, L. A.; Rouillier, P.; Matton, P.; Glencross, R. G.; Beard, A. J.; Knight, P. G. 1993: Relationships between the level of atresia and inhibin contents (alpha subunit and alpha-beta dimer) in morphologically dominant follicles during their growing and regressing phases of development in cattle. *Biology of Reproduction* **48**: 268-276.
- Gümen, A.; Guenther, J. N.; Wiltbank, M. C. 2003: Follicular size and response to Ovsynch versus detection of estrus in anovular and ovular lactating dairy cows. *Journal of Dairy Science* **86**: 3184-3194.
- Hafs, H. D.; Armstrong, D. T. 1968: Corpus luteum growth and progesterone synthesis during the bovine estrous cycle. *Journal of Animal Science* **27**: 134-141.

- Hafs, H. D.; Manns, J. G.; Drew, B. 1975: Onset of oestrus and fertility of dairy heifers and suckled beef cows treated with prostaglandin F_{2α}. *Animal Science* **21**: 13-20.
- Hammond, J. M.; Hsu, C. J.; Mondschein, J. S.; Canning, S. F. 1988: Paracrine and autocrine functions of growth factors in the ovarian follicle. *Journal of Animal Science* **66**: 21-31.
- Hansel, W. 1966: Luteotrophic and luteolytic mechanisms in bovine corpora lutea. *Journal of Reproduction and Fertility* **1**: 33-48.
- Hansel, W.; Echternkamp, S. E. 1972: Control of ovarian function in domestic animals. *American Zoologist* **12**: 225-243.
- Hansel, W.; Beal, W. E. 1979: Ovulation control in cattle. In: Hawk, H. H. ed. *Animal Reproduction 3.Beltsville Symposium in Agricultural Research*. Pp. 91-110.
- Hansel, W.; Convey, E. M. 1983: Physiology of the estrous cycle. *Journal of Animal Science* **57**: 404-424.
- Hansel, W.; Concannon, P. W.; Lukaszewska, J. H. 1973: Corpora lutea of the large domestic animals. *Biology of Reproduction* **8**: 222-245.
- Hanzen, C.; Pieterse, M.; Scenczi, O.; Drost, M. 2000: Relative accuracy of the identification of ovarian structures in the cow by ultrasonography and palpation *per rectum*. *The Veterinary Journal* **159**: 161-170.
- Harris, B. L.; Kolver, E. S. 2001: Review of holsteinization on intensive pastoral dairy farming in New Zealand. *Journal of Dairy Science* **84**: E56-E61.
- Harris, B. L.; Pryce, J. E.; Xu, Z. Z.; Montgomerie, W. A. 2006: Development of new fertility breeding values in the dairy industry. *Proceedings of the New Zealand Society of Animal Production*. Pp. 107-112.
- Haughian, J. M.; Wiltbank, M. C. 2002: GnRH: from physiology to ‘Synch’-ology. *Proceedings of the Annual Conference of the Society for Theriogenology* Pp. 221-235.
- Heersche, G. J.; Kiracofe, G. H.; McKee, R. M.; Davis, D. L.; Brower, G. R. 1974: Control of estrus in heifers with PGF_{2α} and Syncro-Mate. *Journal of Animal Science* **38**: 225 (Abstract).
- Henricks, D. M.; Dicky, J. F.; Niswender, G. D. 1970: Serum luteinizing hormone and plasma progesterone levels during the estrous cycle and early pregnancy in cows. *Biology of Reproduction* **2**: 346-351.
- Herlihy, M. M.; Crowe, M. A.; Diskin, M. G.; Butler, S. T. 2012: Effects of synchronization treatments on ovarian follicular dynamics, corpus luteum growth, and circulating steroid hormone concentrations in lactating dairy cows. *Journal of Dairy Science* **95**: 743-754.
- Hittinger, M. A.; Ambrose, J. D.; Kastelic, J. P. 2004: Luteolysis, onset of estrus, and ovulation in Holstein heifers given prostaglandin F_{2α} concurrent with, or 24 hours prior to, removal of an intravaginal, progesterone-releasing device. *The Canadian Journal of Veterinary Research* **68**: 283-287.
- Holmes, C. W.; Brookes, I. M.; Garrick, D. J.; Mackenzie, D. D. S.; Parkinson, T. J.; Wilson, G. F. 2002: Milk production from pasture - principles and practices. Massey University Palmerston North New Zealand.

- Ireland, J.; Milvae, R.; Martin, T.; Aten, R.; Behrman, H. 1990: Effect of histone H2A on progesterone production by bovine luteal cells. *Biology of Reproduction* **43**: 1058-1063.
- Ireland, J. J. 1987: Control of follicular growth and development. *Journal of Reproduction and Fertility* **34**: 39-54.
- Ireland, J. J.; Roche, J. F. 1983: Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. *Endocrinology* **112**: 150-156.
- Ireland, J. J.; Roche, J. F. 1987: Hypotheses regarding development of dominant follicles during a bovine estrous cycle. In: Roche, J. F.; O'Callaghan, D. ed. *Follicular Growth and Ovulation Rate in Farm Animals*, Martinus Nijhoff, Dordrecht, The Netherlands. Pp. 1-18.
- Ireland, J. J.; Mihm, M.; Austin, E.; Diskin, M. G.; Roche, J. F. 2000: Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements, and terms. *Journal of Dairy Science* **83**: 1648-1658.
- Jaiswal, R. S.; Singh, J.; Marshall, L.; Adams, G. P. 2009: Repeatability of 2-wave and 3-wave patterns of ovarian follicular development during the bovine estrous cycle. *Theriogenology* **72**: 81-90.
- Jinks, E. M.; Smith, M. F.; Atkins, J. A.; Pohler, K. G.; Perry, G. A.; MacNeil, M. D.; Roberts, A. J.; Waterman, R. C.; Alexander, L. J.; Geary, T. W. 2013: Preovulatory estradiol and the establishment and maintenance of pregnancy in suckled beef cows. *Journal of Animal Science* **91**: 1176-1185.
- Jorritsma, R.; César, M. L.; Hermans, J. T.; Kruitwagen, C. L. J. J.; Vos, P. L. A. M.; Kruip, T. A. M. 2004: Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Animal Reproduction Science* **81**: 225-235.
- Judson, J.; Wyk, V.; GravesS, D. C.; Casella, S. J.; Jacob, S. 1985: Evidence from monoclonal antibody studies that insulin stimulates deoxyribonucleic acid synthesis through the type I somatomedin receptor. *Journal of Clinical Endocrinology & Metabolism* **61**: 639-643.
- Juengel, J. L.; Garverick, H. A.; Johnson, A. L.; Youngquist, R. S.; Smith, M. F. 1993: Apoptosis during luteal regression in cattle. *Endocrinology* **132**: 249-254.
- Kacsoh, B. 2000: Endocrine physiology. *Reproductive endocrinology*. New York, New York, McGraw Hill Book Co. Pp. 559-560.
- Kadivar, A.; Ahmadi, M.; Gheisari, H.; Nazifi, S. 2012: Assessment of IGF-I as a factor influencing postpartum reproductive performance and ovarian condition in dairy cattle. *Comparative Clinical Pathology* **21**: 589-596.
- Kaim, M.; Rosenberg, M.; Folman, Y. 1990: Management of reproduction in dairy heifers based on the synchronization of estrous cycles. *Theriogenology* **34**: 537-547.
- Kaneko, H.; Terada, T.; Taya, K.; Watanabe, G.; Sasamoto, S.; Hasegawa, Y.; Igarashi, M. 1991: Ovarian follicular dynamics and concentrations of oestradiol-17 beta, progesterone, luteinizing hormone and follicle stimulating hormone during the periovulatory phase of the oestrous cycle in the cow. *Reproduction, Fertility and Development* **3**: 529-535.

- Kastelic, J. P.; Ginther, O. J. 1991: Factors affecting the origin of the ovulatory follicle in heifers with induced luteolysis. *Animal Reproduction Science* **26**: 13-24.
- Kastelic, J. P.; Bergfelt, D. R.; Ginther, O. J. 1990: Relationship between ultrasonic assessment of the corpus luteum and plasma progesterone concentration in heifers. *Theriogenology* **33**: 1269-1278.
- Kawashima, C.; Fukihara, S.; Maeda, M.; Kaneko, E.; Montoya, C. A.; Matsui, M.; Shimizu, T.; Matsunaga, N.; Kida, K.; Miyake, Y.-I. and others 2007: Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave post-partum in high-producing dairy cows. *Reproduction* **133**: 155-163.
- Kay, J. K.; Phyn, C. V. C.; Roche, J. R.; Kolver, E. S. 2009: Extending lactation in pasture-based dairy cows. II: Effect of genetic strain and diet on plasma hormone and metabolite concentrations. *Journal of Dairy Science* **92**: 3704-3713.
- Kesler, D.; Garverick, H.; Youngquist, R.; Elmore, R.; Bierschwal, C. 1977: Effect of days postpartum and endogenous reproductive hormones on GnRH-induced LH release in dairy cows. *Journal of Animal Science* **45**: 797-803.
- Kesner, J. S.; Convey, E. M.; Anderson, C. R. 1981: Evidence that estradiol induces the preovulatory LH surge in cattle by increasing pituitary sensitivity to LHRH and then increasing LHRH release. *Endocrinology* **108**: 1386-1391.
- Kim, I. H.; Suh, G. H.; Son, D. S. 2003: A progesterone-based timed AI protocol more effectively prevents premature estrus and incomplete luteal regression than an Ovsynch protocol in lactating Holstein cows. *Theriogenology* **60**: 809-817.
- Kim, U. H.; Suh, G. H.; Nam, H. W.; Kang, H. G.; Kim, I. H. 2005: Follicular wave emergence, luteal function and synchrony of ovulation following GnRH or estradiol benzoate in a CIDR-treated, lactating Holstein cows. *Theriogenology* **63**: 260-268.
- Knickerbocker, J. J.; Wiltbank, M. C.; Niswender, G. D. 1988: Mechanisms of luteolysis in domestic livestock. *Domestic Animal Endocrinology* **5**: 91-107.
- Kojima, N.; Stumpf, T.; Cupp, A. S.; Werth, L.; Roberson, M.; Wolfe, M.; Kittok, R. J.; Kinder, J. E. 1992: Exogenous progesterone and progestins as used in estrous synchrony regimens do not mimic the corpus luteum in regulation of luteinizing hormone and 17 beta-estradiol in circulation of cows. *Biology of Reproduction* **47**: 1009-1017.
- Komaragiri, M. V. S.; Casper, D. P.; Erdman, R. A. 1998: Factors affecting body tissue mobilization in early lactation dairy cows. 2. Effect of dietary fat on mobilization of body fat and protein. *Journal of Dairy Science* **81**: 169-175.
- Koos, R. D.; Hansel, W. 1981: The large and small cells of the bovine corpus luteum: ultrastructural and functional differences. *Dynamics of Ovarian Function*.
- Kulick, L. J.; Kot, K.; Wiltbank, M. C.; Ginther, O. J. 1999: Follicular and hormonal dynamics during the first follicular wave in heifers. *Theriogenology* **52**: 913-921.
- Kyle, S. D.; Callahan, C. J.; Allrich, R. D. 1992: Effect of progesterone on the expression of estrus at the first postpartum ovulation in dairy cattle. *Journal of Dairy Science* **75**: 1456-1460.
- Lamb, G. C.; Stevenson, J. S.; Kesler, D. J.; Garverick, H. A.; Brown, D. R.; Salfen, B. E. 2001: Inclusion of an intravaginal progesterone insert plus GnRH and prostaglandin F2 alpha

