AN INVESTIGATION INTO THE PRACTICAL APPLICATION OF THE RADIOIMMUNOASSAY (RIA) TEST OF MILK PROGESTERONE TO IMPROVE ARTIFICIAL INSEMINATION (AI) MANAGEMENT IN DAIRY CATTLE

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Dissertation submitted in fulfilment of the requirements for the Degree

D.TECH: AGRICULTURE

in the

School of Agriculture and Environmental Sciences
Faculty of Health

at the

Technikon Free State

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BLOEMFONTEIN
February 2004

ACKNOWLEDGEMENTS

The Author wishes to express his sincere gratitude and appreciation to the following persons:

- Prof. Johan Greyling, for his encouragement and guidance throughout this study. Without his continual encouragement, competent guidance and constructive criticism, this study would not have been possible.
- Prof. Carlu van der Westhuizen. A special word of thanks for initiating this study and guidance and showing vision and giving direction.
- The Van der Walt family for their wonderful friendship, encouragement, faith and belief in my abilities.
- A special word of thanks to Hester Linde, for the friendliness and able typing of the manuscript.
- The Free State Technikon, for the financial support.

DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, ANDREW STEPHEN VAN DER WALT, identity number 7311305059085 and student number 20155980, do hereby declare that this research project submitted to the Technikon Free State for the Degree D.TECH: AGRICULTURE, is my own independent work, and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Technikon Free State, and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT

DATE

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	Artificial insemination
BCS	Body condition score
BSA	Bovine serum albumin
BST	Bovine somatotropin
BW	Body weight
CIDR	Controlled internal drug release
CL	Corpus Luteum
CLA	Commencement of luteal activity
CR	Conception rate
CV	Coefficient of variation
DMI	Dry matter intake
DOV	Delayed ovulation
EM	Embryonic mortality
EPD	Estimated pregnancy diagnosis
$F_{2\alpha}$	Prostaglandin $F_{2\alpha}$
FSH	Follicle stimulating hormone
GH	Growth hormone
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
IAEA	International Atomic Energy Agency
ICC	Interval from calving to conception
ICS	Interval from calving to first service
IFN	Trophoblast interferon
IGF	Insulin-like growth hormone
IQC	Internal quality control
LH	Luteinizing hormone
MP	Milk progesterone
MPPS	Milk progesterone test
MUN	Milk urea nitrogen
NCYC	New cycle node treatment
ODR	Oestrous detection rate

OIS	Oestrous induction and synchronization
P4	Progesterone
PBS	Phosphate buffered saline
PCL	Persistent corpus luteum
PD	Pregnancy diagnosis
PGF	Prostaglandin
PMSG	Pregnant mare serum gonadotrophin
PR	Pregnancy rate
PRGT	Pregnancy diagnosis node
PUN	Plasma urea nitrogen
QC	Quality control
RIA	Radioimmunoassay
SD	Standard deviation
SERV	Service node
TC	Total counts
UPL	Uterine palpation per rectum

CHAPTER 1

INTRODUCTION

Reproductive performance is one of the most important factors determining profitability in dairy herds (Lamming & Darwash, 1998). Current intercalving intervals (an indicator of reproductive performance) are generally too long for optimal milk production and the number of progeny born per year. These long intercalving intervals may be associated with managerial influences, such as breeding policy or the detection of oestrus or fertility problems, such as acyclia or "repeat breeders" i.e. the problem cow syndrome.

Esslemont and Kossaibati (1998) suggested repeat breeding to be caused by an inadequate supply of the hormone progesterone, during the luteal phase of the oestrous cycle and Erb *et al.* (1976) found that unsuccessful inseminations were often accompanied by asynchronies in the hormonal profiles. Levels of and patterns of especially progesterone secretion could thus be one of the underlying causes of repeat breeding in problem cows.

The hormone progesterone is produced by a transient organ, which develops in the ovary, after ovulation, called the corpus luteum (CL). The CL functions for a specific period of time (19-22 days after AI) and, if conception does not occur, undergoes regression. If conception does occur, however, the CL continues to function and secrete progesterone throughout the gestation period. Thus the concentration of progesterone in the body fluids can vary according to the reproductive status of the animal (Ball & McEwen, 1998) and the measurement of progesterone levels in plasma is a specific indicator of luteal function (Beck *et al.*, 1996).

The presence of progesterone in cow milk was first documented by Mcleod and Williams (1991) and Bloomfield *et al.* (1986). Laing and Heap (1971) realised that, if progesterone levels in milk were closely correlated with those in the plasma, great benefits would accrue to studies of the ovarian

physiology, because of the relative ease of collecting milk rather than blood samples.

Progesterone in milk was positively identified by Heap *et al.* (1973) and high correlations (r = 0.91) with progesterone levels in milk and plasma were recorded by Hoffman *et al.* (1976) and Peters (1984). This led to the development of sophisticated techniques to measure milk progesterone levels by Cox *et al.* (1978) and identify the physiological status of the animal. As a result milk progesterone profiles and individual milk sample measurements can now be used to monitor the luteal and hence ovarian activity of pregnant cows (Pursley *et al.*, 1997b).

Milk progesterone analysis may thus been seen as a most convenient method of monitoring ovarian activity in dairy cows and the possibility to use this technique as a routine procedure for pregnancy diagnosis service to commercial dairy producers may be possible.

The objective of this study was to:

a) test the accuracy of pregnancy diagnosis with the aid of a milk progesterone concentration assay
b) use the milk progesterone assay to evaluate the progesterone profiles of cows classified as problem (low fertile) cows and
c) determine a strategy to solve the incidence of problem low fertile cows, relating to information acquired from the milk progesterone assay.

CHAPTER 2

LITERATURE REVIEW

2.1 FACTORS AFFECTING MILK PROGESTERONE LEVELS AND ITS DIAGNOSTIC VALUE TO MONITOR FERTILITY IN COMMERCIAL DAIRY HERDS

2.2.1 Introduction

Management of the dairy herd for high productive efficiency is an important economic factor in the success of the dairy enterprise. Calving interval is a major indicator of reproductive efficiency and is primarily a function of the number of days from calving to the initiation of the next pregnancy - referred to here as days open, together with the fixed effect of gestation length (Oltenacu *et al.*, 1980). The days open depend on the interval from calving to the first insemination or mating (referred to here as first service), and associated conception rate, along with the interval to and conception rate following repeat breedings. Numerous protocols designed to reduce the interval from calving to conception, have been developed (Stevenson & Pursley, 1994). Risco *et al.* (1995) and Thatcher and Wilcox (1973) emphasised that for AI to be effective, any program of drug use to control ovulation must combine good reproductive management with drug application, consistent with well established physiological concepts. The synchronised ovulation regimens (controlled breeding) reduce the time required to check cows for oestrus, but approximately 60% of the synchronised cows do not conceive at first service. Subsequently most of these non-pregnant cows are managed in conventional programmes of visual detection of oestrus and insemination.

The importance of an accurate detection of oestrus has been emphasised by Kinsel and Etherington (1998), who surveyed 45 herds, using conventional oestrous detection techniques following GnRH and/or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in their breeding programmes. The effectiveness of the

synchronization program was determined by the detection of oestrus, while the conception rate obtained had the greatest impact on the subsequent calving interval. Various aids for detecting oestrus in cattle are available. Studies by Nebel and Jobst (1998), indicated a high proportion of cows to be inseminated following visual observed behavioural changes, associated with oestrus.

Many oestrous behavioural characteristics in cows, such as nervousness and mounting of other cows, are used in commercial herds as an indication of oestrus. These can however frequently be falsely interpreted as indicating oestrus. Nebel and Jobst (1998) reported detection of oestrus to be a problem in 30% of the herds, with up to 46% of the cows inseminated when milk progesterone (P_4) levels are high. These procedures result in low conception rates, while insemination of pregnant cows could cause abortion. Both events prolong the calving interval. A preliminary evaluation of commercial artificial insemination records revealed that cows are artificially inseminated at an interval substantially divergent from the normal oestrous cycle length of approximately 21 days. This suggests that many cows are inseminated in the luteal phase of the estrous cycle or when pregnant (Lamming & Darwash, 1998).

The early diagnosis of pregnancy is an important aspect of reproductive management of a dairy herd or any cattle enterprise for that matter. It can affect the profit via factors such as the quantity milk produced per day of cow herd life, decisions on culling and nutritional regimes and the identification of fertility problems (Ropstad & Refsdal, 1987).

When measured in milk samples collected at appropriate times in relation to AI, progesterone values can be used in conjunction with related physiological data to evaluate the causes of poor reproductive performance more accurately. So for example, it has been reported that in some herds experiencing poor fertility, 15 to 20% of the cows are inseminated when they have elevated serum progesterone levels - indicative of a non-oestrous state. Contrary, low progesterone concentration levels 22-24 days post-insemination

indicate that the cow is not pregnant and, if not returning to oestrus, is indicative of poor oestrous detection (Woolliams, 1997). However, AI technicians are not always able to diagnose the status of ovarian activity correctly and sometimes administer inappropriate therapies - resulting in a loss of money and time. If progesterone determinations can be introduced as a routine method into dairy farms, AI technicians and veterinarians would possess a powerful and practical tool for effective dairy reproductive management and efficient reproduction, with economic benefits also being further exploited. This determination of the progesterone profile could thus provide valuable information regarding the improvement of reproductive management in the dairy herd.

Artificial insemination (AI) is important in achieving faster genetic progress in cattle. In 1959 a national programme was initiated to increase milk and beef production through genetic improvement. By using this breeding technique (AI), the aim was to improve the conception rate (CR) at first service to between 40% and 60%. However, during the past two decades the CR in AI programmes has been recorded below 45% (Esslemont & Kossaibati, 1998). Recent reports have suggested the main cause of low CR following AI to be the lack of accuracy in oestrous detection (Heersche & Nebel, 1994). In South Africa 30% of cattle bred by AI are subject to oestrous induction and synchronisation (OIS) programmes (Haulon *et al.*, 1996). However, the CR is still below 50%. The aim of this project is thus to identify some factors affecting the efficiency of AI in controlled breeding programmes, using progesterone determinations in the blood or milk - in order to determine the proportion of acyclic females or cows with abnormal oestrous cycles. In addition some strategies for improving the CR will be discussed.

The main causes of low fertility are oestrous detection failure, inseminations at inappropriate times, poor semen quality, embryonic mortalities, seasonal influences and factors related to management on farms (Haulon *et al.*, 1996).

2.1.2 Using milk progesterone tests to monitor and improve fertility in commercial dairy herds

The successful use of artificial insemination relies on the accurate detection of oestrus and the correct timing of insemination. It has been shown that poor reproductive performance in dairy herds is largely due to poor oestrous detection. So for example, in a survey on 22 herds, involving 1644 cows, it was found that oestrous detection was the measure best correlated with calving interval (Schopper *et al.*, 1993).

Normal oestrous cycles are often not used when inseminating in a dairy herd, because even in the best managed herds, oestrous detection rates rarely exceed 70 % and average 55 % (Ball & Jackson, 1979; Esslemont & Peeler, 1993; Schopper *et al.*, 1993). Furthermore, 10 to 20 % of non-oestrous cows may be wrongly detected in oestrus and inseminated when conception is not possible (Lamming & Bulman, 1976; Schopper *et al.*, 1993). These errors suggest that a significant improvement in herd reproductive performance could be made if every ovulation was associated with a correctly timed insemination.

One effective approach to obtain this goal, would be to monitor the natural oestrous cycle and thereby predict the time of ovulation. The principle of identifying the stage of the oestrous cycle by monitoring changes in progesterone concentrations in milk has been demonstrated (Laing & Heap, 1971; Ball & Jackson, 1979). By monitoring the frequency distribution of behavioural oestrus in relation to the fall in milk progesterone concentration in cow milk that was sampled daily, Peters and Ball (1987) illustrated that the occurrence of oestrus could be set as the third day of low milk progesterone concentration.

Eldon (1991) used the decrease in milk progesterone concentration as the sole indicator for the timing of AI. The percentage of successful inseminations obtained in this way was higher between day 2 and day 3 of low progesterone, and a significant fall in conception rate following insemination

was recorded on the fourth day of low progesterone concentration. These results suggest that an exact timing of insemination is not essential to ensure fertilization - provided that it is not delayed beyond the third day of low progesterone levels. This finding is in accordance with surveys of 8214 cows by Zaied *et al.*, (1979) and of 6007 cows by Plaizier and King (1996) who found no significant difference in the fertility rate of cows inseminated between one hour and more than 24 hours after the initial detection of oestrus.

It was demonstrated by Butterfield *et al.* (1988) that the dairy herd's reproductive performance can be significantly improved by following an insemination protocol based on the changes in milk progesterone concentrations. When milk was sampled daily for 30 days and cows inseminated on the third day of low progesterone concentration, higher submission rates and conception rates were achieved than in a control group inseminated on the basis of oestrous detection alone (97.5 vs 70.7 % for submission rates and 62.5 vs 47.5 % for conception rates). In addition the number of days from calving to first service were reduced from 91.7 (controls) to 78.3 (progesterone monitored) days.

Mahaputra *et al.* (1990) monitored progesterone concentrations in milk samples taken daily from day 17 to day 24 after insemination, and found an improvement in oestrous detection rates and a reduction in the calving to conception interval. Similar improvements were recorded when milk sampling was reduced to every second day from day 18 or 19 to day 23 or day 24 after insemination, which would be a more practical option for commercial dairy farmers. However, this trial did not include a control group and the herd's reproductive performance was compared with that of the previous years. Consequently, it was not possible to determine whether the improvement was due to the milk progesterone monitoring or to variations between years.

It has been suggested by Peters and Ball (1987) that progesterone determination of frequently taken milk samples can be used to identify the reproductive status of individual cows. The weekly evaluation of milk

samples from cows which have not been observed in oestrus by 35 days after calving will determine whether a cow is cycling normally or not. In a normally cycling cow, 3 or 4 consecutive weekly samples will include at least one sample with a low progesterone concentration, interposed between samples with high progesterone concentrations.

The above reports suggest that milk progesterone can be used to predict the time of ovulation in cattle accurately for practical pregnancy diagnoses. All the above studies made use of laboratory assays for measuring the milk progesterone concentrations, assays which are not readily available to farmers.

2.1.3 Genetic selection and reproductive traits

Many indicators have been used as genetic parameters of reproductive traits and, in particular, of genetic correlations between reproductive traits and milk yield at various stages of lactation (Boyd *et al.*, 1954; Butler & Smith, 1989). Several researchers have indicated a substantial genetic antagonism between milk yield and reproduction as such. Estimates of genetic correlations between reproduction and milk yield have been quoted to range from -0.064 to +0.1. However, other researchers have shown little or no relationship (Boyd *et al.*, 1954).

As milk yield is paramount in dairy cattle selection, many researchers and dairy producers are concerned with the fact that the long-term selection for milk yield could decrease the reproductive performance in dairy cows. Many researchers have reported heritabilities close to zero for reproductive traits and concluded additive genetic variation to be very small in proportion to the phenotypic variation obtained and thus that selection for improved fertility would not be worthwhile (Boyd *et al.*, 1954; Butler *et al.*, 1981; Butler & Smith, 1989; Hansen, 1997). Certain researchers have suggested that the decrease in fertility may be possible through consideration of various fertility indexes in selection decisions (Butler & Smith, 1989; Hansen, 1997). Reimers *et al.* (1990), recorded the heritabilities of days open and age at first calving to be 0.12 and 0.05 respectively, and suggested that direct selection

for fertility through progeny testing may be justified. It was also projected that a 5 to 10 day increase in days open could result from a genetic increase in 1000 kg of milk produced.