- for ovulation control in postpartum suckled beef cows. *Journal of Animal Science* **79**: 2253-2259.
- Lamming, G.; Wathes, D. C.; Peters, A. 1981: Endocrine patterns of the post-partum cow. *Journal of Reproduction and Fertility* **30**: 155.
- Lammond, D. R. 1967a: Synchronization of ovarian function in the cow. *Bovine infertility; proceedings of a postgraduate refresher course conducted at Awahuri in June, 1967, by the Veterinary Services Council and the New Zealand Veterinary Association*. Pp. 51-60.
- Lammond, D. R. 1967b: Control of ovarian function, New Zealand Veterinary Association. 39-50 p.
- Lamothe, P.; Bousquet, D.; Guay, P. 1977: Cyclic variation of F prostaglandins in the uterine fluids of the cow. *Journal of Reproduction and Fertility* **50**: 381-382.
- Laven, R. A. 2008: Life without oestradiol benzoate - AI synchrony in cattle. *Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 173-177.
- Laven, R. A.; Stewart, J.; Dyer, C. 2006: Factors affecting the monetary return from programmes for synchronising oestrus in dairy cows with gonadotrophin-releasing hormone and prostaglandin F_{2α}. *Veterinary Record* **158**: 367-72.
- Lean, I. J.; Galland, J. C.; Scott, J. L. 1989: Relationships between fertility, peak milk yields and lactational persistency in dairy cows. *Theriogenology* **31**: 1093-1103.
- Lean, I. J.; Rabiee, A. R.; Stevenson, M. A. 2003: Meta-analysis: a review of hormonal manipulations of reproduction. *Proceedings of the Australian and New Zealand Combined Dairy Cattle Veterinarians Conference - incorporating the 20th Annual Seminar of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 327-351.
- LeBlanc, S. J.; Leslie, K. E. 2003: Short communication: presynchronization using a single injection of PGF2[alpha] before synchronized ovulation and first timed artificial insemination in dairy cows. *Journal of Dairy Science* **86**: 3215-3217.
- Leroy, J. L. M. R.; Vanholder, T.; Mateusen, B.; Christophe, A.; Opsomer, G.; de Kruif, A.; Genicot, G.; Van Soom, A. 2005: Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro*. *Reproduction* **130**: 485-495.
- Li, X.; Li, X.; Bai, G.; Chen, H.; Deng, Q.; Liu, Z.; Zhang, L.; Liu, G.; Wang, Z. 2012: Effects of non-esterified fatty acids on the gluconeogenesis in bovine hepatocytes. *Molecular and Cellular Biochemistry* **359**: 385-388.
- Liefers, S. C.; Veerkamp, R. F.; te Pas, M. F. W.; Delavaud, C.; Chilliard, Y.; van der Lende, T. 2003: Leptin concentrations in relation to energy balance, milk yield, intake, live weight, and estrus in dairy cows. *Journal of Dairy Science* **86**: 799-807.
- Lima, J. R.; Rivera, F. A.; Narciso, C. D.; Oliveira, R.; Chebel, R. C.; Santos, J. E. P. 2009: Effect of increasing amounts of supplemental progesterone in a timed artificial insemination protocol on fertility of lactating dairy cows. *Journal of Dairy Science* **92**: 5436-5446.

- Littell, R. C.; Milliken, G. A.; Stroup, W. W.; Wolfinger, R. D. 1996: SAS system for mixed models. Cary, NC, USA, SAS Institute.
- Lopes, A. S.; Butler, S. T.; Gilbert, R. O.; Butler, W. R. 2007: Relationship of pre-ovulatory follicle size, estradiol concentrations and season to pregnancy outcome in dairy cows. *Animal Reproduction Science* **99**: 34-43.
- Lopez, H.; Satter, L. D.; Wiltbank, M. C. 2004: Relationship between level of milk production and estrous behavior of lactating dairy cows. *Animal Reproduction Science* **81**: 209-223.
- Lucy, M. C. 2001: Reproductive loss in high-producing dairy cattle: Where will it end? *Journal of Dairy Science* **84**: 1277-1293.
- Lucy, M. C. 2007: Fertility in high-producing dairy cows: Reasons for decline and corrective strategies for sustainable improvement. *Reproduction in Domestic Ruminants* **6**: 237-254.
- Lucy, M. C.; Jiang, H.; Kobayashi, Y. 2001a: Changes in the somatotrophic axis associated with the initiation of lactation. *Journal of Dairy Science* **84, Supplement**: E113-E119.
- Lucy, M. C.; McDougall, S.; Nation, D. P. 2004: The use of hormonal treatments to improve the reproductive performance of lactating dairy cows in feedlot or pasture-based management systems. *Animal Reproduction Science* **82-83**: 495-512.
- Lucy, M. C.; Staples, C. R.; Michel, F. M.; Thatcher, W. W. 1991: Energy balance and size and number of ovarian follicles detected by ultrasonography in early postpartum dairy cows. *Journal of Dairy Science* **74**: 473 - 482.
- Lucy, M. C.; Staples, C. R.; Thatcher, W. W.; Erickson, P. S.; Cleale, R. M.; Firkins, J. L.; Clark, J. H.; Murphy, M. R.; Brodie, B. O. 1992: Influence of diet composition, dry-matter intake, milk production and energy balance on time of post-partum ovulation and fertility in dairy cows. *Animal Science* **54**: 323-331.
- Lucy, M. C.; Billings, H. J.; Butler, W. R.; Ehnis, L. R.; Fields, M. J.; Kesler, D. J.; Kinder, J. E.; Mattos, R. C.; Short, R. E.; Thatcher, W. W. 2001b: Efficacy of an intravaginal progesterone insert and an injection of PGF2alpha for synchronizing estrus and shortening the interval to pregnancy in postpartum beef cows, peripubertal beef heifers, and dairy heifers. *Journal of Animal Science* **79**: 982.
- Luo, W.; Gumen, A.; Haughian, J. M.; Wiltbank, M. C. 2010: The role of luteinizing hormone in regulating gene expression during selection of a dominant follicle in cattle1. *Biology of Reproduction* **84**: 369-378.
- Macdonald, K. A.; Roche, J. R. 2004: Condition scoring made easy. Condition scoring dairy herds 1st edition. Hamilton, New Zealand, Dexcel Ltd. ISBN 0-476-00217-6.
- Macdonald, K. A.; Verkerk, G. A.; Thorrold, B. S.; Pryce, J. E.; Penno, J. W.; McNaughton, L. R.; Burton, L. J.; Lancaster, J. A. S.; Williamson, J. H.; Holmes, C. W. 2008: A comparison of three strains of Holstein-Friesian grazed on pasture and managed under different feed allowances. *Journal of Dairy Science* **91**: 1693-1707.
- Macmillan, K. L. 1978: Oestrus synchronisation with a prostaglandin analogue III. Special aspects of synchronisation 26: 104-108. *New Zealand Veterinary Journal* **26**: 104-108.
- Macmillan, K. L. 1979: Calving patterns and herd production in seasonal dairy herds, New Zealand Society of Animal Production. 168-174 p.

- Macmillan, K. L. 1990: Manipulating the length of bovine oestrous cycle. *Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 141-147.
- Macmillan, K. L. 1997: Why don't cows cycle? . *49th Ruakura Dairy Farmers Conference*. Pp. 52 - 56.
- Macmillan, K. L. 1998: Reproductive management of dairy cattle. In: Fielden, E. D.; Smith, J. F. ed. *Reproductive management of grazing ruminants in New Zealand*, occasional publication 12 Hamilton, New Zealand, Society of Animal Production (Inc), C-/AgResearch Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand. Pp. 91-112.
- Macmillan, K. L. 2002: Advances in bovine theriogenology in New Zealand, 1. Pregnancy, parturition and the postpartum period. *New Zealand Veterinary Journal* **50**: 67-73.
- Macmillan, K. L.; Clayton, D. G. 1980: Factors influencing the interval to post-partum oestrus, conception date and empty rate in an intensively managed dairy herd. *Proceedings of the New Zealand Society of Animal Production*. Pp. 236-239.
- Macmillan, K. L.; Henderson, H. V. 1984: Analyses of the variation in the interval from an injection of prostaglandin F2 α to oestrus as a method of studying patterns of follicle development during dioestrus in dairy cows. *Animal Reproduction Science* **6**: 245-254.
- Macmillan, K. L.; Thatcher, W. W. 1991: Effects of an agonist of gonadotropin-releasing hormone on ovarian follicles in cattle. *Biology of Reproduction* **45**: 883-889.
- Macmillan, K. L.; Curnow, R. J.; Morris, G. R. 1978: Oestrus synchronisation with a prostaglandin analogue. II. Systems in maiden heifers. *New Zealand Veterinary Journal* **26**: 96-103.
- Macmillan, K. L.; Lean, I. J.; Westwood, C. T. 1996: The effects of lactation on the fertility of dairy cows. *Australian Veterinary Journal* **73**: 141-147.
- MacMillan, K. L.; Day, A. M.; Taufa, V. K.; Gibb, M.; Pearce, M. G. 1985: Effects of an agonist of gonadotrophin releasing hormone in cattle. I. Hormone concentrations and oestrous cycle length. *Animal Reproduction Science* **8**: 203-212.
- Mapletoft, R. J.; Steward, K. B.; Adams, G. P. 2002: Recent advances in the superovulation in cattle. *Reproduction Nutrition Development* **42**: 601-611.
- Martin, P. A.; Crump, M. H. 2003: The endocrine pancreas. In: Mauricio, H. P.; Michael, P. D. ed. *McDonald's veterinary endocrinology and reproduction Fifth Edition*. Iowa, Blackwell Publishing Company, Iowa State Press. Pp. 141-163.
- Martinez, M. F.; Adams, G. P.; Bergfelt, D. R.; Kastelic, J. P.; Mapletoft, R. J. 1999: Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. *Animal Reproduction Science* **57**: 23-33.
- Martínez, M. F.; Kastelic, J. P.; Adams, G. P.; Cook, B.; Olson, W. O.; Mapletoft, R. J. 2002: The use of progestins in regimens for fixed-time artificial insemination in beef cattle. *Theriogenology* **57**: 1049-1059.
- Matton, P.; Adelakoun, V.; Couture, Y.; Dufour, J. J. 1981: Growth and replacement of the bovine ovarian follicles during the estrous cycle. *Journal of Animal Science* **52**: 813-820.

- McArdle, C. A.; Holtorf, A. P. 1989: Oxytocin and progesterone release from bovine corpus luteal cells in culture: effects of insulin-like growth factor I, insulin, and prostaglandins. *Endocrinology* **124**: 1278-1286.
- McCall, D. G.; Smith, J. F. 1998: Seasonal factors influencing reproductive management. In: Fielden, E. D.; Smith, J. F. ed. Reproductive management of grazing ruminants in New Zealand Occasional publication 12. Hamilton, New Zealand Society of Animal Production (Inc), C/- AgResearch Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand. Pp. 65.
- McCracken, J. A.; Custer, E. E.; Lamsa, J. C. 1999: Luteolysis: a neuroendocrine-mediated event. *Physiological Reviews* **79**: 263-323.
- McDougall, S. 1992: The effect of stocking rate, breed and pre-partum nutrition on post-partum anoestrus. *Proceedings of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association Annual Conference*. Pp. 49-67.
- McDougall, S. 2001: Effects of periparturient diseases and conditions on the reproductive performance of New Zealand dairy cows. *New Zealand Veterinary Journal* **49**: 60-67.
- McDougall, S. 2010a: Effects of treatment of anestrous dairy cows with gonadotropin-releasing hormone, prostaglandin, and progesterone. *Journal of Dairy Science* **93**: 1944-1959.
- McDougall, S. 2010b: Comparison of diagnostic approaches, and a cost-benefit analysis of different diagnostic approaches and treatments of anoestrous dairy cows. *New Zealand Veterinary Journal* **58**: 81-89.
- McDougall, S.; Macmillan, K. L. Year: of Conference. Anovulatory dairy cows have lower LH pulse frequency and intrafollicular concentrations of oestradiol (E2) and testosterone (T) than cyclic cows. . *Australian Society for Reproductive Biology*.
- McDougall, S.; Rhodes, F. M. 1999: Detection of a corpus luteum in apparently anoestrous cows by manual palpation, transrectal ultrasonography and plasma progesterone concentration. *New Zealand Veterinary Journal* **47**: 47-52.
- McDougall, S.; Compton, C. 2005: Reproductive performance of anestrous dairy cows treated with progesterone and estradiol benzoate. *Journal of Dairy Science* **88**: 2388-2400.
- McDougall, S.; Compton, C. 2008: Effects of treatment of 'not - detected in oestrus' cows with gonadotrophin releasing hormone, prostaglandin and progesterone. *Proceedings of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association Annual Conference*. Pp. 35-50.
- McDougall, S.; Rhodes, F. M.; Compton, C. W. R. 2013: Evaluation of three synchrony programs for pasture-based dairy heifers. *Theriogenology* **79**: 882-889.
- McDougall, S.; Burke, C. R.; Macmillan, K. L.; Williamson, N. B. 1995: Patterns of follicular development during periods of anovulation in pasture-fed dairy cows after calving. *Research in Veterinary Science* **58**: 212-216.
- McDougall, S.; Cullum, A. A.; Annis, F. M.; Rhodes, F. M. 2001: Treatment of anovulatory anoestrous postpartum dairy cows with a gonadotropin-releasing hormone (GnRH), prostaglandin F2 alpha, GnRH regimen or with progesterone and oestradiol benzoate. *New Zealand Veterinary Journal* **49**: 168-172.

- McDougall, S.; Heuer, C.; Morton, J.; Brownlie, T. 2014: Use of herd management programmes to improve the reproductive performance of dairy cattle. *Animal* **8**: 199-210.
- McDougall, S.; Leijnse, P.; Day, A. M.; Macmillan, K. L.; Williamson, N. B. 1993: A case control study of anoestrus in New Zealand dairy cows. *Proceedings of the New Zealand Society of Animal Production*. Pp. 101-104.
- McGuire, M. A.; Dwyer, D. A.; Harrell, R. J.; Bauman, D. E. 1995: Insulin regulates circulating insulin-like growth factors and some of their binding proteins in lactating cows. *American Journal of Physiology - Endocrinology And Metabolism* **269**: E723-E730.
- McMillan, W. H.; Shackell, G. H.; Vishwanath, R. 1998: Comparative reproductive performance. In: Fielden, E. D.; Smith, J. F. ed. Reproductive management of grazing ruminants in New Zealand. Hamilton, New Zealand, New Zealand Society of Animal Production (Inc.) C/- AgResearch Ruakura Research Centre, Private Bag 3123. Pp. 43-64.
- McNaughton, L. R. 2003: A comparison of reproductive performance and physiology of three genotypes of Holstein Friesian dairy cattle. Thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Science. Massey University, Palmerston North, New Zealand.
- McNaughton, L. R.; Sanders, K. M.; Pryce, J. E.; Bracefield, G. E.; Harcourt, S. J.; Spelman, R. J. 2007: Phenotypic relationships between the interval from calving to first luteal activity and fertility in a seasonal dairy production system. *Animal Reproduction Science* **102**: 98-110.
- McNeill, R. E.; Diskin, M. G.; Sreenan, J. M.; Morris, D. G. 2006: Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. *Theriogenology* **65**: 1435-1441.
- Melendez, P.; Gonzalez, G.; Aguilar, E.; Loera, O.; Risco, C.; Archbald, L. F. 2006: Comparison of two estrus-synchronization protocols and timed artificial insemination in dairy cattle. *Journal of Dairy Science* **89**: 4567-4572.
- Mihm, M.; Austin, E. J. 2002: The final stages of dominant follicle selection in cattle. *Domestic Animal Endocrinology* **23**: 155-166.
- Mihm, M.; Baguisi, A.; Boland, M. P.; Roche, J. F. 1994: Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. *Journal of Reproduction and Fertility* **102**: 123-130.
- Mihm, M.; Curran, N.; Hyttel, P.; Knight, P. G.; Boland, M. P.; Roche, J. F. 1999: Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. *Journal of Reproduction and Fertility* **116**: 293-304.
- Mihm, M.; Baker, P. J.; Ireland, J. L. H.; Smith, G. W.; Coussens, P. M.; Evans, A. C. O.; Ireland, J. J. 2006: Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. *Biology of Reproduction* **74**: 1051-1059.
- Mikscha, E. D.; LeFever, D. G.; Mukembo, G.; Spitzer, J. C.; Wiltbank, J. N. 1978: Synchronization of estrus in beef cattle: II. Effect of an injection of norgestomet and an estrogen in conjunction with a norgestomet implant in heifers and cows. *Theriogenology* **10**: 201-221.

Ministry of Primary Industries 2013: Situation and outlook for primary industries 2013 (online version). Ministry for Primary Industries Pastoral House, 25 The Terrace PO Box 2526, Wellington 6140, New Zealand.

Moreira, F.; de la Sota, R. L.; Diaz, T.; Thatcher, W. W. 2000a: Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. *Journal of Animal Science* **78**: 1568-1576.

Moreira, F.; Risco, C. A.; Pires, M. F. A.; Ambrose, J. D.; Drost, M.; Thatcher, W. W. 2000b: Use of bovine somatotropin in lactating dairy cows receiving timed artificial insemination. *Journal of Dairy Science* **83**: 1237-1247.

Moreira, F.; Orlandi, C.; Risco, C. A.; Mattos, R.; Lopes, F.; Thatcher, W. W. 2001: Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. *Journal of Dairy Science* **84**: 1646-1659.

Murondoti, A.; Jorritsma, R.; Beynen, A. C.; Wensing, T.; Geelen, M. J. H. 2004: Unrestricted feed intake during the dry period impairs the postpartum oxidation and synthesis of fatty acids in the liver of dairy cows. *Journal of Dairy Science* **87**: 672-679.