Analyses of milk progesterone data has revealed that certain endocrine abnormalities are heritable (Darwash *et al.*, 1997). In the past fertility traits have generally been considered to be lowly heritable. However, the traits examined were "traditional fertility parameters", which do not solely reflect the physiological characteristics of the cow - due to the impact of managerial decisions. Recent re-examination of these traditional parameters confirms their low heritability (typically <0.05) (Pryce *et al.*, 1997). Although relatively high coefficients of genetic variation are displayed, which indicates room for genetic improvement. Genetic improvement based on "traditional fertility parameters" would thus be slow, as in addition to low heritability the use of these traits are limited by biological (sex and age limitations) and structural (accuracy/ reliability of data recording) constraints (Darwash *et al.*, 1999).

One particular measure of ovarian function, the commencement of luteal activity (CLA) post partum, has been shown to be moderately heritable (range for various transformations, h^2 = 0.13 to 0.28) and repeatable (r^2 = 0.28) in British Friesian cows (Darwash *et al.*, 1997). The CLA has also been shown to be heritable (h^2 = 0.16) in Dutch Holstein-Friesian cows in their first lactation. In the British Friesians, the mean interval to CLA was 28.7 days versus 29.5 days in Dutch Holstein-Friesians (Veerkamp *et al.*, 2000). Shorter intervals were positively correlated to increased fertility in British Friesians, as for each day by which CLA increased, was related to a delay of 0.24 to 0.41 days - regarding the interval to the first service and conception rates respectively (Darwash *et al.*, 1997). Early re-establishment of post partum ovarian activity has been established as an important pre-requisite for high fertility (Thatcher & Wilcox, 1973; Whitmore *et* al., 1974; Stevenson *et al.*, 1983; Silva *et al.*, 1992). However, this remains a controversial topic, as these reports are in disagreement with Ball and McEwen (1998) and Smith and Wallace (1998) and require confirmation in the UK dairy herds of Holstein-Frieslands. Other endocrinopathies associated with sub-fertility

include the duration of the inter-luteal period and persistent corpora lutea (PCL - reflecting delayed luteolysis), but it is not clear to what extent these conditions affect subsequent fertility (Moffitt, 1995).

Although the use of physiological measures (CLA) of fertility, such as defined in breeding programmes are ultimately the preferred parameters to help halt and reverse the decline in fertility, these parameters need to be further investigated. So for example, estimates of heritability and phenotypic or genetic correlations with other economical important traits need clarification for the modern dairy cow population, before successful incorporation into selection indices can be achieved. This work is currently being researched at Nottingham University in collaboration with the Roslin Institute (Bo *et al.*, 1994). However, in the mean time, the low heritability of traditional fertility measures do not prevent their meaningful inclusion in breeding programmes (Woolliams, 1997). In fact, the genetic correlation between the one traditional parameter namely calving interval and body condition score (BCS), is currently being investigated at the Scottish Agricultural College (Edinburgh) and the University of Edinburgh (Haulon *et al.,* 1996). BCS has been recorded by HUKI (Holstein UK and Ireland) since 1996 as part of its linear type classification scheme. As calving dates are reliably recorded, most cows that survive to their second lactation have calving interval data. BCS is believed to be related to the energy balance (Veerkamp *et al.*, 1998) and has a heritability component much greater than most traditional fertility parameters of approximately 0.2 to 0.3 (Jones *et al.*, 1999). With the assumption that energy balance and fertility are related, BCS is a possible selection criterion for fertility. The results of this research suggest that there may be an opportunity to select indirectly for fertility using a combination of calving interval and BCS, which could be implemented at low cost, in the near future.

Although the physiological fertility parameters discussed would be an improvement to the traditional parameters used, a further improvement in response to selection for female fertility could be gained by the use of indirect selection criteria for these parameters. This might involve a heritable trait in the male, which is measurable in early life and genetically correlated to female

fertility. The LH secretory response to a GnRH challenge is one possible parameter, which is heritable in tropical beef bulls, as judged by testosterone responses (MacKinnon *et al.*, 1991).

The genetic reversal of the trend in sub-fertility is a long-term goal. It is unlikely that substantial genetic progress shall be made regarding fertility for at least a decade. In the meantime, milk progesterone analysis allows an alternative to the problem. The most immediately feasible solution is probably endocrine therapy.

2.1.4 Endocrine therapy

Having successfully initiated a return to cyclic activity post partum, the cow must meet two other endocrine criteria. Firstly, the cow must express oestrous behaviour sufficiently to be detected, not only by the bull but also by the stockman. Stockmen are not so sensitive in monitoring the onset of oestrus e.g. as bulls (and the stockmen are probably considerably busier), so a particularly strong manifestation of oestrus is essential. Secondly, the cow must ovulate appropriately and develop a corpus luteum, able to secrete sufficient progesterone. The conceptus must induce the maternal recognition of pregnancy (trophoblast interferon; IFN-τ) by day 16 post-insemination, in order to prevent uterine prostaglandin secretion and luteolysis and the embryo must have undergone elongation in order to produce the signal in sufficient quantities (Mann & Lamming, 1999). When the conceptus growth is compromised by poor uterine secretory function, luteolysis (progesterone withdrawal) occurs and pregnancy fails. Conceptus growth is progesterone-dependant, and a low level of progesterone effectively results in the starvation of the conceptus. Mann and Lamming (1999) recorded that a late post-ovulatory serum progesterone increase in a low luteal phase results in poor embryo development and little or no interferon secretion on day 16. The concentration of interferon-τ in the uterine flushes on day 16 of gestation is related to circulating serum concentration of progesterone between days 4 and 5 of the oestrous cycle ($r^2 = 0.4$; $P<0.05$) (Mann *et al.*, 1998). Therefore, in this context, sufficient progesterone levels mean a concentration high

enough to trigger a normal pattern of uterine secretory function by day 5 after insemination. Cows with circulating serum progesterone concentrations below this threshold, due to a late post-ovulatory rise in progesterone secretion, have higher unsuccessful conception rates or embryonic losses than those above the threshold. The threshold progesterone concentration in the systemic circulation is reflected by the threshold concentration in milk (which varies between assays in the range of 2 to 7ng/ml), and cows suffering from luteal inadequacy can be identified by a milk progesterone assay on day 5 following AI.

There are 3 hormonal therapies that can be applied to correct these endocrinopathies. A low milk progesterone level on day 5 following AI can be treated by progesterone administration via an intravaginal progesterone-releasing device (CIDR). A meta-analysis of numerous studies using progesterone supplementation recorded an overall 5.2% improvement in day 5 pregnancy rate following treatment, compared to a 10.3% improvement where progesterone was administered at day 6 after AI. A further improvement in pregnancy rate of 19.3% was recorded when exogenous progesterone was administered to cows exhibiting low fertility (Mann & Lamming, 1999). Early progesterone supplementation increases the rate of conceptus development. Garrett *et al.* (1988) showed that progesterone administered between day 2 and 5 of gestation increases the conceptus length 10-fold by day 14. In view of the dependence of conceptus growth following progesterone stimulated uterine secretion, it is not surprising that progesterone supplementation should be so effective. Although a 10% improvement in viability following early supplementation may seem a small gain, it is worth noting that this would return conception rates to those considered normal. In other words, it would buy 10 years in the race against sub-fertility in cattle. Manipulation of circulating progesterone concentrations during early pregnancy has resulted in increased rates of conceptus survival in sows, in which decreasing the plane of nutrition reduces the hepatic progesterone catabolism (Ashworth & Pickard, 1998).

The only theoretical disadvantage of administering exogenous progesterone is that it may reduce pituitary gonadotropin secretion, upon which the corpus luteum depends. Although this is not significant while progesterone is being administered, it may become so when the treatment is terminated and the corpus luteum is reduced in size or secretory capability, during the period of administration. In practice, this only occurs with relatively high rates of progesterone administration and not with the CIDR therapy currently in use for this purpose.

A second therapeutic approach to exogenous progesterone supplementation is to block luteal regression by reducing the luteolytic drive associated with follicular oestrogen secretion. This is the principle underlying the use of Buserelin (Receptal, Hoechst Roussel Vet Limited), a GnRH analogue which induces ovulation or atresia of follicles, thus lowering circulating oestrogen concentrations (Mann & Lamming, 1995; Beck *et al.*, 1996). This approach is based on the fact that uterine secretion of prostaglandin $F_{2\alpha}$, which causes luteolysis, is stimulated by circulating oxytocin, and the uterine expression of the oxcytocin receptor is triggered by oestrogen. Reduced follicular secretion of oestrogen leads to reduced uterine secretion of prostaglandin $F_{2\alpha}$ (Mann & Picton, 1995). Treatment at the first service post partum with GnRH given on the day of the insemination recorded a 6% improvement in pregnancy rate. Later administration (days 11 to 13 after service) produced an even greater increase of 10 to 12% (Mee *et al.*, 1990; Peters *et al.*, 1992).

The third hormonal therapeutic strategy should be focussed on treating animals that ovulate, but fail to show oestrus. It has recently been estimated that by using a combination of milk progesterone analysis and Kamar heatmount detectors (Cox Surgical), 72% of first post partum ovulation are silent (i.e. not accompanied by oestrus). This percentage decreases to a mean of 25% at subsequent ovulations. This illustrates that in practice, failure to observe oestrus can be corrected by milk progesterone analysis, which will show ovarian activity in the absence of insemination. Underlying endocrine causes of undetected oestruses may include the absence of ovarian function,

delayed ovulation (DOV) resulting in various types of ovarian cysts or persistent CL's (PCL). Alternatively, there may be normal ovarian activity, but poor behavioural response to the endocrine triggers responsible for the onset of oestrus. These conditions are commonly treated by the administration of progesterone via the CIDR and/or a prostaglandin $F_{2\alpha}$ analogue injection. In the case of a PCL or a luteal cyst, this treatment induces luteolysis and ovulation. Where there is normal ovarian activity, but a failure to show overt signs of oestrus, progesterone treatment and withdrawal may enhance the behavioural manifestation of oestrus. Progesterone administration also has the advantage that it induces follicular development and ovulation in the case of prolonged post partum anoestrous periods (Veerkamp *et al.*, 1998).

In view of the availability of treatment for some of the causes of failure to show oestrus in cows, it would be appropriate to develop optimised therapeutic protocols. These could be based on strategic progesterone monitoring. Application of Kamar heatmount detectors to all cows not observed in oestrus by day 35 to 40, would also give valuable information as to an animal's physiological and behavioural status. Although it is not possible to accurately predict the benefits of these treatments, it may be that an optimised combination of treatments would raise the efficiency of oestrous detection considerably.

2.1.5 Strategies towards treating sub-fertility in dairy cows

The three approaches in treating dairy cow sub-fertility, via endocrinology, nutrition and genetics, are likely to be effective within different time scales. A strategy for changing the trend in dairy cow sub-fertility must clearly include all the cost effective options open. In the short-term, endocrine therapies should be introduced immediately, although their widespread use may be criticised on welfare and ethical grounds. In the long-term (as it involves the administration of hormones), therapy may provide immediate gains. There will always be a proportion of problem cows in the herd that require endocrine treatments. By continuing to breed these animals, the potential heritability of these reproductive abnormalities should always be borne in mind.

Whilst endocrine therapy can be used immediately, the nutritional and genetic approaches are likely to take some time to develop and show dividends. The genetic approach is the most long-term strategy, largely because of the lack of and cost of collecting substantial quantitative and reliable data to analyse. There is however one approach to the genetic improvement of fertility, which may be introduced relatively fast. By using milk progesterone data, Veerkamp *et al.* (1998) and Darwash *et al.* (1999) suggested that it may be possible to exploit the heritability of corpus luteum activity (CLA) and the relationship between CLA and fertility. It would be able to develop a selection index that could be incorporated into progeny testing for bulls. These methods could exploit the fact that a large number of daughters are tested per sire for milk quality and yield. If the milk samples collected were also analysed for progesterone at monthly intervals or during a critical interval post partum period (e.g. 17 to 25 days post partum), a bull could be assigned a breeding value for female fertility. This being estimated from the average fertility of the bull's daughters, as fertile daughters ovulate earlier post partum than those that are less fertile (Darwash *et al.*, 1997). The advantage of this approach is that it could be implemented at the time of the progeny testing, using milk samples obtained for fat and protein analysis. The information would then also be available at the same time as the milk data upon which the value of the sire is currently based. Success of this strategy would require breeding companies to develop a market for the semen of bulls with high male, but more specifically, high female breeding values for fertility. However, this is largely dependent upon the farmer perceiving an advantage in paying a premium for female fertility. Once bulls with a high breeding value for female fertility are identified, and their semen marketed, genetic improvement in female fertility could potentially begin. Although the response to selection depends upon the weight attributed to fertility within the selection index, the selection intensity imposed on both the sire and the dam, the heritability of CLA (which is moderate) and the long generation interval of the cow needs to be recognized (Stevenson *et al.*, 1983).

2.2 STRATEGIES FOR CHANGING THE TREND TOWARDS FERTILITY IN DAIRY CATTLE

2.2.1 Introduction

The three principal problems facing the South African dairy farmer are sub-fertility, mastitis and lameness in the cow herd. Of these, sub-fertility has the greatest cost implication and is the most difficult to treat. Sub-fertility is broadly defined as any condition leading to failure to establish pregnancy following completion of uterine involution 40 to 50 days post partum. Failure to establish pregnancy at the expected time may reflect a number of abnormalities, including the failure to ovulate, failure to show oestrus, inappropriate patterns of ovarian cyclicity and the loss of pregnancy. These physiological conditions may reflect dysfunction at the hypothalamic, pituitary, ovarian or uterine level or even in the conceptus embryonic development. Therefore, sub-fertility in the dairy cow appears more complex than at first appears, and has many potential remedies. The complexity of the condition makes it difficult to analyse, diagnose and treat. However, cures must urgently be found, as the problem of sub-fertility is increasing rapidly in the dairy industry (Esslemont & Peeler, 1993).

Pregnancy rate at first service have reached an all time low of 40% (Darwash *et al.*, 1999; Royal *et al.*, 2000). This has a major impact on dairy farming economics, as the cost of temporary infertility include a loss in income from milk sales, increased feed costs, increased semen costs, where animals are repeatedly artificially inseminated. These losses amount to about 3 cows per day and 60% of all cows can be assumed to be infertile for 30 days per year (Esslemont & Peeler, 1993). To this must be added the cost of culling and replacing a sub-fertile cow. One in 10 cows is culled due to the difficulty of getting the cow into calf, at a total cost (milk losses plus replacement costs) of 600 l each. Given that there are approximately 2.5 million dairy cows in the UK national herd, producing 14 billion kg of milk per annum, these costs add approximately 2 pounds per litre to the price of milk. At a milk price to the farmer of approximately 18p per litre, this represents 10% of the value of the product at the farm gate. In addition to its economic value, sub-fertility also

has important animal welfare implications in terms of treatments to the cow and premature culling of persistently infertile animals. Furthermore, time spent with sub-fertility detracts from time available for other cow welfare concerns. It is surprising to note that the impact of sub-fertility on the dairy economics has only recently been estimated (Esslemont & Peeler, 1993; Stott *et al.*, 1999).

2.2.2 Assessment of sub-fertility in dairy cattle

There are a number of ways to monitor fertility in dairy cattle. These include recording the interval to first service, days open, inter-calving interval, conception rate at first service and number of services per conception. However, these indicators, which may be described as "traditional fertility parameters" are traits that are biased by management decisions and inadvertent data manipulation (e.g. culling animals before being inseminated) (Fortune, 1993).

An alternative range of fertility parameters are provided by the measurement of milk (or serum) progesterone levels and may be called "physiological fertility parameters". These indicate when and if an animal ovulates, reflects the formation and life span of the corpus luteum, as well as whether oestrous cyclicity is normal and pregnancy is maintained. Although physiological function, as monitored by endocrine measures such as circulating progesterone concentrations, may be affected by an animal's nutritional status (body condition score), stress or by environmental factors, these factors are not likely to be affected by management decisions (Anderson & Day, 1994).