Murphy, M.; Boland, M.; Roche, J. 1990: Pattern of follicular growth and resumption of ovarian activity in post-partum beef suckler cows. *Journal of Reproduction and Fertility* **90**: 523-533.

Nation, D. P.; Macmillan, K. L.; Taufa, V. K. 1997: Efficacy of an anoestrous treatment for dairy cows is reduced if administered within 3 weeks of calving. *Proceedings of the New Zealand Society of Animal Production*. Pp. 228-230.

Nation, D. P.; Burke, C. R.; Parton, G.; Stevenson, R.; Macmillan, K. L. 2000: Hormonal and ovarian responses to a 5-day progesterone treatment in anoestrous dairy cows in the third week post-partum. *Animal Reproduction Science* **63**: 13-25.

National Office of Animal Health 2012: NOAH compendium of data sheets for animal medicines (web version). At: <http://www.noahcompendium.co.uk>. Retrieved 19/07/2013.

Navanukraw, C.; Redmer, D. A.; Reynolds, L. P.; Kirsch, J. D.; Grazul-Bilska, A. T.; Fricke, P. M. 2004: A modified presynchronization protocol improves fertility to timed artificial insemination in lactating dairy cows. *Journal of Dairy Science* **87**: 1551-1557.

Nett, T. M. 1987: Function of the hypothalamic-hypophysial axis during the post-partum period in ewes and cows. *Journal of Reproduction and Fertility* **34**: 201-213.

Noakes, D. 2009: Endogenous and exogenous control of ovarian activity. In: Noakes, D. E.; Parkinson, T. J.; England, G. C. W. ed. *Veterinary Reproduction and Obstetrics Ninth Edition* ed, Saunders Elsevier. Pp. 3-58.

Noseir, W. M. 2003: Ovarian follicular activity and hormonal profile during estrous cycle in cows: the development of 2 versus 3 waves. *Reproductive Biology and Endocrinology* **1**: 50.

O'Shea, J. D.; Rodgers, R. J.; D'Occhio, M. J. 1989: Cellular composition of the cyclic corpus luteum of the cow. *Journal of Reproduction and Fertility* **85**: 483-487.

Odde, K. G. 1990: A review of synchronization of estrus in postpartum cattle. *Journal of Animal Science* **68**: 817-830.

- Okuda, K.; Miyamoto, A.; Sauerwein, H.; Schweigert, F. J.; Schams, D. 1992: Evidence for oxytocin receptors in cultured bovine luteal cells. *Biology of Reproduction* **46**: 1001-1006.
- Ortega, H. H.; Palomar, M. M.; Acosta, J. C.; Salvetti, N. R.; Dallard, B. E.; Lorente, J. A.; Barbeito, C. G.; Gimeno, E. J. 2008: Insulin-like growth factor I in sera, ovarian follicles and follicular fluid of cows with spontaneous or induced cystic ovarian disease. *Research in Veterinary Science* **84**: 419-427.
- Ott, R.; Bretzlaff, K.; Hixon, J. 1986: Comparison of palpable corpora lutea with serum progesterone concentrations in cows. *Journal of the American Veterinary Medical Association* **188**: 1417-1419.
- Palmer, M. A.; Olmos, G.; Boyle, L. A.; Mee, J. F. 2010: Estrus detection and estrus characteristics in housed and pastured Holstein-Friesian cows. *Theriogenology* **74**: 255-264.
- Pancarci, S. M.; Jordan, E. R.; Risco, C. A.; Schouten, M. J.; Lopes, F. L.; Moreira, F.; Thatcher, W. W. 2002: Use of estradiol cypionate in a presynchronized timed artificial insemination program for lactating dairy cattle. *Journal of Dairy Science* **85**: 122.
- Parker, K. I.; Compton, C. W. R.; Anniss, F. M.; Weir, A. M.; McDougall, S. 2007: Management of dairy heifers and its relationships with the incidence of clinical mastitis. *New Zealand Veterinary Journal* **55**: 208-216.
- Parkinson, T. 2007: Principles and practice of manipulation of the oestrous cycle in large animals. *Proceedings of the New Zealand Veterinary Nursing Association, Large Animal Nurse and Technician Stream*. Pp. 135-150.
- Parry, D. M.; Willcox, D. L.; Thorburn, G. D. 1980: Ultrastructural and cytochemical study of the bovine corpus luteum. *Journal of Reproduction and Fertility* **60**: 349-357.
- Pate, J. L. 1995: Involvement of immune cells in regulation of ovarian function. *Journal of Reproduction and Fertility* **49**: 365-77.
- Pate, J. L.; Townson, D. H. 1994: Novel local regulators in luteal regression. *Journal of Animal Science* **72**: 31-42.
- Peeler, I. D.; Nebel, R. L.; Pearson, R. E.; Swecker, W. S.; Garcia, A. 2004: Pregnancy rates after timed AI of heifers following removal of intravaginal progesterone inserts. *Journal of Dairy Science* **87**: 2868-2873.
- Perry, G. A.; Smith, M. F.; Roberts, A. J.; MacNeil, M. D.; Geary, T. W. 2007: Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. *Journal of Animal Science* **85**: 684-689.
- Perry, G. A.; Smith, M. F.; Lucy, M. C.; Green, J. A.; Parks, T. E.; MacNeil, M. D.; Roberts, A. J.; Geary, T. W. 2005: Relationship between follicle size at insemination and pregnancy success. *Proceedings of the National Academy of Sciences of the United States of America*. Pp. 5268-5273.
- Peter, A. T.; Vos, P. L. A. M.; Ambrose, D. J. 2009: Review: postpartum anestrus in dairy cattle. *Theriogenology* **71**: 1333-1342.

- Peters, A. R.; Ward, S. J.; Warren, M. J.; Gordon, P. J.; Mann, G. E.; Webb, R. 1999a: Ovarian and hormonal responses of cows to treatment with an analogue of gonadotrophin releasing hormone and prostaglandin F_{2α}. *Veterinary Record* **144**: 343-346.
- Peters, A. R.; Mawhinney, I.; Drew, S. B.; Ward, S. J.; Warren, M. J.; Gordon, P. J. 1999b: Development of a gonadotrophin-releasing hormone and prostaglandin regimen for the planned breeding of dairy cows. *Veterinary Record* **145**: 516-521.
- Peters, M. W.; Pursley, J. R. 2002: Fertility of lactating dairy cows treated with ovsynch after presynchronization injections of PGF2[alpha] and GnRH. *Journal of Dairy Science* **85**: 2403-2406.
- Pfaff, D. 2005: Hormone-driven mechanisms in the central nervous system facilitate the analysis of mammalian behaviours. *Journal of Endocrinology* **184**: 447-453.
- Pickering, J. 2002: The use of Cue-Mate in oestrus synchronisation programs for fixed-time insemination of heifers in New Zealand. *Proceedings of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association Annual Conference*. Pp. 159-166.
- Pickering, J. 2008: AI synchrony in dairy heifers. *Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 183-189.
- Pierson, R. A.; Ginther, O. J. 1984: Ultrasonography of the bovine ovary. *Theriogenology* **21**: 495-504.
- Pieterse, M.; Taverne, M.; Kruip, T.; Willemse, A. 1990: Detection of corpora lutea and follicles in cows: a comparison of transvaginal ultrasonography and rectal palpation. *Veterinary Record* **126**: 552-554.
- Plunkett, S. S.; Stevenson, J. S.; Call, E. P. 1984: Prostaglandin F_{2α} for lactating dairy cows with a palpable corpus luteum but unobserved estrus. *Journal of Dairy Science* **67**: 380-387.
- Poretsky, L.; Kalin, M. F. 1987: The gonadotropic function of insulin. *Endocrine Reviews* **8**: 132-141.
- Pursley, J. R.; Mee, M. O.; Wiltbank, M. C. 1995: Synchronization of ovulation in dairy cows using PGF_{2α} and GnRH. *Theriogenology* **44**: 915-923.
- Pursley, J. R.; Wiltbank, M. C.; Stevenson, J. S.; Ottobre, J. S.; Garverick, H. A.; Anderson, L. L. 1997: Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. *Journal of Dairy Science* **80**: 295-300.
- Pursley, R. J.; Silcox, R. W.; Wiltbank, M. C. 1998: Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. *Journal of Dairy Science* **81**: 2139-2144.
- Rabiee, A. R.; Lean, I. J.; Stevenson, M. A. 2004: A Bayesian meta-analysis of the effects of administering an intra-vaginal (CIDR) device in combination with other hormones on the reproductive performance of cycling, anoestrous and inseminated cows. *New Zealand Veterinary Journal* **52**: 384-393.
- Rahe, C. H.; Owens, R. E.; Fleeger, J. L.; Newton, H. J.; Harms, P. G. 1980: Pattern of plasma luteinizing hormone in the cyclic cow: Dependence upon the period of the cycle. *Endocrinology* **107**: 498-503.

- Rajakoski, E. 1960: The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. *Acta Endocrinol* **52**: 1-68.
- Rajamahendran, R.; Taylor, C. 1990: Characterization of ovarian activity in postpartum dairy cows using ultrasound imaging and progesterone profiles. *Animal Reproduction Science* **22**: 171-180.
- Rajamahendran, R.; Robinson, J.; Desbottes, S.; Walton, J. S. 1989: Temporal relationships among estrus, body temperature, milk yield, progesterone and luteinizing hormone levels, and ovulation in dairy cows. *Theriogenology* **31**: 1173-1182.
- Ray, D. E.; Halbach, T. J.; Armstrong, D. V. 1992: Season and lactation number effects on milk production and reproduction of dairy cattle in Arizona. *Journal of Dairy Science* **75**: 2976-2983.
- Redding, T. W. 1972: Stimulation of release and synthesis of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in tissue culture of rat pituitaries in response to natural and synthetic LH and FSH releasing hormone. *Endocrinology* **90**: 764.
- Redmer, D. A.; Reynolds, L. P. 1996: Angiogenesis in the ovary. *Reviews of Reproduction*: 182-192.
- Reeves, J. J.; Arimura, A.; Schally, A. V. 1971: Changes in pituitary responsiveness to lutenizing hormone-releasing hormone (LH-RH) in anestrus ewes pretreated with estradiol benzoate. *Biology of Reproduction* **4**: 88-92.
- Renaville, R.; Hammadi, M.; Portetelle, D. 2002: Role of the somatotropic axis in the mammalian metabolism. *Domestic Animal Endocrinology* **23**: 351-360.
- Reynolds, L. P.; Redmer, D. A. 1999: Growth and development of the corpus luteum. . *Journal of Reproduction and Fertility* 181-191.
- Rhodes, F. M.; McDougall, S.; Verkerk, G.; Xu, Z. Z.; Cullum, A. 2000: Treatment of the non-cycling cow. *Proceedings of the Australian and New Zealand Combined Dairy Cattle Veterinarians Conference - incorporating the 17th Annual Seminar of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 125-134.
- Rhodes, F. M.; McDougall, S.; Burke, C. R.; Verkerk, G. A.; Macmillan, K. L. 2003: Invited review: treatment of cows with an extended postpartum anestrous interval. *Journal of Dairy Science* **86**: 1876-1894.
- Rhodes, F. M.; Clark, B. A.; Nation, D. P.; Taufa, V. K.; Macmillan, K. L.; Day, M. L.; Day, A. M.; McDougall, S. 1998: Factors influencing the prevalence of postpartum anoestrus in New Zealand dairy cows. *Proceedings of the New Zealand Society of Animal Production*. Pp. 79-81.
- Rinderknecht, E.; Humbel, R. E. 1978: The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *Journal of Biological Chemistry* **253**: 2769-2776.
- Rivera, H.; Lopez, H.; Fricke, P. M. 2004: Fertility of Holstein dairy heifers after synchronization of ovulation and timed AI or AI after removed tail chalk. *Journal of Dairy Science* **87**: 2051.

- Roberson, M. S.; Wolfe, M. W.; Stumpf, T. T.; Kittok, R. J.; Kinder, J. E. 1989: Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. *Biology of Reproduction* **41**: 997-1003.
- Robertson, H. A. 1969: The endogenous control of estrus and ovulation in sheep, cattle and swine. *Vitamins and Hormones* **27**: 91-130.
- Roche, J. F. 1976: Fertility in cows after treatment with a prostaglandin analogue with or without progesterone. *Journal of Reproduction and Fertility* **46**: 341-345.
- Roche, J. F. 1996: Control and regulation of folliculogenesis - a symposium in perspective. *Reviews of Reproduction* **1**: 19-27.
- Roche, J. F. 2006: The effect of nutritional management of the dairy cow on reproductive efficiency. *Animal Reproduction Science* **96**: 282-296.
- Roche, J. R.; Macdonald, K. A.; Burke, C. R.; Lee, J. M.; Berry, D. P. 2007: Associations among body condition score, body weight, and reproductive performance in seasonal-calving dairy cattle. *Journal of Dairy Science* **90**: 376 - 391.
- Rosenberg, M.; Kaim, M.; Herz, Z.; Folman, Y. 1990: Comparison of methods for the synchronization of estrous cycles in dairy cows. 1. Effects on plasma progesterone and manifestation of estrus. *Journal of Dairy Science* **73**: 2807-2816.
- Roth, Z.; Meidan, R.; Shaham-Albalancy, A.; Braw-Tal, R.; Wolfenson, D. 2001: Delayed effect of heat stress on steroid production in medium-sized and preovulatory bovine follicles. *Reproduction* **121**: 745-751.
- Rowson, L.; Tervit, R.; Brand, A. 1972: The use of prostaglandins for synchronization of oestrus in cattle. *Journal of Reproduction and Fertility* **29**: 145 (abstract).
- Roy, G. L.; Twagiramungu, H. 1996: A fixed-time AI program using the GnRH-PGF_{2α}-GnRH method for beef females. *Journal of Animal Science* **74**: 462 (Abstract).
- Roy, G. L.; Twagiramungu, H. 1999: Time interval between GnRH and prostaglandin injections influences the precision of estrus in synchronized cattle. *Theriogenology* **51**: 413.
- Royal, M. D.; Darwash, A. O.; Flint, A. P. F.; Webb, R.; Woolliams, J. A.; Lamming, G. E. 2000: Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. *Journal of Animal Science* **70**: 487 - 501.
- Saiduddin, S.; Quevedo, M. M.; Foote, W. D. 1968: Response of beef cows to exogenous progesterone and estradiol at various stages postpartum. *Journal of Animal Science* **27**: 1015-1020.
- Santos, J. E. P.; Thatcher, W. W.; Chebel, R. C.; Cerri, R. L. A.; Galvão, K. N. 2004: The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. *Animal Reproduction Science* **82-83**: 513-535.
- Sartori, R.; Haughian, J. M.; Shaver, R. D.; Rosa, G. J. M.; Wiltbank, M. C. 2004: Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *Journal of Dairy Science* **87**: 905-920.
- Sartori, R.; Guardieiro, M.; Surjus, R.; Melo, L.; Prata, A.; Ishiguro, M.; Bastos, M.; Nascimento, A. 2013: Metabolic hormones and reproductive function in cattle. *Animal Reproduction* **10**: 199-205.

- Satheshkumar, S.; Subramanian, A.; Devanathan, T. G.; Kathiresan, D.; Veerapandian, C.; Palanisamy, A. 2012: Follicular and endocrinological turnover associated with GnRH induced follicular wave synchronization in Indian crossbred cows. *Theriogenology* **77**: 1144-1150.
- Savio, J. D.; Boland, M. P.; Roche, J. F. 1990a: Development of dominant follicles and length of ovarian cycles in post-partum dairy cows. *Journal of Reproduction and Fertility* **88**: 581-591.
- Savio, J. D.; Boland, M. P.; Hynes, N.; Mattiacci, M. R.; Roche, J. F. 1990b: Will the first dominant follicle of the estrous cycle of heifers ovulate following luteolysis on day 7? *Theriogenology* **33**: 677-687.
- Savio, J. D.; Thatcher, W. W.; Badinga, L.; de la Sota, R. L.; Wolfenson, D. 1993a: Regulation of dominant follicle turnover during the oestrous cycle in cows. *Journal of Reproduction and Fertility* **97**: 197-203.
- Savio, J. D.; Thatcher, W. W.; Morris, G. R.; Entwistle, K.; Drost, M.; Mattiacci, M. R. 1993b: Effects of induction of low plasma progesterone concentrations with a progesterone-releasing intravaginal device on follicular turnover and fertility in cattle. *Journal of Reproduction and Fertility* **98**: 77-84.
- Schally, A. V.; Arimura, A.; Kastin, A. J.; Matsuo, H.; Baba, Y.; Redding, T. W.; Nair, R. M. G.; Debeljuk, L.; White, W. F. 1971: Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. *Science* **173**: 1036-1038.
- Schams, D. 1987: Luteal peptides and intercellular communication. *Journal of Reproduction and Fertility* **34**: 87-99.
- Schams, D.; Koll, R.; Li, C. H. 1988: Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa cells in culture. *Journal of Endocrinology* **116**: 97-100.
- Schams, D.; Schallenberger, E.; Hoffmann, B.; Karg, H. 1977: The oestrous cycle of the cow: hormonal parameters and time relationships concerning oestrus, ovulation, and electrical resistance of the vaginal mucus. *Acta Endocrinologica* **86**: 180-192.
- Schams, D.; Schallenberger, E.; Menzer, C.; Stangl, J.; Zottmeier, K.; Hoffmann, B.; Karg, H. 1978: Profiles of LH, FSH and progesterone in postpartum dairy cows and their relationship to the commencement of cyclic functions. *Theriogenology* **10**: 453-468.
- Schmitt, E. J.; Diaz, T.; Barros, C. M.; de la Sota, R. L.; Drost, M.; Fredriksson, E. W.; Staples, C. R.; Thorner, R.; Thatcher, W. W. 1996a: Differential response of the luteal phase and fertility in cattle following ovulation of the first-wave follicle with human chorionic gonadotropin or an agonist of gonadotropin-releasing hormone. *Journal of Animal Science* **74**: 1074-1083.
- Schmitt, E. J. P.; Drost, M.; Diaz, T.; Roomes, C.; Thatcher, W. W. 1996b: Effect of a gonadotropin-releasing hormone agonist on follicle recruitment and pregnancy rate in cattle. *Journal of Animal Science* **74**: 154-161.
- Sheffel, C.; Pratt, B.; Ferrell, W.; Inskeep, E. 1982: Induced corpora lutea in the postpartum beef cow. II. Effects of treatment with progestogen and gonadotropins. *Journal of Animal Science* **54**: 830-836.