The analysis of reproductive parameters based on milk progesterone levels, identifies a number of abnormalities in ovarian function, associated with reduced fertility. These include a long period of post partum anoestrus (a delay between calving and ovulation), a lengthy anoestrous period between cycles (which may reflect delayed ovulation, presence of a cystic follicle or absence of ovarian function), and a persistent corpus luteum in the first or subsequent cycles post partum. Each of these atypical patterns has been defined by Lamming and Darwash (1998). The total number of cows

experiencing one or more of these atypical ovarian characteristics has risen significantly over the past 20 years (Royal *et al.*, 2000). However, what is particularly evident is the incidence of persistent corpora lutea in the first cycle post partum - rising from 7.3% (Darwash *et al.*, 1999) to 18.2% in Holstein Friesians (Royal *et al.*, 2000). The interval to first service extended from 73.9 to 77.6 days and the calving rate to first service declined from 55.6% to 39.7%. A figure of approximately 40% for the current rate of calving at first service is supported by the independent studies of Darwash *et al.* (1999). Reproduction data for the subsequent period suggest a calving first service success rate of 50% (Esslemont, 1992). The calving rate at first service therefore declined between the two data recording periods in 1975-82 and 1995-98 by approximately 1% per annum. This compares well with the decline in conception rate at first service in the USA of approximately 0.45% per annum between 1975 and 1997 (Butler & Smith, 1989; Beam & Butler, 1999).

2.2.3 Relationship between plasma (PUN), milk urea nitrogen (MUN) concentrations and fertility around AI

As the genetic capacity for milk production has increased, so the conception rate in dairy cattle has decreased (Butler & Smith, 1989). High dietary protein intake stimulates milk production (Grings *et al.*, 1991), but has also been associated with decreased fertility (Kaim *et al.*, 1983; Canfield *et al.*, 1990). Cows fed excess dietary protein recorded increased blood urea, altered urine fluid composition, decreased uterine pH and reduced conception rates (Jordan *et al.*, 1983; Elrod & Butler, 1993; Elrod *et al.*, 1993). Plasma progesterone concentrations were reportedly lower in cows fed high levels of dietary protein (Sonderman & Larson, 1989). In relating dietary protein degradability to fertility, Ferguson *et al.* (1988; 1993) reported blood urea nitrogen concentrations exceeding 20 mg/dl to be associated with reduced conception rates in lactating cows.

Plasma urea nitrogen (PUN) and milk urea nitrogen (MUN) are useful indicators of the protein metabolism and nutritional status of the cows and is particularly advantageous for field metabolic monitoring purposes (Roseler *et*

al., 1993). High dietary protein, resulting in high concentrations of urea nitrogen in plasma and milk, have been associated with decreased fertility in dairy cattle (Kaim *et al.*, 1983; Ropstad & Refsdal, 1987; Canfield *et al.*, 1990; Elrod & Butler, 1993).

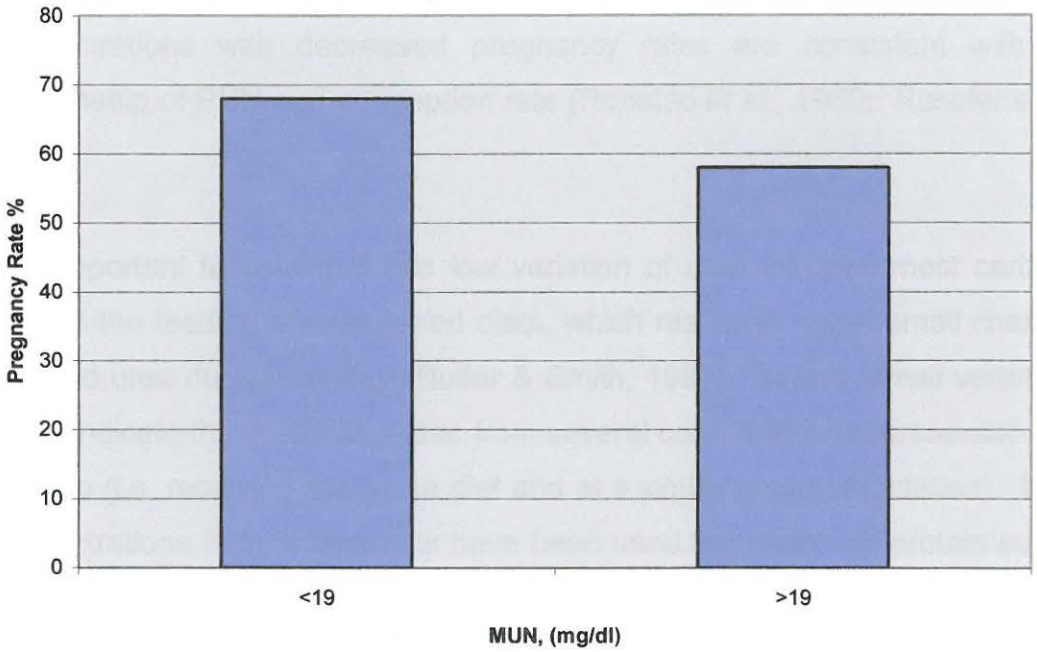

Figure 2.1 **The relationship of milk urea nitrogen (MUN) with pregnancy rate at first AI in lactating dairy cows (n = 155) (Canfield *et al.*, 1990)**

The pregnancy rate has been reported to be significantly reduced in cows with a MUN of >19mg/dl with the number of cows becoming pregnant to AI being indicated within each MUN category (Figure 2.1). As these cows were fed typical diets for lactating cows, the measured urea concentrations in the milk should reflect normal values for the dairy population. Previously, Ferguson *et al.* (1988; 1993) reported a similar range of urea concentrations in cows and also decreased conception rates. The magnitude of the associated decrease in pregnancy rates associated with high blood urea nitrogen seems to be related to the underlying reproductive performance among herds (Ferguson *et al.*, 1993). Kinsel and Etherington (1998) recorded no detrimental effect of high dietary protein and claimed that the discrepancies between studies in relating decreased fertility with the amount of dietary protein could be

attributed to differences in the reproductive management systems - rather than protein intake or to urea production.

As urea equilibrates within body fluids, MUN should be similar to plasma urea nitrogen (PUN) as an indicator of the urea nitrogen status in dairy cows and is more convenient to monitor. The relationship of increased MUN concentrations with decreased pregnancy rates are consistent with the relationship of PUN and conception rate (Ropstad *et al.*, 1989; Roseler *et al.*, 1993).

It is important to note that this low variation of urea nitrogen most certainly reflects the feeding of total mixed diets, which results in rather small changes in blood urea during the day (Butler & Smith, 1989). In turn, small variations could indicate that MUN analyses from several cows may be representative of a group (i.e. receiving the same diet and at a similar stage of lactation). Urea concentrations in bulk tank milk have been used to predict the protein supply (Refsdal *et al.*, 1985; Ropstad *et al.*, 1989) and fertility differences between herds (Ropstad & Refsdal, 1987).

2.2.4 The interaction of high milk yield and reproductive performance

The determination of oestrus prior to breeding and early pregnancy diagnosis are essential factors in successful dairy management programmes (Badinga *et al.*, 1985). Due to the fact that fluctuations in progesterone levels in milk are highly correlated with changes in the reproductive status of the animal, these changes can be used to assess the optimal time for artificial insemination in the animals - in order to ensure the establishment of pregnancy (Barnes *et al.*, 1985; Bonczek *et al.*,1988). Milk or serum progesterone concentrations can be used to predict pregnancy with an accuracy of 75 to 96% and the efficiency of predicting the non-pregnant status of the cow approaches 100%, when compared with that of rectal uterine palpation 60 to 90 days post insemination (Butler *et al.*, 1981; Butler & Smith, 1989).

The milk yield per day of the cow follows a predictable curvilinear function that peaks at 6 to 9 weeks of lactation and then declines at a constant rate. The total milk income within a lactation period is thus determined by the peak and consistency of milk yield. The economic returns of the individual cow represent an integration of several performance traits, in addition to milk yield. However, to maximise profit over a herd lifetime, economic models recommend an optimal calving interval of between 12 to 13.5 months. Increasingly, dairy producers and researchers have questioned the biological feasibility and economic justification of a 12- to 13.5 month calving interval in cows with milk yields exceeding 13 500 kg milk per lactation (Dhaliwal *et al.*, 1996).

Various reproductive traits have been measured and recorded in investigations for a possible relationship between milk yield and reproductive performance. Days open, the period from calving to conception, is subject to management policy - such as e.g. the voluntary waiting period prior to breeding, and is influenced by the conception rate and efficiency of oestrous detection. Therefore, days open and intercalving interval are unsatisfactory indicators of reproductive performance for determining potential correlation between milk yield and reproduction. Although influenced by many factors, conception rate or the number of breedings per conception is more inherently associated with physiological functions and it is a more desirable indicator of reproductive function. Specifically in cows yielding more milk, which generally record a lower conception rate (Etherington *et al.*, 1994).

As dairy cattle achieve higher milk yields, the relationship between yield and reproduction become more apparent (Canfield & Butler, 1990). Research indicates an antagonistic relationship between high milk yield and reproductive performance. An important similarity in milk yield and reproductive efficiency is that management and environment account for the majority of variation (Butler & Smith, 1989). In addition, interactions between nutrition and reproductive performance are apparent, e.g. the detrimental effects of a prolonged negative energy balance (Canfield & Butler, 1991).

2.3 NUTRITION AND MILK PRODUCTION AS CAUSES OF SUB-FERTILITY

In addition to endocrine and genetic focuses on cattle sub-fertility, a nutritional approach also offers considerable potential in addressing the problem of sub-fertility in cattle. High-yielding dairy cows are selected for their ability to partition energy into milk production at the expense of body fat early in lactation, and a cow's body condition score during lactation is heritable (Coffey, 1998; Jones *et al.*, 1999). As a result dairy cows enter a state of negative energy balance at a time when they are expected to return to ovarian cyclicity. The normal development of the ovarian follicle involves the production of both local and systemic growth factors, in particular insulin-like growth factor I (IGF-I) (Guidice, 1992; Jones & Clemmons, 1995). Hepatic IGF-I production (which determines systemic levels of IGF-I) is dependant on growth hormone (GH). However systemic IGF-I levels fall under these conditions, although GH levels are elevated (Van Eederburg *et al.*, 1996) because under-nutrition or a state of negative energy balance lowers insulin secretion - which reduces hepatic growth hormone receptor levels (Pell & Bates, 1990). The local availability of IGF also declines, through its effect on the levels of the IGF binding proteins (IGFBPs). In the bovine follicle, as it approaches ovulation, the expression of the gene for one of these IGFBPs (IGFBP-2) decreases, allowing IGF to exert its effect by increasing the granulosa cell growth (Armstrong *et al.*, 1998). The expression of IGFBP-2 is reduced by insulin, so a high circulating level of insulin effectively increases the availability of IGF within the follicle and a low insulin level decreases the availability. As a result of these systemic and local relationships between metabolic hormones and growth factors, milk yield compromises follicular growth (Webb *et al.*, 1999). This is exhibited in the positive relationship between the change of body condition score during early lactation and CLA (Canfield & Butler, 1990; Beam & Butler, 1997). Regression analyses show that CLA is unfavourably correlated to milk, fat and protein yields, whereas energy balance, live weight during lactation and weight gain all have a favourable genetic correlation with CLA (range of $r = -0.40$ to -0.80) (Veerkamp *et al.*, 2000).

Changes in the systemic IGF-I levels may alter the function of the uterine IGF system in addition to its effects on the ovary. The endometrium produces IGF-II during early pregnancy in ruminants, but IGF-I in the uterus is probably derived from the circulation. The endometrial glands express IGF receptors during pregnancy, so IGF-I in the uterus may affect the uterine glandular secretion of embryotrophic factors. IGFBP's are also expressed in the uterus and the expression of at least one of these proteins (IGFBP-3) is reduced in the bovine uterus in the presence of a conceptus. The operation of the IGF system in both the ovary and the uterus therefore points to the benefits of maintaining high IGF-I levels. If a nutritional regime could be formulated that would maintain high insulin levels at this critical time of lactation, thereby maintaining the hepatic expression of the growth hormone receptor and IGF-I secretion, the adverse effects of negative energy balance on fertility may be overcome (Wathes *et al.*, 1998).

A second relationship between diet and fertility involves that of circulating concentrations of ammonia and/ or urea in the dairy cow. The rapid digestible diets fed for maximum milk yield generally contain high levels of rumen degradable protein and this results in elevated conversion of dietary protein into ammonia and urea. In some cases ammonia is produced at rates exceeding the hepatic clearance ability for urea. As a result, these diets can lead to elevated circulating levels of ammonia and urea, which have deleterious effects on the follicle, the embryo and the uterus. One approach to improving fertility is therefore to provide a diet that raises insulin levels and lowers ammonia/ urea concentrations during a critical period of follicular development. However, as follicle development from the preantral stage to ovulation takes approximately 4 months, it is not certain at present when such a diet should be fed, without compromising milk yield. Further research is essential in this area (Butler *et al.*, 1996; McEvoy *et al.*, 1997; Sinclair *et al.*, 1999).

Trials with high milk producing (top 5% UK herds) and low producing (average for UK herds) dairy cows, show that in both groups, animals are lactating to about 75% of their metabolic capacity (Knight, 1999). This means that, if

cows are given experimental galactopoietic stimuli, such as frequent milking, recombinant bovine somatotropin (BST) and thyroxin, cows with both high and low genetic milk production merit lines will increase their yield by 25%. However, when stimulated in this way, ovarian activity (and in fact all follicular development) ceases and milk production soon drops (Webb *et al.*, 1999). It would therefore seem that within the 25% "unused" metabolic capacity, there is a compromise between reproduction and milk yield. One aim of dairy nutrition research should be to investigate how to maintain milk yield without compromising fertility. Alternatively, it must be accepted that a high peak milk yield is not always compatible with a short intercalving interval.

2.3.1 Energy balance and body condition loss

Interrelationships between energy balance and post partum reproductive function in dairy cows have been extensively researched (Butler & Smith, 1989). The present review focuses more on the recent literature. The energy requirements of a lactating cow are met via a combination of dietary intake and the mobilisation of body reserves. High yielding dairy cows cannot maintain a positive dietary energy balance (consume enough energy to satisfy maintenance and production requirements) during early lactation and must therefore mobilise body reserves. Butler and Smith (1989) reported a negative energy balance usually reaches its maximum during week 1 to 2 of lactation, and then recovers at a variable rate. A direct relationship ($r = -0.80$) has been recorded between milk yield and energy balance. It was further determined that the correlation between milk yield and days to first ovulation only become significant after a lactation period of 40 days, the period when most cows have already ovulated. It is suggested that factors other than milk yield alone are involved in determining the interval to first ovulation. Butler and Smith (1989) concluded that the effects of high milk yield on conception rates were caused by a delayed resumption in ovarian activity, thereby limiting the number of estrous cycles before breeding, with subsequent reduced conception rates (Reimers *et al.*, 1990).

Inclusion of fats in dairy diets can increase the calorie concentration, without reducing fibre content - thus increasing energy intake and efficiency of energy

utilisation, especially during early lactation, when cows fail to consume sufficient feed. Few studies have reported the use of fats in dairy diets to enhance reproductive performance. Ferguson *et al.* (1993), reported no differences in days to first service between cows supplemented with fat and control cows. However, cows receiving a fat supplementation had a higher conception rate at first service (59.1 vs. 42.6%) and higher conception rates for all services (59.3 vs. 40.7%). In a more recent study, cows supplemented with calcium salts commenced ovarian cyclicity later than controls. However, once ovarian cyclicity commenced, fat supplemented cows had more frequent normal cycle lengths (18 to 26 days) compared to the controls. Serum progesterone concentrations were higher for supplemented cows in the luteal phase prior to service and were higher 9 and 24 days after service in cows that conceived. For these cows conception rates were not different at first service, but were higher in cows fat supplemented for the second to forth services ($P<0.05$) by 34 days. Milk yield responses to fat supplementation appeared to be variable across herds, but fat included in diets of high yielding cows enhanced conception rates and reduced the number of days open. Higher serum progesterone concentrations accompanied the increased reproductive performance (Ferguson *et al.*, 1993).