- Sheldon, I. M.; Cronin, J.; Borges, A. 2011: The postpartum period and dairy cow fertility Part 2: Ovarian function. *Livestock* **16**: 20-24.
- Silcox, R. W.; Powell, K. L.; Kiser, T. E. 1993: Ability of dominant follicles (DF) to respond to exogenous GnRH administration is dependent on their stage of development. *Journal of Animal Science* **71**: 513 (Abstract).
- Siqueira, L. G. B.; Torres, C. A. A.; Amorim, L. S.; Souza, E. D.; Camargo, L. S. A.; Fernandes, C. A. C.; Viana, J. H. M. 2009: Interrelationships among morphology, echotexture, and function of the bovine corpus luteum during the estrous cycle. *Animal Reproduction Science* **115**: 18-28.
- Sirois, J.; Fortune, J. E. 1988: Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. *Biology of Reproduction* **39**: 308-317.
- Sirois, J.; Fortune, J. E. 1990: Lengthening the bovine estrous cycle with low levels of exogenous progesterone: A model for studying ovarian follicular dominance. *Endocrinology* **127**: 916-925.
- Smeaton, T. C.; Robertson, H. A. 1971: Studies on the growth and atresia of graafian follicles in the ovary of the sheep. *Journal of Reproduction and Fertility* **25**: 243-252.
- Smith, J. F. 1976: Use of a synthetic prostaglandin analogue for synchronization of oestrus in heifers. *New Zealand Veterinary Journal* **24**: 71-73.
- Smith, J. F.; Fairclough, R. J.; Payne, E.; Peterson, A. J. 1975: Plasma hormone levels in the cow. *New Zealand Journal of Agricultural Research* **18**: 123-129.
- Smith, M. W.; Stevenson, J. S. 1995: Fate of the dominant follicle, embryonal survival, and pregnancy rates in dairy cattle treated with prostaglandin F2 alpha and progestins in the absence or presence of a functional corpus luteum. *Journal of Animal Science* **73**: 3743-51.
- Smith, R. D.; Pomerantz, A. J.; Beal, W. E.; McCann, J. P.; Pilbeam, T. E.; Hansel, W. 1984: Insemination of holstein heifers at a preset time after estrous cycle synchronization using progesterone and prostaglandin. *Journal of Animal Science* **58**: 792-800.
- Souza, A. H.; Ayres, H.; Ferreira, R. M.; Wiltbank, M. C. 2008: A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. *Theriogenology* **70**: 208-215.
- Souza, A. H.; Gümen, A.; Silva, E. P. B.; Cunha, A. P.; Guenther, J. N.; Peto, C. M.; Caraviello, D. Z.; Wiltbank, M. C. 2007: Supplementation with estradiol-17 β before the last gonadotropin-releasing hormone injection of the ovsynch protocol in lactating dairy cows. *Journal of Dairy Science* **90**: 4623-4634.
- Souza, A. H.; Silva, E. P. B.; Cunha, A. P.; Gümen, A.; Ayres, H.; Brusveen, D. J.; Guenther, J. N.; Wiltbank, M. C. 2011: Ultrasonographic evaluation of endometrial thickness near timed AI as a predictor of fertility in high-producing dairy cows. *Theriogenology* **75**: 722-733.
- Spencer, T. E.; Burghardt, R. C.; Johnson, G. A.; Bazer, F. W. 2004: Conceptus signals for establishment and maintenance of pregnancy. *Animal Reproduction Science* **82-83**: 537-550.

- Spicer, L. J.; Echternkamp, S. E. 1986: Ovarian follicular growth, function and turnover in cattle: a review. *Journal of Animal Science* **62**: 428-451.
- Spicer, L. J.; Echternkamp, S. E. 1995: The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. *Domestic Animal Endocrinology* **12**: 223-245.
- Spicer, L. J.; Tucker, W. B.; Adams, G. D. 1990: Insulin-like growth factor-I in dairy cows: relationships among energy balance, body condition, ovarian activity, and estrous behavior. *Journal of Dairy Science* **73**: 929-937.
- Sprecher, D.; Nebel, R.; Whitman, S. 1989: The predictive value, sensitivity and specificity of palpation per rectum and transrectal ultrasonography for the determination of bovine luteal status. *Theriogenology* **31**: 1165-1172.
- Stabenfeldt, G.; Ewing, L.; McDonald, L. 1969: Peripheral plasma progesterone levels during the bovine oestrous cycle. *Journal of Reproduction and Fertility* **19**: 433-442.
- Statistics NZ 2010: Global New Zealand international trade, investment and travel profile year ended December 2009. Tatauranga Aotearoa Wellington, New Zealand Ministry of Foreign Affairs and Trade. Pp. 1.
- Statistics NZ 2014: New Zealand is home to 3 million people and 60 million sheep. At: http://www.stats.govt.nz/browse_for_stats/population/mythbusters/3million-people-60million-sheep.aspx. Retrieved 08/09/2014.
- Stevenson, J.; Call, E. 1983: Influence of early estrus, ovulation, and insemination on fertility in postpartum Holstein cows. *Theriogenology* **19**: 367-375.
- Stevenson, J. L.; Dalton, J. C.; Santos, J. E. P.; Sartori, R.; Ahmadzadeh, A.; Chebel, R. C. 2008a: Effect of synchronization protocols on follicular development and estradiol and progesterone concentrations of dairy heifers. *Journal of Dairy Science* **91**: 3045-3056.
- Stevenson, J. S. 2008: Progesterone, follicular, and estrual responses to progesterone-based estrus and ovulation synchronization protocols at five stages of the estrous cycle. *Journal of Dairy Science* **91**: 4640-4650.
- Stevenson, J. S. 2011: Alternative programs to presynchronize estrous cycles in dairy cattle before a timed artificial insemination program. *Journal of Dairy Science* **94**: 205-217.
- Stevenson, J. S. 2014: To presynch or not to presynch? At: http://www.hoards.com/E_reproduction/REP33. Retrieved 01/02/2014.
- Stevenson, J. S.; Lucy, M. C.; Call, E. P. 1987: Failure of timed inseminations and associated luteal function in dairy cattle after two injections of prostaglandin F₂-alpha. *Theriogenology* **28**: 937-946.
- Stevenson, J. S.; Kobayashi, Y.; Thompson, K. E. 1999: Reproductive performance of dairy cows in various programmed breeding systems including Ovsynch and combinations of gonadotropin-releasing hormone and prostaglandin F_{2a}. *Journal of Dairy Science* **82**: 506-515.
- Stevenson, J. S.; Pursley, J. R.; Garverick, H. A.; Fricke, P. M.; Kesler, D. J.; Ottobre, J. S.; Wiltbank, M. C. 2006: Treatment of cycling and noncycling lactating dairy cows with progesterone during Ovsynch. *Journal of Dairy Science* **89**: 2567-2578.

- Stevenson, J. S.; Lamb, G. C.; Johnson, S. K.; Medina-Britos, M. A.; Grieger, D. M.; Harmoney, K. R.; Cartmill, J. A.; El-Zarkouny, S. Z.; Dahlen, C. R.; Marple, T. J. 2003: Supplemental norgestomet, progesterone, or melengestrol acetate increases pregnancy rates in suckled beef cows after timed inseminations. *Journal of Animal Science* **81**: 571-586.
- Stevenson, J. S.; Tenhouse, D. E.; Krisher, R. L.; Lamb, G. C.; Larson, J. E.; Dahlen, C. R.; Pursley, J. R.; Bello, N. M.; Fricke, P. M.; Wiltbank, M. C. and others 2008b: Detection of anovulation by heatmount detectors and transrectal ultrasonography before treatment with progesterone in a timed insemination protocol. *Journal of Dairy Science* **91**: 2901-2915.
- Stock, A. E.; Fortune, J. E. 1993: Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. *Endocrinology* **132**: 1108-1114.
- Stronge, A. J. H.; Sreenan, J. M.; Diskin, M. G.; Mee, J. F.; Kenny, D. A.; Morris, D. G. 2005: Post-insemination milk progesterone concentration and embryo survival in dairy cows. *Theriogenology* **64**: 1212-1224.
- Swanson, L. V.; Hafs, H. D. 1971: LH and Prolactin in Blood Serum from Estrus to Ovulation in Holstein Heifers. *Journal of animal science* **33**: 1038-1041.
- Swanson, L. V.; Hafs, H. D.; Morrow, D. A. 1972: Ovarian characteristics and serum LH, prolactin, progesterone and glucocorticoid from first estrus to breeding size in Holstein heifers. *Journal of Animal Science* **34**: 284-293.
- Tenhagen, B. A.; Kuchenbuch, S.; Heuwieser, W. 2005: Timing of ovulation and fertility of heifers after synchronization of oestrus with GnRH and prostaglandin F-2 alpha. *Reproduction in Domestic Animals* **40**: 62-67.
- Thatcher, W. W.; Macmillan, K. L.; Hansen, P. J.; Drost, M. 1989: Concepts for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. *Theriogenology* **31**: 149-164.
- Townson, D. H.; Tsang, P. C.; Butler, W. R.; Frajblat, M.; Griell, L. C.; Johnson, C. J.; Milvae, R. A.; Niksic, G. M.; Pate, J. L. 2002: Relationship of fertility to ovarian follicular waves before breeding in dairy cows. *Journal of Animal Science* **80**: 1053-1058.
- Trimberger, G. W. 1944: Conception rate in dairy cattle by artificial insemination at various intervals before and after ovulation. *Journal of Dairy Science* **27**: 659-660.
- Turzillo, A. M.; Fortune, J. E. 1990: Suppression of the secondary FSH surge with bovine follicular fluid is associated with delayed ovarian follicular development in heifers. *Journal of Reproduction and Fertility* **89**: 643-653.
- Twagiramungu, H.; Guilbault, L. A.; Dufour, J. J. 1995: Synchronization of ovarian follicular waves with a gonadotropin-releasing hormone agonist to increase the precision of estrus in cattle: a review. *Journal of Animal Science* **73**: 3141-3151.
- Ulberg, L. C.; Lindley, C. E. 1960: Use of progesterone and estrogen in the control of reproductive activities in beef cattle. *Journal of Animal Science* **19**: 1132-1142.
- Ursely, J.; Leymarie, P. 1979: Varying response to luteinizing hormone of two luteal cell types isolated from bovine corpus luteum. *Journal of Endocrinology* **83**: 303-NP.