In the past the change in body condition of cows during early lactation has been used to indicate how much body reserves is mobilised to compensate for milk yield. Changes in body condition have been useful similar to body weight (BW) changes, because BW changes are influenced by factors such as feed intake, growth rate and frame size. Albright and Avare (1997) proposed a body condition scoring system independent of frame size, age and DMI and which has proved to be useful for monitoring the depletion and repletion of body tissue reserves in dairy cows.

2.3.2 Hormonal Influences on fertility rate in dairy cows

When the incidence of infectious pathology is omitted, the apparent fertility rate in the dairy cow lies between 70 and 80% - when based on the EPD value of milk progesterone concentration 3 weeks after AI, but this is much lower (approximately 50%) than when based on actual calving rates. It is now

clear that this variation results essentially from early embryonic death and not from the failure of fertilisation. Three quarters of unsuccessful pregnancies fail during the first 18 days following breeding. Furthermore, the majority of term failures occur at, or before, the time of embryo hatching in the uterus. This corresponds with day 9 or in the middle of the luteal phase of the estrous cycle (Berger *et al.*, 1981; Barnes *et al.*,1985).

Numerous attempts have been made to compensate for these embryonic losses. Hormonal manipulation of the sexual cycle prior to AI has been attempted by several researchers. Repeat breeding cows have for example been treated with GnRH or an agonist in the mid-luteal phase, before AI, with success (Holtz *et al.*, 1986; Antal *et al.*, 1987). However, results from experiments have been variable, especially in animals with no history of reproductive disorders. This would suggest that such modes of treatment, prior to ovulation, may not be advisable for inhibiting embryonic mortality, especially in normally cycling females prior to AI (Antal *et al.*, 1987; Butterfield *et al.*, 1988).

2.3.3 Relationship between the concentration of progesterone in serum and milk

A clear relationship exists between the concentration of progesterone in serum and milk. The measurement of progesterone in blood and milk at various stages in the estrous cycle is an accurate indicator of the reproductive status of the animal (Laing & Heap, 1971; Rajamahendran *et al.*, 1976; Bulman & Lamming, 1978). Milk progesterone determination has many potential applications, for example to detect or confirm oestrus (Shemesh *et al.*, 1978), to diagnose pregnancy (Robertson & Sarda, 1971), monitor post partum ovarian activity (King *et al.*, 1978), early embryonic mortality (Cox *et al.*, 1978) and ovarian disorders (Lamming & Bulman, 1976).

2.3.4 Occurrence of progesterone in bovine milk

Progesterone concentration in milk is similar to the concentration of the hormone in blood during the oestrous cycle and pregnancy. Due to the non-invasive nature and simplicity of sample collection, as well as the availability

of suitable methodology, i.e. radio- and enzyme immunoassay, the determination of progesterone in milk has become an important tool to study the reproductive endocrinology and for improving reproduction in dairy cows (Hoffman *et al.*, 1976).

Due to its liposolubility, progesterone accumulates in the lipid fraction of milk (Heap *et al.*, 1976). Therefore, variability in milk progesterone concentration can be explained by fluctuations in the fat content of milk. The fat content of milk is in turn affected by many factors e.g. the intervals between milking, the completeness of milking, the time of milking during the day, the time at which the milk sample is collected during the milking session and the quarter being milked (Rosenberg *et al.*, 1990). Generally, it has been suggested that the determination of progesterone in defatted milk or milk fat will eliminate the variation caused by varying milk fat concentrations and thus eliminate the effect of the time of sampling (Rhodes *et al.*, 1995). Nevertheless, in some studies, skim milk samples of the foremilk were recorded to show lower levels, compared with progesterone values in samples collected at later stages of milking (Etherington *et al.*, 1996). Partition studies with ^{3}H-progesterone and milk fat measurements revealed that the quantity of progesterone in the aqueous phase of milk was negatively correlated with the fat concentration of the milk sample. However, this finding does not explain the low progesterone concentrations recorded in defatted foremilk samples, as milk fat concentrations at this stage of sampling are generally low (Staples *et al.*, 1990).

Milk is secreted continuously by the mammary secretory cells, but removed only periodically (once, twice or thrice a day). Between periods of milk removal, milk storage occurs in two interconnected compartments within the udder. The cisternal compartment consists of the space occupied by the teat canal, gland cistern and the major milk ducts. The alveolar storage compartment consists of small ducts and alveolar lumen. Cisternal milk is considered to be milk that can be removed from the udder by overcoming the teat sphincter barrier. The alveolar milk on the other hand, is the milk that subsequently remains in the udder and requires oxcytocin-induced contraction

of the mammary alveoli for evacuation of the gland via the cisternal space. Filling of the cisternal compartment starts shortly after milking. Consequently milk samples collected before milking and between milkings correspond to the milk obtained from the gland cistern. Milk samples collected at approximately the middle of the milking session and immediately after milking are subsequently derived from the alveoli. Composite milk thus comprises of a mixture of milk from cisternal and alveolar origin. The effect of storage of milk in these two main compartments of the mammary gland on milk progesterone concentration has not been previously studied (Gatica *et al.*, 1996).

The very distinct cluster of points corresponding to the milk fractions collected prior to and after application of the milking machine and between milking suggest that the milk composition and milk progesterone concentrations are affected by the place of storage of milk in the mammary gland. The determination of progesterone in skimmed milk and in milk fat confirmed this finding. Etherington *et al.* (1996) recorded progesterone levels in defatted foremilk to have lower levels than the values in samples collected at later stages of milking. Results also recorded that progesterone concentrations in fat-free milk were lower in samples before suckling, compared to samples collected after suckling. In other studies defatted, foremilk progesterone concentrations did not differ from those in samples collected at later stages of milking (Peters, 1984; Macmillan *et al.*, 1989). In light of current findings, the reason for the reported discrepancies in defatted milk progesterone concentrations can be explained by the origin of the milk sample in relation to storage of milk in the mammary gland. A milk sample which is obtained immediately before milking can be derived either from the gland cistern or be a mixture of cisternal and alveolar milk - depending on whether milk let-down has occurred before the sample is collected. In a study performed by Macmillan *et al.* (1989), the udder and teats were washed prior to sampling. This could have stimulated the milk ejection reflex and caused filling of the gland cistern with alveolar milk. Thus the samples would no longer have been foremilk samples of cisternal origin. This milk would have contained the same amount of progesterone as for samples collected after milking, or in whole milk collected from the bucket.

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Another hypothesis to explain discrepancies regarding skim milk progesterone concentrations was proposed in partition studies with ^{3}H-progesterone and milk fat measurements. Staples *et al.* (1990) recorded the amount of progesterone in the aqueous phase of milk to be inversely related to the percentage of milk fat and the incubation temperature of whole milk prior to centrifugation. Based on this finding, it was concluded that the decrease in skim milk progesterone concentrations found in after-milk strippings, compared with those in samples obtained during mid-milking, were caused by an increase in milk fat concentration. Skimmed milk progesterone concentrations tended to decline with the last fractions of alveolar milk. However, the pattern of change in skim milk progesterone concentrations followed the same trend as that shown by milk fat progesterone concentrations.

2.3.5 Effect of nutrition on endocrine parameters, ovarian physiology, oocyte and embryo development

Genetic improvement in dairy cows has led to a substantial increase in milk yield, which has been associated with a decrease in reproductive performance (Assey *et al.*, 1994). Reduced fertility is particularly evident in cows where milk yields are above 6 000kg per lactation (Nebel & McGilliard, 1993; Macmillan *et al.*, 1996) and in cows fed in excess during the previous dry period (Kruip *et al.*, 1998). Early embryo mortality is a significant cause or reproductive failure in ruminants and part of this may be related to nutritional influences around the time of mating (Dunne *et al.*, 1999). Although the final manifestation of a detrimental affect of nutrition on fertility may be the death of the embryo, it is not clear if nutrition affects embryo quality by changing the follicular environment, the developmental capacity of the oocyte itself or through changes occurring during early embryo development.

Nutrition can influence the reproductive functions in ruminants. However, the relationship between nutrition and reproduction is complex and responses are often variable and inconsistent. In the case of the lactating dairy cow, inadequate nutrition in the short term, or as a consequence of a prolonged

depletion of body reserves during early lactation, can have significant deleterious effects on the resumption of post partum ovarian activity, conception rates and infertility. Deleterious effects following excessive nutrition, around the time of mating on embryo development, are becoming evident both in non-superovulated (Dunne *et al.*, 1997) and in superovulated cattle (Mantovani *et al.*, 1993; Yaakub *et al.*, 1996; Nolan *et al.*, 1999).

2.3.6 Effect of dietary urea on fertility

High dietary protein, resulting in high plasma concentrations of urea nitrogen and in milk (>190 mg/l) has been associated with decreased fertility in dairy cattle (Jordan *et al.*, 1983; Elrod & Butler, 1993; Butler *et al.*, 1996). One suggestion is that this decrease is due to an altered uterine environment (Elrod & Butler, 1993). In sheep, Fahey *et al.* (1998) reported that despite high dietary urea and blood urea concentrations, no effect of dietary urea on ovulation rate was recorded in donor or recipient ewes . However, embryo quality in donors was reduced, as fewer embryos of more than 8 cells were recovered at day 4 from urea-treated ewes. The diet offered to recipients had no effect on embryo survival. Thus, it was suggested that the effects of urea on embryo quality are likely to be due to alterations in the oviduct environment or deleterious changes in the follicle, rather than changes in the uterine environment. This has been supported by recent data showing no difference in pregnancy rates at day 35 after transfer of good quality in-vitro produced embryos to recipients on different levels of dietary urea (Garth *et al.*, 1999).

Another manifestation due to excess urea in the circulation, is the birth of abnormally large offspring (Young *et al.*, 1998). Ewes fed excess amounts of urea from 21 days before mating to day 63 of gestation recorded oversized lambs at birth (McEvoy *et al.*, 1997). This effect occurred via embryonic exposure to high concentrations ammonia in the reproductive tract. Including these effects, in-vivo links have also been established between high levels of ammonia and poor embryo development in-vitro in some culture systems (Gardner & Lane, 1993).

The result of these and other studies indicate that the determination of plasma urea concentration around the time of insemination or embryo transfer is of little value in predicting subsequent fertility (O'Callaghan *et al.*, 2000a). Adequate energy intake, in association with dietary crude protein intake may be critical and is an area that requires further investigation. These studies suggest that the deleterious effects of increased plasma urea identified are likely to occur at the level of the oocyte within the follicle. It is suggested that this results in the development of poor quality embryos - rather than urea being primarily responsible for disruptions in the uterine environment. This highlights the importance of nutrition in the pre-ovulation period (possibly many weeks) on the fertility performance (O'Callaghan *et al.*, 2000a).

2.3.7 Nutrition and gonadotropin secretion

Energy status is generally considered to be the major nutritional factor that influences the reproductive processes - with prolonged low energy intake impairing fertility. In sheep, poor nutrition, which results in lower ovulation rates, is associated with a decreased LH pulse frequency, which is likely due to inadequate hypothalamic GnRH secretion (Rhind *et al.*, 1989a). In cattle, a strong correlation between negative energy balance in early lactation and the resumption of ovulation post partum is evident (Canfield & Butler, 1990). While ovulation may not occur in animals with low feed intake, follicle growth and atresia will occur. Such follicular wave turnover without ovulation, is often evident in post partum beef cows in a poor body condition (Stagg *et al.*, 1995). The practical significance of this occurrence is a lengthening of the calving to first ovulation interval, and often an extension in the calving to conception interval. Long term restriction in feed intake has been show to induce anoestrus in cattle, due to insufficient quantities of circulating LH. These effects, are however, not immediately evident and dietary restriction of several months may be required to prevent follicle growth and ovulation (O'Callaghan *et al.*, 2000b).

In contrast to the situation in monogastrics, the effect of short term dietary restriction on LH pulse patterns are more difficult to observe in ruminants. In ewes, restricted dietary intake resulted in no change in LH secretion (Abecia

et al., 1995) or a relatively low reduction in LH pulse frequency when diets were restricted for approximately 3 weeks (Rhind *et al.*, 1989a). FSH is essential for follicle growth and ovulation, yet there is little evidence of the effect of nutrition on plasma FSH concentrations (Findlay & Clarke, 1987). Mackey *et al.* (1997) however, demonstrated that a short term restriction in dietary intake to approximately 40% of maintenance energy requirements, increased serum FSH in heifers, compared to those offered diets of twice the maintenance energy requirements. This trend was repeated in ovariectomized heifers on similar diets, suggesting that the effects are mediated at least, in part, by changes at the level of the pituitary and not solely due to an alteration in the steroid feedback. Thus, nutritional effects on gonadotropin secretion in cattle are relatively minor, unless dietary restrictions persist for extended periods of time.

2.3.8 Nutrition and serum progesterone concentrations

Feed intake in cows can influence the concentration of progesterone, with a strong negative correlation between dietary intake and serum progesterone concentrations (McEvoy *et al.*, 1995; Rhind *et al.*, 1989b). This effect of feed intake on circulating progesterone levels may be due to an increase in the rate of catabolism of progesterone and in hepatic circulation at higher feeding levels (Parr *et al.*, 1993). Serum progesterone, due to its negative feedback, can affect the LH pulse frequency and is also thought to play an important role in the oocyte maturation and early embryo development (McCann & Hansel, 1986; Kleemann *et al.*, 1994).

Feeding sheep ad lib consistently reduces the serum progesterone concentration, compared to restricted feeding, but the results obtained in cattle indicate that this effect to be more variable (O'Challaghan *et al.*, 2000c). Ad lib feeding in heifers increased (McEvoy *et al.*, 1997), decreased (Villa-Godoy *et al.*, 1990) or had no effect (Spitzer *et al.*, 1978) on serum progesterone concentrations when compared to restricted feeding. Low serum progesterone levels post breeding, can thus reduce fertility (Larson *et al.*, 1997). However, as steroids are selectively stored in fat, any dietary regimen that results in fat mobilisation will result in the release of stored

progesterone. This may account for some of the increased serum progesterone levels evident in animals on low dietary intakes. Recently, serum progesterone concentrations and embryonic interferon-tau have been positively correlated (Mann *et al.*, 1996). Thus, minor changes in maternal progesterone concentration during the initial period of embryo development may alter the secretion of this anti-luteolytic agent and may be critical to embryo survival.

In sheep, overfeeding caused a reduction in circulating serum progesterone concentrations and also decreased pregnancy rates (Parr *et al.*, 1987) and also decreased both the rate of development and viability of the embryos (Creed *et al.*, 1994). In cattle, Mann *et al.* (1998) claimed that the timing of the serum progesterone increase after ovulation is of importance to the development of the embryo. These researchers also recorded a delayed rise in progesterone level to be associated with smaller and potentially less viable embryos on day 16 after mating. A more recent trial in beef heifers aiming to show the detrimental effect of an acute reduction in energy intake immediately after insemination on embryo survival, failed to show a relationship between serum progesterone concentrations early in the oestrous cycle and embryo survival (Dunne *et al.*, 1999). Peripheral progesterone concentrations on day 0 and 1 after the LH peak are important for embryo survival in cows (Ashworth *et al.*, 1989). This progesterone level presumably modifies follicular maturation and oocyte quality. Others have suggested that the effect of progesterone on embryo development acts primarily through the effect of progesterone on the uterus (Abecia *et al.*, 1996; Lozano *et al.*, 1998). Increased concentrations of progesterone during the luteal phase before and after breeding have been associated with higher pregnancy rates (Butler *et al.*, 1996). Most of these experimental results are based on the evaluation of peripheral progesterone concentrations. However, when studying the effect of nutrition on peripheral (jugular) and local (ovarian vein and endometrium) progesterone concentration trials could not demonstrate any relationship between these measurements (Abecia *et al.*, 1997; Lozano *et al.*, 1998). Thus, the use of jugular vein progesterone concentration alone as an indicator of the effect of nutrition on embryo development must be employed with

caution, as embryo survival may be more related to concentrations of progesterone in the ovarian vein and the endometrium, than circulating serum progesterone concentrations as such.