- Vandehaar, M. J.; Sharma, B. K.; Fogwell, R. L. 1995: Effect of dietary energy restriction on the expression of insulin-like growth factor-I in liver and corpus luteum of heifers 1. *Journal of Dairy Science* **78**: 832-841.
- Vanholder, T.; Leroy, J. L. M. R.; Soom, A. V.; Opsomer, G.; Maes, D.; Coryn, M.; Kruif, A. d. 2005: Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. *Animal Reproduction Science* **87**: 33-44.
- Vanholder, T.; Leroy, J. L. M. R.; Van Soom, A.; Maes, D.; Coryn, M.; Fiers, T.; de Kruif, A.; Opsomer, G. 2006: Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. *Animal Reproduction Science* **92**: 51-63.
- Vasconcelos, J. L. M.; Silcox, R. W.; Rosa, G. J. M.; Pursley, J. R.; Wiltbank, M. C. 1999: Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology* **52**: 1067-1078.
- Vasconcelos, J. L. M.; Sartori, R.; Oliveira, H. N.; Guenther, J. G.; Wiltbank, M. C. 2001: Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. *Theriogenology* **56**: 307-314.
- Villa-Godoy, A.; Hughes, T. L.; Emery, R. S.; Enright, W. J.; Ealy, A. D.; Zinn, S. A.; Fogwell, R. L. 1990: Energy balance and body condition influence luteal function in holstein heifers. *Domestic Animal Endocrinology* **7**: 135-148.
- Wade, G. N.; Jones, J. E. 2004: Neuroendocrinology of nutritional infertility. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **287**: R1277-R1296.
- Waldmann, A.; Reksen, O.; Landsverk, K.; Kommisrud, E.; Dahl, E.; Refsdal, A. O.; Ropstad, E. 2001: Progesterone concentrations in milk fat at first insemination — effects on non-return and repeat-breeding. *Animal Reproduction Science* **65**: 33-41.
- Walsh, S. W.; Williams, E. J.; Evans, A. C. O. 2011: A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science* **123**: 127-138.
- Wathees, D. C.; Swann, R. W. 1982: Is oxytocin an ovarian hormone? *Nature* **297**: 225-227.
- Wathees, D. C.; Swann, R. W.; Birkett, S. D.; Porter, D. G.; Pickering, B. T. 1983: Characterization of oxytocin, vasopressin, and neurophysin from the bovine corpus luteum. *Endocrinology* **113**: 693-698.
- Wathees, D. C.; Fenwick, M.; Cheng, Z.; Bourne, N.; Llewellyn, S.; Morris, D. G.; Kenny, D.; Murphy, J.; Fitzpatrick, R. 2007: Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. *Theriogenology* **68**: S232-S241.
- Webb, R.; Woad, K. J.; Armstrong, D. G. 2002: Corpus luteum (CL) function: local control mechanisms. *Domestic Animal Endocrinology* **23**: 277-285.
- Webb, R.; Gosden, R. G.; Telfer, E. E.; Moor, R. M. 1999: Factors affecting folliculogenesis in ruminants. *Journal of Animal Science* **68**: 257-284.
- Webb, R.; Garnsworthy, P. C.; Gong, J. G.; Armstrong, D. G. 2004: Control of follicular growth: Local interactions and nutritional influences. *Journal of Animal Science* **82**: E63-E74.

- Whittier, W. D.; Gwazdauskas, F. C.; McGilliard, M. L. 1989: Prostaglandin $F_{2\alpha}$ usage in a dairy reproduction program for treatment of unobserved estrus, pyometra and ovarian luteal cysts. *Theriogenology* **32**: 693-704.
- Wiltbank, M. C.; Souza, A. H.; Carvalho, P. D.; Bender, R. W.; Nascimento, A. B. 2012: Improving fertility to timed artificial insemination by manipulation of circulating progesterone concentrations in lactating dairy cattle. *Reproduction, Fertility and Development* **24**: 238-243.
- Wise, T. H.; Caton, D.; Thatcher, W. W.; Barron, D. H.; Fields, M. J. 1982: Ovarian function during the estrous cycle of the cow: ovarian blood flow and progesterone release rate. *Journal of Animal Science* **55**: 627-637.
- Wishart, D. F. 1972: Observations on the estrous cycle of the Friesian heifer. *Veterinary Record* **90**: 595-597.
- Wishart, D. F. 1974: Synchronisation of oestrus in cattle using a potent progestin (SC21009) and PGF $_2\alpha$. *Theriogenology* **1**: 87-90.
- Wolfenson, D.; Thatcher, W. W.; Savio, J. D.; Badinga, L.; Lucy, M. C. 1994: The effect of a GnRH analogue on the dynamics of follicular development and synchronization of estrus in lactating cyclic dairy cows. *Theriogenology* **42**: 633-644.
- Wright, I. A.; Russel, A. J. F. 1984: Partition of fat, body composition and body condition score in mature cows. *Animal Production* **38**: 23-32.
- Wynne, K.; Stanley, S.; McGowan, B.; Bloom, S. 2005: Appetite control. *Journal of Endocrinology* **184**: 291-318.
- Xu, C.; Wang, Z.; Liu, G. W.; Li, X. B.; Xie, G. H.; Xia, C.; Zhang, H. Y. 2008: Metabolic characteristic of the liver of dairy cows during ketosis based on comparative proteomics. *Asian-Australasian Journal of Animal sciences* **21**: 1003-1010.
- Xu, Z. Z.; Burton, L. J. 1999a: Reproductive performance of dairy heifers after estrus synchronization and fixed-time artificial insemination. *Journal of Dairy Science* **82**: 910-917.
- Xu, Z. Z.; Burton, L. J. 1999b: Effects of oestrus synchronisation and fixed-time artificial insemination on the reproductive performance of dairy heifers. *New Zealand Veterinary Journal* **47**: 101-104.
- Xu, Z. Z.; Burton, L. J. 2000: Reproductive performance of dairy cows in New Zealand. *Proceedings of the Australian and New Zealand Combined Dairy Cattle Veterinarians Conference - incorporating the 17th Annual Seminar of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*. Pp. 23-41.
- Xu, Z. Z.; Burton, L. J. 2003: Reproductive performance of dairy cows in New Zealand: final report on the monitoring fertility project. Occasional Publication of the Livestock Improvement Corporation. Hamilton, New Zealand, Livestock Improvement Corporation. Pp. 1-51.
- Xu, Z. Z.; Burton, L. J.; Macmillan, K. L. 1997: Reproductive performance of lactating dairy cows following estrus synchronization regimens with PGF $_2\alpha$ and progesterone. *Theriogenology* **47**: 687-701.

- Zolman, J.; Convey, E. M.; H., B. J.; Hafs, H. D. P. 1973: Release of bovine luteinizing hormone by purified porcine and synthetic gonadotropin releasing hormone. *Proceedings of the Society for Experimental Biology and Medicine*. Pp. 142-189.
- Zulu, V. C.; Nakao, T.; Sawamukai, Y. 2002a: Insulin-like growth factor-I as a possible hormonal mediator of nutritional regulation of reproduction in cattle. *The Journal of Veterinary Medical Science / The Japanese Society of Veterinary Science* **64**: 657-65.
- Zulu, V. C.; Sawamukai, Y.; Nakada, K.; Kida, K.; Moriyoshi, M. 2002b: Relationship among insulin-like growth factor-I, blood metabolites and postpartum ovarian function in dairy cows. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science* **64**: 879-885.

Appendix 1

Radioimmunoassay of progesterone

Plasma progesterone concentrations were measured in duplicate 10 µL aliquots by radioimmunoassay, using the ImmuChem Double Antibody Progesterone ^{125}I RIA kit for in vitro diagnostic use (MP Biomedicals, USA). The sensitivity of the assay was 0.14ng progesterone/mL. The intra-assay coefficients of variation at 80, 50 and 20% binding on the standard curve were 16.1%, 8.4% and 9.9% respectively; the inter-assay coefficients of variation were 19.1%, 14.4% and 15.7% for low, medium and high solutions, respectively. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer

Progesterone concentration in the plasma was measured by RIA. All samples were assayed in duplicate and the final result was presented as the average of the two readings. Samples were assayed undiluted. Ten µL of plasma was incubated with 20 µL of iodinated progesterone and 50 µL of antiserum (^{125}I -progesterone and antiserum ImmuChem Double Antibody Progesterone ^{125}I RIA kit for in vitro diagnostic use, MP Biomedicals, USA; 4 000 cpm, (counts per minute)) for 1 hour at 37°C. 50 µL of precipitant solution (MP Biomedicals, USA) was added and each sample was vortexed thoroughly, then centrifuged for 15 minutes at 2000 g at 4°C. Twenty µL of 50 g/L starch (Sigma-Aldrich, St. Louis, Missouri, USA) plus 0.1 g/l neutral red (BDH) in 0.1M phosphate-buffered saline (PBSG) pH 7.0 with 10% gelatine was then added to increase adhesion of the pellet to the tube, samples centrifuged for a further 15 minutes at 2000 g at 4°C and the supernatant aspirated off. The pellets were counted on a Wallac 1470 automatic gamma counter (Perkin Elmer, USA) for 5 minutes each.

The cross-reactivity (at 50% displacement as compared to the curve) of the progesterone antibody with other steroids was tested by MP Biomedicals. Cross-reactions were as follows: 20 α dihydroprogesterone (5.41%), desoxycorticosterone (3.80%), corticosterone (0.70%), 17 α -hydroxyprogesterone (0.67%), pregnenolone (0.41%), androstenedione (0.23%), testosterone (0.16%) and 11-desoxycortisol, pregnenolone sulphate, cholesterol, dehydroepiandrosterone (DHEA), ethiocholanolone, oestradiol-17 α , oestradiol-17 β , oestrone, oestriol, andosterone, aldosterone, cortisol and DHEA-S (<0.1%).

Parallelism and hormone additions

Plasma

A serial dilution of plasma in PBSG was parallel to the progesterone standard curve. The quantitative recovery of progesterone in plasma extracts was measured by adding different amounts of standard progesterone to two plasma extracts in PBSG. The recoveries of added progesterone were $98.0 \pm 3.0\%$ and $101.4 \pm 5.4\%$.

Assay sensitivity

The sensitivity of the progesterone assay was determined as the hormone concentration at the mean ± 2 standard deviations from the zero hormone point on the standard curves. Assay sensitivity was 0.14ng progesterone/mL.