2.3.9 Effect of nutrition on ovarian function

The ability of nutrition to alter the ovulation rate and calving rate in cows is well documented, with a rapid improvement in body condition usually being associated with an increased ovulation rate (Coop, 1966). Alterations in ovulation rate may be related to the entry rate of glucose into the cells of animals on a high plane of nutrition. Dietary supplements containing high energy and protein components have been shown to increase the ovulation rate in cows (Downing *et al.*, 1995a). Similarly, increases in ovulation rate were reported when glucose was infused directly into the vascular system (Downing *et al.*, 1995b; Williams *et al.*, 1997). Thus, it is likely that a short term energy supply is directly related to follicle recruitment and perhaps also to follicle growth. However, this effect may be of short duration when the diet level is altered (Gutierrez *et al.*, 1997).

Dietary restrictions have been shown to alter follicular growth characteristics in cattle (Murphy *et al.*, 1991) and in superovulated cows (Yaakub *et al.*, 1997). Murphy *et al.* (1991) reported that heifers on a restricted energy dietary intake have a reduced size and persistence of the dominant follicle, compared to animals offered higher intakes. Acute nutritional restriction (0.4 of maintenance) for about 12 days decreases the growth rate and maximum diameter of the dominant follicle and induced failure of the dominant follicle to ovulate after induced luteolysis with prostaglandin (Mackey *et al.*, 1999). Several studies have shown that feeding fat altered the growth pattern of the ovarian follicles and this effect is somewhat independent of the energy intake as such (Mattos *et al.*, 2000). Supplemental fat in the diet increased the number of follicles (Lucy *et al.*, 1991; Beam & Butler, 1997) and increased the size of the preovulatory follicle (Lucy *et al.*, 1990). This increased follicle size may have beneficial effects on both oocyte quality (Lonergan *et al.*, 1994) and on corpus luteum function, resulting ultimately in higher pregnancy rates (Mattos *et al.*, 2000).

In the case of superovulated heifers, Nolan *et al.* (1999) reported an increase in follicle numbers after stimulation with exogenous FSH, in heifers offered a low dietary intake, compared to heifers on a high dietary intake. This difference in response was predominantly due to an increase in the number of follicles with a size of 7 to 10 mm, when measured around the time of the LH surge. However, this trend was not repeated when ovulation rates were recorded after superovulation. In the case of ewes superovlated with FSH, a lower ovulation rate was recorded in ewes offered diets half of the maintenance energy requirements, compared to ewes offered diets of twice the maintenance energy requirements. In a later trial, the ovulation rates of ewes superovulated on similar diets, but using different gonadotrophin preparations were not different. Thus, it is clear that dietary intake can, under certain conditions, alter the growth characteristics of follicles. However, the effect of dietary intake on the number of follicles growing in response to stimulation with a fixed dose of FSH during superovulation is less consistent (Rieger *et al.*, 1992).

2.4 THE EFFECT OF STAGE OF LACTATION ON DAIRY COW FERTILITY

Lactational effects are associated mainly with the reproductive performance of genetically superior cows – i.e. cows that are being bred during peak lactation in order to maintain a 365-day calving interval. Higher producing cows (±40 liter/cow/day) will partition ingested energy and mobilise body reserves to meet the demands of the increasing milk production. The diet available may be pasture of varying quality and composition, or a concentrate diet that is not specifically balanced for the cow's requirements. Daily milk yield increases after calving, more rapidly than the cow's appetite. Rapid mobilisation of body fat reserves, reflected by the loss of body condition score after calving, may further depress body appetite and increase the difference between energy intake and production output. These effects contribute to reduced fertility in some high-producing herds of genetically superior cows (Esslemont & Peeler, 1993).

Fertility is commonly lower in lactating cows, compared to younger, non-parous heifers. The difference may vary according to the standard of heifer rearing, as well as the level of milk production at peak lactation in the cows. So for example, well reared American Holstein heifers mated at an average body weight of 350kg have pregnancy rates following first insemination (percentage of heifers pregnant to the first insemination/ heifers inseminated) of over 70% (Plaizier, 1993). Whereas Friesian heifers reared in New Zealand under conditions of extensive grazing and mated at about 250kg recorded pregnancy rates which were 10% - 15% lower (Macmillan & Asher, 1990). These results obtained in lactating cows are reversed, and differing responses in different production systems suggest that other potentially confounding factors, such as age and post parturient health, are probably not dominant factors influencing fertility. Pregnancy rates to first insemination for cows in New Zealand herds average 60% and 65% (MacMillan & Day, 1982), whereas the average calving rates in American herds are 20% lower (Nebel & McGilliard, 1993). Results obtained for dairy cattle in herds in Great Britain (Esslemont & Peeler, 1993) and Australia (Cavestany *et al.*, 1990) are between these extremes for heifers and cows, respectively.

The lower pregnancy rate at first insemination recorded in heifers in New Zealand may reflect the effects of under-nutrition, especially during the first winter, when heifers are 10 to 12 months of age. Well managed Friesian heifers reared solely on pastures can reach puberty before 12 months of age and average 350kg at a breeding age of 15 months. Some contemporary animals subjected to restricted feeding and only averaging 250kg at 15 months, had not reached puberty at this age (Hansen, 1997). Calving patterns showing a failure of heifers to calve as two-year-olds and extended calving to conception intervals in two-year-old lactating heifers and the low body weights of New Zealand heifers at mating and calving, suggests that under-nutrition delays puberty and affects the reproductive performance of heifers in some New Zealand herds (Macmillan & Asher, 1990).

Levels of nutrition that restrict peak milk yield and extend the period of post partum anoestrus do not prevent New Zealand cows from achieving

comparatively high pregnancy rates of 60% to 65% at the first insemination - especially when compared with the results obtained with American Holsteins of only 45% (Macmillan & Asher, 1990; Nebel & MacGilliard, 1993). The difference in pregnancy rates for cows and heifers in American herds (45% vs 70%) suggests that the American Holstein in inherently fertile, but has its reproductive performance significantly compromised by comparatively high levels of milk production in early lactation (Nebel & MacGilliard, 1993; Plaizier, 1993).

This general perception that the negative relationship that exists between the levels of milk production and the reproductive performance is confirmed by comparative figures for Holstein or Friesian cows in the USA and New Zealand, as well as Great Britain and Australia. This information is summarised in Table 2.1 and does not take into account differences in the efficiency of oestrous detection and errors in detection, or aspects of insemination practices and semen processing. Oestrous detection rates reflect the efficiency and the sensitivity of oestrous detection. The results presented in Table 2.1 were not obtained from randomly selected herds, but are probably a fair reflection of the differences obtained in the different production systems. Differences in average herd size and calving pattern need to be considered in these comparisons. Larger herds generally have lower fertility and seasonal calving patterns favour a greater sensitivity in oestrous detection, because there will be a greater prevalence of cows in oestrus allowing greater behavioural interaction.

Oestrous detection rates in American herds (40 to 45%) are similar to the pregnancy rates (Nebel & McGilliard, 1993). Comparable estimates of oestrous detection rates were obtained in Great Britain, Australia and New Zealand with 50% (Esslemont & Peeler, 1993), 70 to 80% (Larcombe, 2002 - personal communication) and 90% (Macmillan & Asher, 1990), respectively. These differences in detection rates may have a greater impact on the intercalving intervals and embryonic survival rates, compared to differences in pregnancy rates (Table 2.1). So for example, the probability of conception to a single oestrus/ ovulation sequence for cows in American herds is estimated

to be about 20% (i.e. 0.45 x 0.45), compared to 54% for cows in New Zealand herds (i.e. 0.90 x 0.60). It is not clear, however, whether the specificity or sensitivity of oestrous detection is affected by increased milk production. Medina *et al.* (1994) recorded the expression of oestrus to be less obvious in Holstein than in Jersey cattle, but high producing cows did not express signs of oestrus differently to lower milk producers. If the predictive value of oestrous detection, that is, the proportion of the cows that are truly in oestrus out of those which the herdsman determines to be in oestrus, is the same for higher producers and lower producers - then the lower conception rates recorded for the higher producing dairy cows would have resulted from lower fertility and not from miss-diagnosis of oestrus. Further, differences in sensitivity of heat detection between higher and lower producers would be reflected in increases in the calving to conception interval, but not in first-service pregnancy rates.

Table 2.1 Relationship between milk yield and conception rates in some American dairy herds

Milk yield range (kg)	Conception rate (%)	
	Study 1 (n = 5461)	Study 2 (n = 141 000)
<5900	47	49
<5900 – 6800	43	45
<6800 – 7730	39	41
<7730 – 8640	38	39
>8640	30	38

From: Mahaputra *et al.* (1990)

Table 2.2 Milk production and selected reproductive characteristics in two groups of cows with low (n = 9) or high milk yield (n = 10)

	Group	
Reproductive characteristics	**Low**	**High**
Milk yield in the first 75 day of lactation (kg/cow)	2043	2438
Interval from calving to uterine involution (days)	27	24
Interval from calving to 1st ovulation (days)	29	31
Interval from calving to 1st luteal phase (days)	14	11
Interval from calving to 1st detected oestrus (days)	43	66
Interval from calving to conception (days)	74	217

From: Rajamahendran *et al.* (1993)

CHAPTER 3

PROTOCOL FOR THE COLLECTION AND PROCESSING OF SAMPLES FOR MILK AND PLASMA PROGESTERONE AND CERTAIN MILK COMPONENT ASSAYS

3.1 INTRODUCTION

In order to ensure the sustained use of radioimmunoassay (RIA) technology for milk or plasma progesterone determinations, a greater degree of self-reliance in the preparation of the RIA components, is considered important. Thus, the IAEA self-coating RIA method for progesterone determinations in plasma (PScRIA) was developed whereby bulk quantities of progesterone antibody was supplied by the IAEA Laboratories, Vienna and the remaining materials (^{125}I labelled progesterone, tubes, buffers, standards and IQC) - either purchased directly or prepared by the end user. However, in the initial phase of transferring this technology to selected laboratories in developing countries, all required material was supplied by IAEA. This protocol enables counterparts to perform the antibody self-coating procedure and the assay for plasma and milk progesterone. It also verifies the validation of the RIA procedure for progesterone determination.

The anti-progesterone monoclonal antibody (6H11/14) used in the progesterone assay used in this study, was developed in collaboration with the Agricultural Biotechnology Centre, Gödöllo, Hungary. The antibody has a high level of specificity for the 11-∝-hydroxyprogesterone-hemisuccinate analogue of progesterone. Substitution of the antibody preparation or any other specified reagent will potentially alter the performance characteristics of this assay. If substitution is attempted, the user should evaluate the assay performance characteristics against established progesterone standards.

3.2 MATERIALS AND METHODS

Milk or plasma sampling and sample processing has a major influence on the progesterone concentrations recorded in the milk or plasma. In order to

achieve reliable results, it is of the utmost importance to apply as consistent and repeatable a sampling technique as possible.

3.3 MILK PROGESTERONE DETERMINATION

(i) Milk samples (10 ml) from the same milking stage were used, i.e. strippings for day 0, composite milk sample of days 10 to 12 and days 22 to 24.

(ii) Preservative was added to whole milk [1 sodium-azide tablet (100mg) or 1 dichromate tablet (100mg) per 10ml of milk].

(iii) Milk was centrifuged at 700G for 10 minutes (for skimming of fat) at the same temperature (4°C) and at each sampling. Centrifugation was performed as soon as possible after sample collection.

(iv) Skim milk was aspirated as follows:

a) Milk was centrifuged and placed in a refrigerator (4°C) for 15 minutes to harden the fat layer.

b) A glass rod was used to pierce the fat layer.

c) The entire skim milk sample was transferred to a storage vial, using a Pasteur pipette.

Skim milk samples with preservative had the following storage capabilities:

- Room temperature: Stable at 37°C for 1-2 weeks
- 4°C: Stable for at least 3 months.
- Deep frozen: Indefinitely

3.3.1 Chemical reagents and materials

- Monoclonal antibody 6H11/14; 0.25ml vial of 1:10 dilution, lyophilised (Stored at –20°C).
- Radioactive tracer: progesterone – 11-α-hemisuccinate-2- [^{125}I] iodohistamine (10 µl); (Amersham: #IM-139) ratio of methanol:water (9:1).

- Milk standards – lyophilised skim-milk standards with progesterone concentrations of 0, 1.25, 2.5, 5, 10, 20 and 40 nM (nmol/l) (Stored at 4°C).
- Carbonate/bicarbonate tablets (coating buffer; EC Diagnostics, Sweden; #LD8922) (Stored dry at room temperature).
- Phosphate buffered saline (PBS) tablets (diluent buffer; EC Diagnostics Sweden; #LD9402) (Stored dry at room temperature).
- Bovine serum albumin (BSA), RIA grade (Sigma; #A9647) (Stored at 4°C).
- Tween 20 (Sigma; #P-1379) (Stored at room temperature).
- Sterile filtered distilled water/ glycerol (50:50) solution, for reconstituting antibody (Stored at room temperature).
- Nanopure water for reconstitution of standards (Stored at room temperature).
- Nunc 1 ml cryovials (Nunc; #366656), for storage of the antibody stock solution. (Stored dry at room temperature).

3.3.2 Preparation of reagents and samples

i) Coating buffer

70 ml distilled/de-ionised water was placed in a 100 ml volumetric flask, to which one carbonate/bicarbonate tablet was added and shaken until completely dissolved. Distilled water was added to the 100 ml mark (0.05M carbonate buffer with a pH 9.6 ± 0.05).

ii) Diluent buffer (PBS)

500 ml distilled/de-ionised water was placed in a beaker to which one PBS tablet (0.14M NaCl, 3mM KCl) was added and stirred until completely dissolved. The solution was then transferred to a 1 l volumetric flask and filled with distilled water. This represented a 0.01 M phosphate buffered saline solution with a pH of 7.4 ± 0.2.

iii) Antibody stock solution

One vial of the lyophilised monoclonal antibody was reconstituted with 0.25 ml of the supplied diluent of sterile filtered distilled water/glycerol to obtain the antibody stock solution (1:10 dilution). This solution was aliquoted in 25 µl volumes into 1 ml Nunc cryovials.

iv) Milk standards

Lyophilised milk standards were reconstituted with 1 ml distilled water added to each vial.

v) Washing solution

1 ml Tween 20, was mixed with 1 l distilled water to obtain a 0.1% washing solution.

3.3.3 Coating and assay procedures

i) Label assay tubes

Label Nunc "star" tubes were used in duplicate for Total counts (TC), Zero binding (B0), Standards and quality control (QC).

ii) Preparation of antibody coating solution

25 µl (one aliquot) of the antibody stock solution was transferred to a 50ml volumetric flask. Complete transfer of the antibody was ensured by repeated rinsing of the aliquot tube with the coating buffer (0.05 M carbonate buffer). This gave an antibody coating solution dilution of 1:20000.

iii) Coat tubes

300 µl Antibody coating solution was dispensed into each tube, except for the total count (TC) tubes. Tubes were covered with parafilm and incubated overnight (12 hours at 4°C).

iv) Radioactive tracer working solution

33 mg BSA (bovine serum albumin) was transferred into a glass beaker with 33 ml diluent buffer (PBS). 20 µl of the stock tracer solution (^{125}I-progesterone) was added and mixed well to obtain the tracer working solution. 200 µl of the tracer working solution would give a count of 2500 – 30000 cpm in the gamma counter.

v) Assay Set up

All working solutions were allowed to reach room temperature before setting up the milk assay. Progesterone samples were also gently mixed before sampling.

40 µl of the standard QC solution was added to respective antibody coated tubes. It was compulsory that the pipette tip should touch a vane at the bottom of the star tube to guarantee complete expulsion. Using a repeater pipette, 200 µl of tracer working solution was added to each tube (including the TC). Tubes were covered with parafilm and incubated overnight (at least 12 hours) at 4°C.

After incubation the TC tubes were removed from the rack and decanted. All remaining tubes were decanted into an appropriate radio-active disposal container. 500 µl washing solution was added to each tube (except the TC tubes) and decanted to remove the solution. The tubes were then rinsed a second time with 500 µl washing solution to ensure complete milk progesterone removal.

vi) Radio-activity measure and milk progesterone determinations

The radio-activity of each tube (including TC) was counted in a gamma counter for a fixed time of 60 seconds. The maximum percentage binding in the assay (Bmax) was determined by dividing the average counts per minute (cpm) of the two zero bindings (B0) by the average cpm of the 2 TC tubes and multiplied by 100, e.g.

$$Bmax = \frac{\text{Average cpm of B0}}{\text{Average cpm of TC}} \times 100$$

The percentage binding was then calculated (%B/B0) for all the standards, samples and quality control tubes by dividing the cpm of each tube with that of the B0 tubes and multiplying by 100.

$$\frac{B}{B0} = \frac{\text{Average cpm of standard/sample/QC} \times 100}{\text{Average cpm of B0}}$$

A logit – log graph paper provided with the kits was used to plot the percentage bound (B/ B0) on the vertical (y) axis and concentration of milk progesterone standards on the horizontal (x) axis. A straight line was drawn through these points of the graph.

vii) Quality control of assay

The accuracy of an assay was defined as "a measure of variation in estimating a sample, e.g. the intra-assay (within assay) coefficient of variation". Alternatively, this may be explained as the range of possible values obtained by an assay for the true value of a sample. If the assay variation was large, then the range of values obtained for the true value would also be large. That is, if the true value of a sample is 10, and the assay varies as much as 20% the assay values obtained may range from 8 to 12 for that particular sample.

The intra-assay coefficient of variation was measured by analysing a number of samples (it could be actual samples, or "quality control" samples) several times and calculating the coefficient of variation of each sample. An acceptable intra-assay variation was set at less than 10%. The coefficient of variation was defined as the standard deviation of multiple estimates of a sample divided by the mean of the multiple estimates 100, e.g.

Mean of 20 replicates	Standard deviation
6.9	0.61

$$\%CV = \frac{0.61}{6.90} \times 100 = 8.8$$

3.4 PLASMA PROGESTERONE DETERMINATION

3.4.1 Handling of the kit

i) Receipt

Record was kept of the lot number and arrival date in a log book and the kit contents inspected for damage during shipping. Kit components were stored at 2 - 8°C in a refrigerator, or at -20°C in a deep freezer (radioactive materials must be stored in a refrigerator designated for the purpose).

ii) Safety considerations

The kit contains radioactive material and other ingredients which necessitate certain precautions:

a) A material safety data sheet relating to the kit components are found in the appendix of the instructions with each kit
b) Laboratory personnel should be familiar with standard precautions when using radioactive materials i.e., radiological protection, handling, disposal and the procedures to implement in the event of spills of such materials.

3.4.2 Procedures for the collection and processing of samples

Sampling and sample processing can have a major effect on the progesterone concentrations in plasma. In order to achieve reliable results, it is of the utmost importance to perform as consistent a sampling procedure as possible:

i) Sample blood from the jugular or tail vein of the cow, which is most conveniently achieved using evacuated tubes with heparin or EDTA as anti-coagulants;

ii) Blood samples for plasma harvesting must be stored at 4°C immediately after collection;

iii) Centrifugation of plasma at 770G (approx. 2000 rpm) for 20 minutes (preferably at the same temperature each time i.e. 4°C, if refrigerated centrifuge is available). Centrifugation should be done within 2 – 4 hours following sample collection;

iv) Aspiration of the plasma using a Pasteur pipette and the transfer to storage vials – stored at -20°C until assayed.

3.4.3 Equipment required

- Pipettes
  - Micropipettes suitable for pipetting 20, 25, 40, 200 and 1 000 µl with disposable plastic tips.
  - Hand held multi-dispenser with combi-tips for dispensing 200, 300 and 500 µl (e.g. Eppendorf 4780 pipette).
  - Pasteur pipettes (23 cm length) and pipet bulbs.
  - Graduated pipette of 10 ml.
- Glassware: A selection of beakers (50, 100 and 500 ml) and volumetric flasks (50, 100 and 1 000 ml).
- Tubes for coating: Polystyrene Nunc "star" tubes (immuno quality), 75 x 12 (Nunc; # 4-70319).
- Decantation rack for the tubes.
- Vortex mixer.
- Analytical balance (4 decimal places).
- A single well or multi-well gamma counter (make, etc.).

It is essential that the recipient laboratory has a constant supply of sterile distilled and deionized water.

3.4.4 Reagents and material supplied

- Monoclonal antibody 6H11/14; 0.25 ml/vial of 1:10 dilution, lyophilized (stored at -20°C).

- Radioactive tracer: progesterone-11-α-hemisuccinate-2-[^{125}I) iodo-histamine (10 μCi/100 μl; Amersham; #IM-138) in methanol:water (9:1;). This is the stock tracer solution and should not be used more than 120 days past the "activity" date. Store in refrigerator at 4°C.

- Plasma standards – Lyophilized bovine plasma standards with progesterone concentrations of 0, 1.25, 2.5, 5, 10, 20 and 40 nM (nmol/l). Store in refrigerator at 4°C.

- Carbonate/bicarbonate tablets (coating buffer; EC Diagnostics, Sweden; #LD8922). Store dry at room temperature.

- Phosphate buffered saline (PBS) tablets (diluent buffer; EC Diagnostics, Sweden; #LD9402). Store dry at room temperature.

- Bovine serum albumin (BSA), RIA grade (Sigma; #A-9647). Store in refrigerator at 4°C.

- Tween 20 (Sigma; #P-1379). Store at room temperature.

- Sterile filtered distilled water/glycerol (50:50) solution, for reconstituting antibody. Store at room temperature.

- Nanopure water for reconstitution of standards. Store at room temperature.

- Nunc 1 ml cryovials (Nunc; #366656), for storage of antibody stock solution. Store dry at room temperature.

3.4.5 Preparation of reagents and samples

(Done one day before commencing a series of assays)

- *Coating buffer*

  Place approximately 70 ml distilled/deionized water in a 100 ml volumetric flask, add one carbonate/bicarbonate tablet and shake until completely dissolved (may take up to 15 min.). Add distilled water to make up to the 100 ml mark (this is 0.05 M carbonate buffer with a pH 9,6 ± 0.05). Label and store at 4°C, for no longer than a month.

- *Diluent buffer (PBS)*

  Place approximately 500 ml distilled.deionized water in a beaker, add one PBS tablet (0.14 M NaC1, 3 mM KCl) and stir until completely dissolved. Transfer the solution to a 1 l volumetric flask and add distilled water to make up to the mark (this is 0.01 M phosphate buffered saline with a pH 7.4 ± 0.2). Label and store at 4°C for no longer than 2 weeks.

- *Antibody stock solution*

  Reconstitute one vial of the lyophilized monoclonal antibody with 0.25 ml of the supplied diluent (sterile filtered distilled water/glycerol) to obtain the antibody stock solution (1:10 dilution). This should be aliquoted in 38.5 µl volumes into 1 ml Nunc cryovials or similar small tubes with caps (there should be 10 aliquots). These vials should be stored upright in a rack at -20°C.

- *Plasma standards*

  To reconstitute lyophilized plasma standards, add 1 ml of distilled water to each vial. Allow to stand overnight in the refrigerator (4°C). Shake gently until completely dissolved. Reconstituted standards should be stored at 4°C for no longer than one week.

Recommendation for the reconstituted plasma standards

After reconstitution of the plasma standards, aliquot into 1 ml Nunc cryovials or similar tubes and store at -20°C.

- *Washing solution*

  Add 1g of Tween 20 to 1l distilled water to obtain a 0.1% washing solution. Mix well until completely dissolved. Label and store at room temperature for no longer than 2 weeks.

3.4.6 Coating and assay procedures

a) Day 1

- ***Labeling of assay tubes***

  Label Nunc "star" tubes in duplicate for total counts (TC) B_{zero} (B_0), standards, QC and unknowns. It is recommended that each assay contains 150 tubes. The reagent volumes and procedures described are adjusted for assays of this size. Laboratories with the necessary resources may run more than one assay (of 150 tubes each) simultaneously.

- ***Preparation of antibody coating solution***

  Transfer 38.5 µl (one aliquot) of the antibody stock solution to a 50 ml volumetric flask. Ensure complete transfer of all antibody by repeated rinsing of the aliquot tube with the coating buffer (0.05 M carbonate buffer). Fill the flask to the mark with the same buffer. This will give an antibody coating solution with a 1:13000 dilution; 50 ml is sufficient for the coating of 150 tubes. Label and store at 4°C for no longer than 2 days.

- ***Coat tubes***

  Dispense 300 µl the antibody coating solution into each tube, except the TC tubes. Cover the tubes with parafilm or similar sealing material and incubate overnight (at least 18 hours) at 4°C (in the refrigerator).

b) Day 2

➢ ***Decantation and washing of tubes***

After incubation, decant the contents of the tubes and tap the mouth of the tubes vigorously on absorbent paper to remove the remaining fluid. Add 500 µl washing solution to each tube. Decant the washing solution from the tubes as described above. Rinse the tubes a second time with 500 µl washing solution and decant.

➢ ***Preparation of radioactive tracer working solution***

Weigh out 33 mg BSA (bovine serum albumin) and transfer into a glass beaker with 33 ml diluent buffer (PBS). Dissolve completely using a clean glass rod or pipette. Add 20 µl of the stock tracer solution (^{125}I-progesterone) and mix well to obtain the tracer working solution. Label and store at 4°C for no longer than one week. Take 2 aliquots of 200µl each from tracer working solution and count in a gamma counter for one minute for a total count (TC). This should be approximately 20000 – 30000 cpm. If total counts are between 10000-15000 cpm, increase the assay count time in order to obtain the required minimum cpm (approx. 1000) for the highest progesterone standards. Do not alter tracer concentration nor volume. If counts are below 10000 cpm, fresh tracer should be obtained.

➢ ***Setting up of assay***

Allow all reagents to reach room temperature (15-20 min) before setting up the assay. Gently mix or lightly vortex all samples before pipetting. Add 40 µl of the standard, QC or unknown sample to the respective antibody coated tubes. The pipette tip should touch a vane on the bottom of the star tube to guarantee complete expulsion. Using a repeater pipette, add 200 µl of the tracer working solution to each tube (including TC). Cover with parafilm and incubate overnight (at least 20 hours) at 4°C.

c) **Day 3**

➢ ***Decantation and washing of tubes***

After incubation, remove the TC tubes from the rack and vigorously decant all the remaining tubes into an appropriate radioactive waste disposal container and allow the tubes to drain for 5 minutes on absorbent paper. Add 500 µl washing solution to each tube (except TC) and decant to remove the fluid as described above. Rinse the tubes a second time with 500 µl washing solution and decant as described above.

➢ ***Determination of radioactivity and plasma progesterone concentration***

Count the radioactivity of each tube (including TC) in a gamma counter for a fixed time (normally 60 sec.). Calculate the maximum percentage binding in the assay (B_{max}) by dividing the average counts per minute (cpm) of the 2 zero binding tubes (B_0) by the average cpm of the 2 TC tubes and multiplying by 100.

$$B_{max} = \frac{Average\ cpm\ of\ B_0}{Average\ pm\ of\ TC} \times 100$$

Calculate the percentage binding (% B/B_0) for all standards, samples and quality control tubes by dividing the cpm of each of these tubes with that of the B_0 tubes and multiplying by 100.

$$\frac{B}{B_0} = \frac{Average\ cpm\ of\ standard/sample/QC}{Average\ cpm\ of\ B_0}$$

Using the logit-log graph paper provided, plot the average percentage bound (b/B_0) on the vertical (y) axis and the concentration progesterone standard on the horizontal (x) axis. Draw a straight line through the points on the graph. Do not draw a straight line between each of the x, y co-ordinates but draw a "best-fit" straight line through

all coordinates. Plasma progesterone concentrations of the unknown samples and QC can then be measured by reading their percentage bound and extrapolating these values from the standard curve to determine the progesterone concentration on the x-axis.

d) Quality control of assays

Precision of an assay is defined as: "A measure of the variation in estimating a sample, e.g. the intra-assay (within assay) coefficient of variation". Alternatively, this may be explained as the range of possible values obtained by an assay for the true concentration of a sample. If the assay variation is large, then the range of concentrations obtained for the true value will be large. So for example if the real concentration of a sample is 10 and the assay varies as much as 20%, the assay values obtained may range from 8 to 12 for that sample. The intra-assay coefficient of variation is usually measured by analyzing a number of samples (it can be actual samples, or "quality control" samples) many times and calculating the coefficient of variation for each of the samples. An acceptable intra-assay variation is less than 10%. The coefficient of variation can thus be defined as: "The standard deviation of multiple estimates of a sample, divided by the mean of the multiple estimates, multiplied by 100".

Mean of 20 replicates — Standard deviation

60 — 0.32

$$\% \text{ CV} = \frac{0.32}{6} \times 100 = 5.3$$

The interassay or between assay variation is extremely important as its control is the only way to ensure that progesterone concentration levels from a series of samples coming from one animal reflect its clinical condition and not the variation due to a drift between assays. To address this, researchers are encouraged to set up interassay IQC charts with the following parameters: Total counts; B_0 – maximum binding; 20%, 50% and 80% binding values

from the logit – log graph slope of the logit – log graph; absolute progesterone concentrations of the IQC I (low) and IQC II (medium) samples.

In order to make internal quality control samples (IQC), 2 large pools of plasma (e.g. 100 ml per pool) should be collected from the specie being studied. Normally, mixed pools should be made from several animals in different physiological conditions - in such a way that the pools are likely to have low or medium concentrations of progesterone. The low pool should have a value which is close to that used to differentiate between the presence and absence of luteal activity. The medium pool should have a value which is close to that expected during the mid-luteal phase of the oestrous cycle.

The material should be treated and centrifuged in the same manner as unknown samples. One ml aliquots of the plasma are then pipetted into suitable capped vials and either: 1) deep frozen (-20°C) or 2) freeze-dried and frozen (-20°C) depending on facilities. Thus, each time an assay is run, a vial of each of these IQC samples could be thawed or reconstituted with 1 ml of distilled water. These samples should then be included in each routine assay (at the beginning and end of the sample tube sequence) and the progesterone values plotted on a simple time series graph.

Clearly, when the internal quality control sample value varies considerably from the average concentration, some doubt must exist regarding the quality of the assay and the assay should therefore be repeated. Acceptable limits (mean ± 2 SD) can only be calculated following a minimum of 10 assay runs having been completed. Thus, large volumes of IQC material needs to be collected, treated and aliquoted to ensure that long term internal quality assurance checks can be performed.

3.4.7 Assay characteristics of plasma progesterone determinations

a) Sensitivity

40 Zero standard (maximum binding-MB) tubes were processed in a single assay along with a set of non-zero standards and quality control samples.

Means and standard deviations were calculated regarding cpm for the 40 zero tubes. From a standard curve obtained using a logit-log plot and using the mean as the zero point, progesterone concentrations were determined, at increasing standard deviations from the mean (Table 3.1).