Intra- and inter-assay variation

Solutions of progesterone in PBSG at concentrations that gave approximately 80, 50 and 20% binding on the standard curve were used as low, medium and high quality controls in every assay. The mean concentrations of progesterone in these solutions were 262 ± 42 , 3005 ± 253 and 13374 ± 1323 ng/mL, respectively. The intra-assay coefficient of variation for each solution was determined by conducting an assay with at least ten duplicates of each solution. The intra-assay coefficients of variation for progesterone were 16.1% ($n = 10$), 8.4% ($n = 15$) and 9.9% ($n = 10$) for low, medium and high solutions, respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for 42 assays were 19.1%, 14.4% and 15.7% for low, medium and high solutions, respectively.

Appendix 2

Radioimmunoassay of oestradiol

Oestradiol concentrations in plasma were measured by RIA. Undiluted samples were assayed in duplicate. 40 µL of plasma was incubated with 20 µL of antiserum for 1 hour at room temperature (18–25°C), then 20 µL of iodinated oestradiol (^{125}I -estradiol and antiserum DSL4800 Ultra-Sensitive Estradiol ^{125}I RIA kit, Immunotech, Czech Republic; 4000 cpm) was added and the mixture incubated for a further 2 h at room temperature. 200 µL of precipitating reagent (Immunotech, Czech Republic) was added and each sample was vortexed thoroughly, incubated for 20 minutes, then centrifuged for 20 minutes at 2000 g at 4°C. The supernatant was aspirated off. The pellets were counted on a Wallac 1470 Automatic gamma counter for 5 minutes each.

The cross-reactivity of the oestradiol antibody with other steroids was tested by Immunotech. Cross-reactions were as follows: oestrone (2.40%), oestrone- β -D-glucuronide (0.20%), oestrone-3-sulphate (0.01%), equilin (0.34%), D-equilenin (3.40%), 17 α -oestradiol (0.21%), 16-keto-oestradiol (0.21%), 17 β -oestradiol-3-glucuronide (2.56%), oestradiol-3-sulphate (0.17%), oestriol (0.64%) and testosterone, DHEA, diethyl stibesterol and 17 β E₂-17-glucuronide (<0.1%).

Parallelism and hormone additions

Plasma

A serial dilution of plasma in the kit zero calibrator (Immunotech) was parallel to the oestradiol standard curve. The quantitative recovery of oestradiol in plasma extracts was measured by adding different amounts of standard oestradiol to two plasma extracts in PBSG. The recoveries of added oestradiol were $103.4 \pm 9.2\%$ and $101.9 \pm 5.1\%$.

Assay sensitivity

The sensitivity of the oestradiol assay was determined as the hormone concentration at the mean ± 2 standard deviations from the zero hormone point on the standard curves. Assay sensitivity was 3.3 pg oestradiol/mL.

Intra and inter-assay variation

Solutions of oestradiol at concentrations that gave approximately 60 and 20% binding on the standard curve were used as low and high quality controls in every assay. The mean concentrations of oestradiol in these solutions were 29.0 ± 1.3 and 214.7 ± 14.8 pg/mL,

respectively. The intra-assay coefficient of variation for each solution was determined by the sum of squares method. The intra-assay coefficients of variation for oestradiol for these assays were 10.8% and 7.1% for low and high solutions, respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for these assays were 14.8% and 4.0% for low and high solutions, respectively.

Appendix 3

Radioimmunoassay of insulin

Insulin concentrations in plasma were measured by radioimmunoassay. Undiluted samples were assayed in duplicate. 20 µL of plasma was incubated with 20 µL of iodinated insulin and 20 µL of antiserum (^{125}I -insulin and antiserum, INSIK-5 kit, DiaSorin Inc., USA; 4000 cpm) for 16 h at 4°C. 200 µL of precipitating reagent (DiaSorin Inc., USA) was added and each sample was vortexed thoroughly, incubated for 15 minutes at room temperature, 20°C, then centrifuged for 20 minutes at 2000 g at 4°C. The pellets were counted on a Perkin Elmer Wallac 1470 (Perkin Elmer, USA) automatic gamma counter for 2 minutes each.

The cross-reactivity of the insulin antibody with other steroids was tested by DiaSorin. Cross-reactions are as follows: human insulin (100%), bovine insulin (100%), porcine insulin (100%), rat insulin (100%), porcine proinsulin (28%), bovine glucagon (0.46%), porcine glucagon (0.03%) and porcine C-peptide and human C-peptide (<0.01%).

Parallelism and hormone additions

Plasma

A serial dilution of plasma in the kit zero calibrator was parallel to the insulin standard curve. The quantitative recovery of insulin was measured by adding different amounts of standard insulin to three plasma samples. The recoveries of added insulin were $103.5 \pm 2.7\%$, $93.9 \pm 7.9\%$, and $95.5 \pm 6.3\%$.

Assay sensitivity

The sensitivity of the insulin assay was the minimum hormone concentration that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean ± 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity, expressed as µU insulin/mL plasma, was 4.4 µU/mL.

Intra and inter-assay variation

Solutions of insulin at concentrations that gave approximately 80 and 50% binding on the standard curve were used as low and high quality controls in every assay. The mean concentrations of insulin in these solutions were 12.3 ± 1.6 and 54.6 ± 6.2 µU/mL, respectively. The intra-assay coefficient of variation for each solution was determined by conducting an assay with twelve duplicates of each solution. The intra-assay coefficients of variation for insulin were 11.2% and 15.5% for low and high solutions, respectively. Inter-assay coefficients

of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for three assays were 12.9 % and 11.3% for low and high solutions, respectively.

Appendix 4

Analysis of IGF-I

Concentrations of IGF-I were measured in duplicate 20 µL aliquots by ELISA, using the DSL-10-2800 ACTIVE IGF-I ELISA (Diagnostic Systems Laboratories, USA). The limit of sensitivity of the assay was 1.5 ng/mL IGF-I/mL. Control solutions of IGF-I were used as low and high quality controls in every plate. The mean concentrations of IGF-I in these solutions were 188.9 ± 8.7 and 353.9 ± 10.8 ng/mL, respectively. The intra-assay coefficients of variation for low (~200 ng/mL) and high (~350 ng/mL) control solutions were 8.1% and 2.5%, respectively, whilst the inter-assay coefficients of variation were 6.5% and 4.3%. The assay was validated for use in bovine plasma by demonstrating parallelism between standards diluted in plasma and assay buffer.

Plasma sample pre-treatment

20.2 µL plasma in a polypropylene culture tube was pre-treated by adding 1000 µL sample buffer I (Diagnostic Systems Laboratories, USA), vortexed then incubated at room temperature (~25°C) for 30 minutes. Samples with high concentration of endogenous IGF-I were diluted in IGF-I standard A/sample diluent (Diagnostic Systems Laboratories, USA) before pre-treatment. 1000 µL of sample buffer II (Diagnostic Systems Laboratories, USA) was added and the mixture vortexed thoroughly. The extracts were refrigerated (4°C) overnight and assayed the next day, or frozen at -20°C if assayed after 24 h.

Assay procedure

IGF-I concentrations in plasma were measured by enzyme-linked immune-sorbent assay (ELISA). Samples were assayed in duplicate. 20 µL of plasma extract was incubated with 100 µL of assay buffer, (DSL-10-2800 ACTIVE IGF-I ELISA, Diagnostic Systems Laboratories, USA) for 2 h at 25°C on an orbital microplate shaker (Grant-bio PMS1000, UK) set at 600 rpm. The plates were washed five times with 300 µL wash solution (Diagnostic Systems Laboratories, USA) using an automatic microplate washer (BioTek ELx50, USA). The plates were incubated with 100 µL IGF-I antibody-enzyme conjugate solution (Diagnostic Systems Laboratories, USA) for 30 minutes at 25°C on the orbital microplate shaker at 600 rpm and then washed five times as above. 100-µL TMB chromogen solution (Diagnostic Systems Laboratories, USA) was added to each well and the plate shaken at 600 rpm for 10 minutes. 100 µL stopping solution (0.5N NaOH, Diagnostic Systems Laboratories, USA) was added and the plates were read on a BioTek PowerWave 340 plate reader, (BioTek, USA) at 450 nm with a

background correction of 620 nm. Plates were read within 30 minutes of adding the stopping solution.

The cross-reactivity of the IGF-I antibody with other steroids was tested by Diagnostic Systems Laboratories. Cross-reactions were as follows: human IGF-I (100%), human IGF-II (non-detectable at 500 ng/mL) and insulin and growth hormone (non-detectable at 200 ng/mL).

Parallelism and hormone additions

Plasma

A serial dilution of plasma extract in the kit zero standard was parallel to the IGF-I standard curve. The quantitative recovery of IGF-I was measured by adding different amounts of standard IGF-I to a plasma extract. The recovery of added IGF-I were $87.5 \pm 3.5\%$, $88.7 \pm 3.8\%$ and $87.9 \pm 3.7\%$.

Assay sensitivity

The sensitivity of the IGF-I assay was the minimum hormone concentration that could be consistently distinguished from zero. It was determined as the hormone concentration at the mean ± 2 standard deviations from the zero hormone point on the standard curves. The assay sensitivity, expressed as ng IGF-I/mL plasma, was 1.5 ng/mL.

Intra and inter-assay variation

Control solutions of IGF-I were used as low and high quality controls in every plate. The mean concentrations of IGF-I in these solutions were 188.9 ± 8.7 and 353.9 ± 10.8 ng/mL, respectively. The intra-assay coefficient of variation for each solution was determined by the sum of squares method. The intra-assay coefficients of variation for IGF-I for twelve plates were 8.1% and 2.5% for low and high solutions, respectively. Inter-assay coefficients of variation were calculated from duplicates of the solutions included at the beginning and end of each assay. The inter-assay coefficients of variation for twelve plates were 6.5% and 4.3% for low and high solutions, respectively.