Table 3.1 Sensitivity of plasma progesterone assay

Mean ± SD of cpm from 40 MB tubes	Number of SD's (cpm)	% B/B_0	P4 conc nM (nmol/l)	Approx. sensitivity nM (nmol/l)
12976 ± 274	1 SD (12702)	98.4	0.01	0.19
	2 SD (12428)	96.3	0.2	
	3 SD (12428)	94.2	0.35	

The detection limit of this assay was 0.19 nmol/l (ca. 0.06ng/ml).

Table 3.2 Intra assay variation of plasma progesterone assay

Intra assay*			
Sample	**Mean nM (nmol/l)**	**SD nM (nmol/l)**	**CV %**
1	4.2	0.3	7.1
2	6.5	0.4	6.8
3	14.1	1.0	7.1
Inter assay**			
Sample	Mean nM (nmol/l)	SD nM (nmol/l)	CV %
1	4.3	0.4	8.9
2	6.5	0.4	5.6
3	13.6	0.6	4.5

* Statistics were calculated for each sample from the results of 20 pairs of tubes in a single assay
** Statistics were calculated for each sample from the results of pairs of tubes in 20 different assays

b) Precision

The reliability of the RIA procedure using the self coating system was assessed by examining its reproducibility on samples with low, intermediate and high levels of progesterone.

c) Method comparison

A comparison of assay parameters between the DPC solid phase coated-tube assay (Coat-a-Count) and IAEA self-coating system was performed. As shown in Table 3.4 a good correlation between these 2 methods was obtained.

Table 3.3 Specificity of 6H11/14 mouse monoclonal antibody used in the RIA assay

Steroids	Cross-reaction (%)
Progesterone	100
11αhydroxyprogesterone-hemisuccinate	108.57
11-α-OH-Progesterone	70.37
11-β-OH-Progesterone	20.00
17α-hydroxy-progesterone	3.62
5α-pregnane-3.20-dione	31.66
5β-pregnane-3.20-dione	97.43
5-pregnen-3β-OL-20 one	6.55
5β-pregnane-3α,20α-diol	ND
4-pregnen-20β-ol-3-one	ND
4-pregnen20αol-3one	ND
17β-oestradiol	ND
17α-oestradiol	ND
Oestrone	0.1
Oestriol	ND
Testosterone	ND
4-androstene-3.17-dione	5.27
Dehydroisoandrosterone	2.92
Corticosterone	ND
Deoxycorticosterone	0.1
Hydrocortisone	ND

ND = not detectable

Table 3.4 A comparison of assay parameters between the DPC assay and IAEA self-coating system

	Values using DPC kit	Values using self coating kit
TC	58551 cpm	28.642 cpm
% NSB	0.2-0.7	0.3-0.8
% MB	49.6	58.5
Corr. Coef.	0.9998	0.9991
Intercept		
20% B/Bo	42.4 n/mol/l	28.5 nmol/l
50% B/Bo	6.0 nmol/l	6.5 nmol/l
80% B/Bo	0.92 nmol/l	1.6 nmol/l
Sensitivity	0.1 nmol/l	0.19 nmol/l
Precision*		
Sample 1	4.7%	5.1%
Sample 2	5.0%	4.4%
Sample 3	3.8%	3.3%

* Data refer to intra-assay comparison of 20 tubes per sample

3.5 MILK COMPONENT (FAT, PROTEIN, LACTOSE AND UREA) DETERMINATIONS

3.5.1 Application

The instrument used is an infrared system which, when used under the conditions defined, estimates the concentration of fat, protein and lactose in milk - expressed in grams per 100 g of milk.

3.5.2 The infrared optical system

Infrared light is passed through the carbonyl groups in the ester bonds of the glyceride at approximately 5.7 μm (traditionally referred to as the B filter), for the determination of milk fat, and through the secondary amide groups of

peptide bonds at approximately 6.5 μm, for the determination of milk protein content. Infrared light is passed through the hydroxyl groups of lactose at approximately 9.6 μm, then estimating the milk lactose content. The amount of infrared light absorbed at any specific μm is thus used to estimate the concentration of the relevant milk components.

3.5.3 Materials and reagents

3.5.3.1 Basic solutions

i) Dye-buffer basic solution: 2.5 g Ethidium bromide (10 tablets) dissolved in 1 l distilled water, heated to 40-60°C. The solution is stirred until the ethidium bromide dissolves. 415 g Fossomatic buffer-powder (one bottle) is then added and stirred until dissolved. Thereafter 4 l of distilled water is added and stirred until all solids dissolve. While stirring 40 ml Triton X-100 concentrate is added. This constitutes the basic dye/buffer solution. The solution is stored in an amber container, airtight and refrigerated for no longer than 90 days.

ii) Triton-X-100: 10 ml Triton-X-100 is dissolved in 1 l distilled water at 40-60°C. Store airtight and cooled for a period of no longer than 30 days.

3.5.3.2 Working solutions

i) Dye-buffer working solution: Add 1 l of dye/buffer basic solution to 9 l distilled water. The fossomatic dye/buffer tank holds 10 l (2700 samples). Refill tank when required.

ii) Cleaning solution: Add 10 ml of 1% Triton-X-100 and 5 ml of 25% ammonia to 10 l distilled water. This constitutes the cleaning solution. The MilkoScan cleaning solution tank holds 50 l (2500 samples). Refill tank when required.

iii) *Zero solution:* For MilkoScan 605 model - Dissolve 50 g Stella non-foaming powder in 10 l distilled water. Use within one week. For the System 4000 model, dissolve 5 ml of Foss Electric S-6060 Zero Liquid concentrate in 5 l distilled water. Use within one week.

iv) *Zero check:* Purge instrument. Select standby option – automatic zero settings will follow. If not, select zero on instrument panel. A set of 5 zero measurements are done to get an average. The average must be between 0.00 and 0.10, if higher, purge instrument again. If the second zero average exceeds 0.10 accept, purge again and zero. The zero settings, if acceptable, must be lower than 0.05.

3.5.4 Calibration adjustments

Traditional calibrations are based on the assumption that absorption of a specific wavelength is proportional to the concentration of a component. As this assumption is not completely true, there are two corrections for the calculated concentration: linearity correction and inter-correction. The linearity correction adjusts the raw data in order to obtain the best possible proportionality between absorption and concentration. The coefficient of the calibration was calculated using the raw data and reference results for the components to be calibrated. This meant that reference values were needed for fat, protein and lactose, even if only one component is calibrated.

i) ***The use of pilot samples***

The purpose of a pilot sample was to confirm the stability of the instrument, e.g. to ensure that the instrument gives the same (repeatable) result on pilot samples throughout the day.

There are two steps when using pilot samples: Define the pilot sample by using the 'Pilot Definition" batch type function. Identify the pilot sample by using metal strips on the sample bottle. The instrument will then recognise these bottles as pilot samples. The instrument will automatically give the deviations between pilot samples and stop when variation exceeds pre-set limits. Limits for pilot sample deviations are given below:

Component	Allowed variation
Fat, Protein, Lactose	0.05%
Urea	3.50 mg/dl

ii) The use of 10% milk powder samples

Powder milk samples are analysed directly after calibration of the instrument to attain a 10% calibration standard - which is then used to check the instrument calibration accuracy at the start. These 10% milk powder samples are made up each week to be used as anchoring samples.

3.5.5 Sample preparation

General guidelines: Samples must be in reasonably good condition and not have started to curdle or separate, and must be free of dirt and other foreign particles.

Pre-heating samples: It is important to pre-heat samples in order to evenly distribute fat globules in the milk. For maximum accuracy, samples must be heated to ± 40°C (37-42°C range), immediately before analysis. Correcting the temperature is especially critical when measuring urea. Avoid prolonged heating or overheating of samples as it can cause the separation of the fat ("oiling off").

Sample shaking: The pipette unit includes a stirrer, which distributes the fat globules evenly in the sample just prior to pipeting, ensuring that the ml aspirated into the analyser is representative of the sample. It is recommended that the samples be thoroughly mixed after heating, in order to dissolve possible deposits of milk solids. Avoid vigorous shaking, as this could cause churning or formation of air bubbles in the sample.

3.5.6 Milk sample preservation

Samples can either be analysed as preserved or non-preserved milk. Preserving samples, however, provides extra storage time and transportation.

Bronopol mixtures (Micro Tabs) were used to preserve the milk, as it does not lower the somatic cell counts, and is not harmful to the environment.

Table 3.5 Preservation time of milk with different preservatives at 5°C and 20°C

Preserving agent	5°C	20°C	IDF recommendation
Bronopol (0.02%)	5 days	5 days	
Potassium dichromate	12 days	3 days	3 days
Sodium azide (0.02%)	2 days		2 days (5°C)
Ortobor acid (0.6%)	2 days		2 days (5°C)

3.5.7 Procedures and methods

A one step procedure for calculating fat B %, protein %, lactose % and milk-urea-nitrogen content in milk was used. Sample bottles were packed in the MilkoScan racks provided and pilot samples included at the end of the batch. Information was recorded on log sheet (owner, herd code, test date, amount, tank) and entered into the MilkoScan computer. Empty, sour or otherwise unsatisfactory samples were also recorded on the log sheet. Milk samples were pre-heated in a water bath (42-45°C) to 38-40°C for 12-15 minutes and mixed by repeatedly inverting samples.

3.5.8 Results

Test reports would show the member's name, member identification number, test date, region and breed in the upper left-hand corner. This is followed by columns with the headings, sample no. fat B, protein and lactose.

Example:

Member name	:	HJ Kemp
Number	:	123456
Test date	:	99/02/22
Region	:	2
Breed	:	298

Sample No	Fat B	Protein	Lactose
1	3.87	3.45	4.98

3.5.9 Repeatability and accuracy

Identical samples, tested at short intervals, should give results not exceeding a mean deviation of more than 0.05% for milk fat, protein and lactose. The pilot or test sample at the end of each batch also served as a repeatability check for the assay.

CHAPTER 4

PREGNANCY DIAGNOSIS BASED ON THE MILK PROGESTERONE CONCENTRATION IN DAIRY CATTLE

4.1 INTRODUCTION

Serum progesterone levels in milk are closely related to the growth and secretory function of the corpus luteum during the normal cycle and pregnancy. These serum progesterone concentrations are however closely related to the milk progesterone levels and can thus be utilized to determine the pregnancy status in a cow. Where corpora lutea are present, progesterone is actively secreted and where pregnancy exists this active corpora lutea and progesterone secretion persists (Royal *et al.*, 2000).

Thus the milk progesterone levels recorded can be used for pregnancy diagnosis. In the pregnant cow, the high levels of milk progesterone reached on approximately day 14 (day 0 = oestrus) of the cycle persists, but in the non-pregnant cow the milk progesterone levels rapidly declines from day 17 of the cycle (Sturman *et al.*, 2000). Between days 20 to 24 following AI the difference recorded in milk progesterone concentration between pregnant and non-pregnant cows are at their greatest. This difference in milk progesterone levels thus forms the basis of milk progesterone pregnancy diagnosis in cattle (Royal *et al.*, 2000).

The pregnancy diagnosis is thus dependent on the presence, or absence of a corpus luteum in the ovary, 20 to 24 days after insemination. Thus discriminatory levels of milk progesterone must be set to enable the differentiation between an active corpus luteum, a regressing corpus luteum or the absence of a corpus luteum (pregnant or non-pregnant) (Wehrman *et al.*, 1993).

Milk progesterone for pregnancy diagnosis was evaluated in dairy cattle to determine the following:

(a) the accuracy of the test, and

(b) the discriminatory limit of milk progesterone indicating the presence of an active corpus luteum and possible pregnancy.

4.2 MATERIALS AND METHODS

4.2.1 Farm animals and records

The survey was conducted on 4 dairy farms in Gauteng and Mpumalanga, South Africa, each with more than 150 cows in milk. Five hundred and eighty two (582) Holstein dairy cows, varying in age from 30 to 65 months, were utilised in this study. The parity of the cows varied between 2 to 5, BCS at calving ranged between 2 and 4.5 and BW ranged between 380 kg to 650 kg. All cows post partum reproductive (uterine involution, ovarian activity and fertility) productive parameters were monitored by the herdsman and veterinarian records, through rectal palpation and visual observations.

Nutrition in the herds was based on improved pastures with supplementation of maize silage (20-25kg/cow/day), hay ad lib and concentrates (10-13kg/cow/day) with 17-19% protein content, with a relative energy value of 12MJ/kg/day fed in the form of a mixed diet throughout the year. Machine milking was carried out twice daily (6:00, 18:00) on 3 farms and 3 times per day (6:00, 14:00, 22:00) on the remaining farm.

The oestrous detection routine used was based on overt signs (cows standing to be mounted) and these observations were carried out twice a day at the time when cows were moved to the milking parlour (6:00 and 18:00). The time devoted to observation of the cows in oestrus was approximately 1 hour in the morning and again an hour in the afternoon. AI (12-14 hours after standing oestrus) was used exclusively on all the farms with no clean up bulls being used in the breeding system.

The data collected included calving date, parity (1 = first-calf heifers and 2 = mature cows), ease of calving (1 = normal, 2 = simple assistance and 3 = major assistance), breeding dates, pregnancy diagnosis, monthly milk

production, body weight, body condition score at calving and at each breeding and milk progesterone levels monitor the efficiency of oestrous detection.

Information was collected in a database designed especially for this purpose. Body weight (BW) and body condition score (BCS) at calving and at AI were recorded. The BCS was assessed on a scale of 1 to 5 as described by Edmonson *et al.* (1989), but for the statistical analysis cows were grouped in low body condition (≤ 2) and moderate (>2) groups, allowing the classing of the cows into 2 respective groups. The milk production of each cow was recorded daily. Reproductive records were recorded until the cow was certified pregnant or culled. The farm's veterinarian performed pregnancy diagnosis every month, starting at 60 days after the onset of AI.

4.2.2 Milk sampling

Milk samples for pregnancy diagnosis were obtained from the 4 dairy herds. In 3 of the herds, a single sample, taken between days 20 and 24 after insemination was analysed. Two milk samples, taken on day 21 and day 22 after AI, were taken from the fourth herd.

Three aspects of milk progesterone pregnancy diagnosis in cows were to be analysed. The discriminatory progesterone limit was set, using discriminant analysis (a statistical package available on the Statistical Packages for Social Sciences) and day 21 milk progesterone concentrations of cows confirmed pregnant. The pregnancy status in the cows was diagnosed and confirmed by rectal palpation 60 days following insemination. The results of 582 milk pregnancy tests i.e. 376 pregnant diagnoses and 206 non-pregnant diagnoses, formed the basis of this analysis.

Using the milk progesterone level of the sample, and the outcome of insemination, the discriminant function was calculated. From the discriminant function, several milk progesterone concentrations were evaluated to calculate at which level the frequency of a false positive and negative

production, body weight, body condition score at calving and at each breeding and milk progesterone levels monitor the efficiency of oestrous detection.

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Using the milk progesterone level of the sample, and the outcome of insemination, the discriminant function was calculated. From the discriminant function, several milk progesterone concentrations were evaluated to calculate at which level the frequency of a false positive and negative

diagnosis was minimised. Logit analysis was then performed to test the findings of discriminant analysis.

A second analysis performed, determined whether the day of sampling affected the accuracy of classification for pregnancy in cows. This analysis utilised the data obtained from complete milk progesterone profiles of 76 cows over 136 inseminations.

Table 4.1 Milk progesterone concentrations of three samples and interpretation in relation to rectal pregnancy diagnosis in dairy cows

Day 0 (day of AI)	Day 9-13	Day 21-24	Rectal palpation results; interpretation
[1]Low	[2]High	High	Pregnant
Low	[3]Intermediate	High	Pregnant; RIA problem, biological variations
Low	High	Low	Non-pregnant; fertilisation failure, early embryonic death, post AI anoestrus
Low	Intermediate	Low	Non-pregnant; fertilisation failure, short luteal phase, RIA problem, biological variation
Intermediate/ High	Low/intermediate/ High	Low	Non-pregnant; AI at incorrect time, post AI anoestrus
Low	High	High	Non-pregnant; late embryonic death (>16 days) luteal cyst, persistent corpus luteum (CL)
High	High	High	Pregnant; AI on pregnant animal
Low	Intermediate	High	Non-pregnant, RIA problem, biological variation, late embryonic death, persistent CL
Low	High	Intermediate	Non-pregnant, fertilisation failure, late embryonic death RIA problem, biological variation
Low	Low	Intermediate	Non-pregnant, AI in anoestrous cow, RIA problem
Intermediate/ High	Low/Intermediate/ High	Intermediate/ High	Non-pregnant, AI at incorrect time, luteal cyst, persistent CL

[1]Low = <1.0nmol/l [2]High = ≥3.0 nmol/l [3]Intermediate = between 1.0 and 3.0 nmol/l

The third feature tested was to establish whether two consecutive milk samples, taken on day 21 and day 22 after insemination gave more precise results than one sample taken between days 20 to 24 after AI. This analysis

was based on 196 pregnancy tests obtained using a single sample and 386 pregnancy tests using the double sampling method.

In addition to these 3 aspects of the test, the overall accuracy of milk progesterone for pregnancy diagnosis, as performed on the milk sample taken, was also determined. In this diagnosis, according to the milk progesterone levels, cows could be classified e.g. as in oestrus; bred at the right time and pregnant; bred at the right time and open; bred at the wrong time; or bred pregnant (Table 4.1).

Milk samples for progesterone (MP) concentration determinations were collected at day 0, 10 and 23 after AI and if the cow returned to oestrus, milk sampling was re-initiated. In this scheme, according to the milk progesterone levels, cows could be classified e.g. as in oestrus (low-low-low milk progesterone levels); bred at the right time and pregnant (low-high-high milk progesterone levels); bred at the right time and open (low-high-low milk progesterone levels); bred at the wrong time (high-low-high- or high-high-low milk progesterone levels); or bred pregnant (high-high-high milk progesterone levels) (Table 4.2).

4.2.3 Milk progesterone analysis

Milk samples were collected at the morning milking (6:00), the same method as before milk stripping, with the exception that the routine mastitis test strip was performed prior to the actual sampling in 20 ml vials containing sodium azide (Merck KgaA, Damstadt, Germany) and stored at 4°C on the farm for later transport to the radioimmunoassay (RIA) laboratory. There the milk samples were centrifuged and the fat free fraction stored at -20°C until analysed for progesterone concentration with the aid of a solid phase RIA kit. The intra-assay coefficient of variation (CV) for samples with progesterone concentrations below 1 nmol/l milk progesterone level was 8.2% and for samples with a progesterone level of above 1nmol/l, 9.8%. The inter-assay coefficient of variance was 11.7 and 4.5% for samples with P4 values below or above 1 nmol/l milk progesterone, respectively. Results and values

determined according to standard inter-assay and assay variation are described in 3.4.7.

Table 4.2 Milk progesterone concentrations on the day of service and on day 9 to 13 with respect to the accuracy of oestrous detection

Day 0 (days of AI)	Day 9-13	Interpretations
[1]Low	[2]High	Milk progesterone concentration within negative range on day 0 and within positive range on day 9-13 indicates an ovulatory cycle-accurate oestrous detection
Low	Low	Milk progesterone concentration within negative range on both days indicates anoestrus, anovulation, or short luteal phase-inaccurate oestrous detection
High	High	Milk progesterone concentration within positive range on both days indicates AI in pregnant animals or in animals with luteal cyst-inaccurate oestrous detection
High	Low	Progesterone concentration within positive range on day 0 and within negative range on day 9-13 indicates that AI was performed during luteal phase-inaccurate oestrous detection

[1]Low = <1.0nmol/l [2]High = ≥3.0 nmol/l [3]Intermediate = between 1.0 and 3.0 nmol/l
Thirty-two (6.7%) services were made in cows with an intermediate level of milk progesterone on day 0, day 9-13 or on both occasions

4.2.4 Inter-oestrous intervals

Another important parameter to determine the efficiency of oestrous detection and to evaluate the embryonic mortality (EM) was by determining the inter-oestrous interval. To calculate this, intervals were classified as: <18 days ("short"), 18 – 24 days ("normal"), 23-35 days ("extended" or EM or incorrect oestrous detection), 36-48 days ("2 x normal", one missed oestrus or EM), and >48 days ("long", two missed oestruses or EM or abortions) (Macmillan & Day, 1982).

4.2.5 Statistical analysis

The statistical analysis of the intervals from calving to first service and from calving to conception, were carried out according to the following general linear model (SAS, 1990):

$$INT_{ijklmnopqrs} = \mu + b_j + c_k + d_l + e_m + f_n + g_o + h_p + i_q + j_r + k_s = \varepsilon_{ijklmnopqrs}$$

Where INT is the interval to first service or to conception; b is the *j*th effect of farm (A, B, C, D and E); c is the *k*th effect of parity (1 and 2); d is the *l*the effect of ease of calving (1, 2, 3 and 4); e is the *m*th effect of BW (≤500 and > 500kg) f is the *n*th effect of BCS at calving (1 and 2); g is the *o*th effect of month of calving (1-7); h is the *p*th effect of month of breeding (5-12); I is the *q*th effect of BW at service (≤ 500 and >500 kg); j is the *r*th effect of BCS at service (1 and 2); k is the *s*th effect of milk production at service (5,10,15,20,25,30l) and $\varepsilon_{jklmnopqrs}$ is the error.

Interactions between variables were tested and mean comparison was done using the LSD method. To analyse conception rate at first service and overall pregnancy rate the CATMOD procedure was utilised.

4.3 RESULTS

4.3.1 Discriminant analysis

The discriminant analysis (Table 4.3) demonstrates no difference in overall classification at a progesterone level of 3.0 nmol/l or 3.5 nmol/l milk. However, the Pearson correlation was higher at 3.0 nmol/l milk progesterone level.

The logit analysis (Table 4.4) provided slightly more sensitive results. With this analysis a level of 3 nmol/l proved to be the most accurate in predicting pregnancy, although only marginally so.

Table 4.3 Accuracy of pregnancy diagnosis in cows at different milk progesterone levels (Discriminant analysis)

Progesterone concentration (nmol/l)	Correct diagnosis of cows (%)			Pearson Correlation
	Non-pregnant	*Pregnant*	*Overall*	
2.0	92.0	99.5	95.8	91.05
2.5	93.2	98.9	96.05	93.23
3.0	97.1	98.9	98.0	96.24
3.5	98.1	97.9	98.0	95.52
4.0	98.1	94.7	96.4	91.30

Table 4.4 Accuracy of pregnancy diagnosis in cows with different milk progesterone concentrations (Logit analysis)

Progesterone concentration (Nmol/l)	Correct diagnosis of cows (%)			Pearson Correlation
	Non-pregnant	*Pregnant*	*Overall*	
2.0	92.0	99.5	95.8	91.05
2.5	96.0	98.9	97.5	93.23
3.0	97.1	98.4	97.9	95.49
3.5	97.1	97.9	97.6	94.75
4.0	98.1	94.7	95.9	91.30

When combining the results of both the discriminant and logit analysis, a "cut off" level for milk progesterone of 3 nmol/l was used to distinguish between pregnant and non-pregnant cows.

4.3.2 Day of sampling

The number of days post insemination when a milk sample was taken had a definite affect on the accuracy of pregnancy classification in pregnant and non-pregnant cows. This variation in the accuracy is set out in Table 4.5.

Table 4.5 Effect of day of milk sampling on the accuracy of pregnant diagnosis

Day on which sample was taken (day 0 = day of AI)	Percentage of cows incorrectly diagnosed
17	23.0
18	18.0
19	7.8
20	7.6
21	5.0
22	2.1
23	1.4
24	1.6
25	2.1
26	2.0
27	3.1
28	4.8

Milk samples taken between days 22 to 26 post AI provided accurate diagnosis of pregnancy, with the 23-day sample proving the most accurate for classification. Outside this time frame, the percentage of cows incorrectly classified as pregnant was high. This could be expected for samples taken before day 18 or after day 27, but not for milk samples collected between 19 and 21 days following AI.

4.3.3 Number of milk samples used in pregnancy test

Before the investigation it was assumed that two milk samples, taken on days 21 and 22 post AI, would provide a more accurate diagnosis of pregnancy than a single sample taken between 20 and 24 days. The extent to which the two procedures differed was of interest and Table 4.6 presents a summary of the results with regard to the number of milk samples used.

Table 4.6 Effect of number of milk samples used on the accuracy of pregnancy test in cows

Number of samples	Sample induced error rates (%)	
	Incorrect pregnant diagnoses	Incorrect non-pregnant diagnoses
1	6.5	3.4
2	4.8	3.8

The results confirm that a double milk sampling routine was more accurate in pregnancy diagnosis. For non-pregnant cows, diagnostic error rates were virtually identical. Overall, the taking of two milk samples proved to improve the accuracy of pregnancy diagnosis by 1.7%, a difference much smaller than expected.

4.3.4 Accuracy of the milk pregnancy test

Using a milk progesterone concentration of 3 nmol/l as the reference level, the reliability of pregnancy diagnosis in dairy cows was determined.

The progesterone test for non-pregnancy in cows was 98.3% accurate and the assay for pregnancy was 90.4% accurate. Overall, an accuracy of 94.4% for diagnosis of pregnancy was achieved using the milk progesterone assay on 21-24 days post AI.

4.4 DISCUSSION

The reference level of milk progesterone of 3 nmol/l used in pregnancy diagnosis was similar in cows to those reported by other researchers (Scipioni & Foote, 1999; Waldmann *et.al.*, 1999; Qureshi *et.al.*, 2000). Reist *et al.* (2000) and Cavestany and Galina (2001) introduced an upper and lower discriminatory reference progesterone limit, thus allowing some flexibility in the diagnosis of pregnancy. Cows recording milk progesterone levels within these limits were classified as "doubtful" pregnancies.

The single discriminatory progesterone reference limit set in this current progesterone assay was shown to be very accurate and repeatable. There was thus no need to set upper and lower limits for confirmation of pregnancy. In addition, only 6% of the milk samples analysed had milk progesterone concentrations of 2 to 4 nmol/l. These few samples were classified using the discriminatory reference limit.

The close relationship between the discriminant levels and logit analysis of the data tended to indicate evidence that the discriminant function could possibly be used to set "cut off" limits in the diagnosis.

The results of these milk analyses emphasized the importance of day of milk sampling on the accuracy of classification. For pregnancy tests in cows, a milk sample is usually taken 20 to 25 days after insemination as this range tends to covers the known variation in oestrous cycle length. It would appear from the present progesterone results that milk samples taken during the latter half of this period (i.e. day 22 to 25), were more accurate for confirming pregnancy than those taken earlier (i.e. days 20 and 21). Båge (1998) also found maximum accuracy to be obtained from a day 23 following AI milk sample, but other research (Davidge, 1987; Waldmann *et al.*, 1999) claimed maximum accuracy to be achieved 21 to 23 days after artificial insemination. However, the day 23 milk sampling procedure has the advantage in that farmers are able to exclude any cows from the pregnancy test that have demonstrated oestrus before this time.

Results also provide important information regarding the advisability of early or late milk sampling for the purpose of pregnancy diagnosis. In some instances it may be desirable to sample for pregnancy earlier than 20 days following AI (e.g. when wanting to inseminate non-pregnant cows at that specific oestrus rather than at the next oestrous period), or later than 26 days following AI (for practical reasons). During these sampling times, the milk progesterone levels may be decreasing, or increasing, as a result of the impending oestrus, but the values still lie above the discriminator reference limit. This makes pregnancy diagnosis difficult to perform, as is evident from

the percentage incorrect diagnosis. Diagnoses based on milk progesterone tests at these times of sampling are therefore not advisable.

The double milk sampling routine on 11-13 and 21-24 days post AI has the advantage over a single milk sample in circumstances when milk progesterone concentration is still decreasing to a basal level. This occurs when the oestrous cycle length is slightly longer, or milk sampling is done too early. The first milk sample may have a progesterone level higher than the discriminatory reference limit, leading to an incorrect pregnancy diagnosis. A second sample on the other hand may indicate that the milk progesterone concentration is still decreasing and thus a correct diagnosis can be made.

Although there was a slight improvement in the accuracy of pregnancy diagnosis using two samples rather than one in the current study, the difference was not sufficient to warrant the taking of a second sample. In practice, a double milk sampling routine would imply more labour, time and financial implications. Thus it would seem that the single sampling technique would be a commercially more viable option.

Accuracy and repeatability of the pregnancy diagnosis are the most important factors that determine the feasibility of the milk progesterone pregnancy test. The RIA in determining the milk progesterone in this study was found to be highly consistent, thus the misdiagnosis of certain cows may have been the result of other compelling factors. A number of "on farm" practices like e.g. oestrous observations and nutritional management can affect the test accuracy.

Milk progesterone concentrations are highly correlated with the milk fat concentration. Thus the quality of the milk sample drawn from the cow for the hormonal test must be consistent with the milk used in the standard curve of the assay. Any deviation from this could lead to erroneous results and complicate the interpretation of the data (Schopper & Claus, 1986).

If an insemination is performed during the luteal phase of the oestrous cycle, the milk test sample will be drawn during the corresponding luteal phase of the subsequent cycle - thus giving a false positive result. The record keeping of herd management is thus important to ensure that inseminations are appropriately performed, and that the test samples are taken from the right cows on the right days. Studies have shown the accuracy of the milk progesterone tests to vary between farms, due to different farm management practices including record keeping accuracy (Kinsel & Etherington, 1998).

Milk progesterone concentration during the oestrous cycles in dairy cows follows the same basic pattern and irregularities and deviations are not really common. However, these deviations may then contribute to test inaccuracies, and must therefore not be ignored. So for example ovulation may have occurred at the normal time in the cow, but fertilisation may have been unsuccessful and as a result, the corpus luteum may have a life span shorter or longer than normal. The subsequent milk progesterone concentration at the time of testing may thus be higher than the discriminatory reference limit, leading to a false positive diagnosis (Ott *et.al.*, 1986). A second irregularity that may occur in milk progesterone concentration, concerns the mechanism whereby the life span of the corpus luteum is terminated. If no conception occurs, the mechanism of corpus luteum degeneration may fail and lead to the occurrence of a persistent secretory corpus luteum – or luteal cyst. The milk progesterone levels thus remain high, although the cow is not pregnant.

There may also be an abnormal delay between the observed oestrous behaviour when insemination was performed and actual ovulation. This would lead to a false pregnancy diagnosis 21 – 24 days later. Without monitoring milk progesterone levels over the entire oestrous cycles in cows, these irregularities in hormonal profile would be difficult to identify. Although such irregularities occur fairly infrequently they generally add to the incidence of incorrect pregnancy determinations in cows, as described in 3.4.7.

Embryonic losses appear to be the main cause of incorrect positive pregnancy predictions (Walker *et al.*, 1996). Early embryonic deaths have been well

documented in lactating cows, and losses of 10 to 25% have been reported in well managed dairy herds. The extent of these embryonic losses could explain the consequences of false positive pregnancy diagnoses (Walton *et al.*, 1987; Xu *et al.*, 1996; Xu *et al.*, 1997). It may not be right to include embryo loss in the false positive category, as the cow could have been pregnant on the day of sampling. If this were the case, the accuracy of pregnancy diagnosis of the cows in this study would increase to 97.4% - leaving a 2.6% fault due to herd management or oestrous cycle abnormalities. However, in the eyes of the farmer the RIA milk progesterone test gave an acceptable result and embryo mortalities must be included when determining the precision of the milk pregnancy assay.

The overall success rate of 90.4% and 98.3% achieved for diagnosis of pregnant and non-pregnant cows is similar to results obtained by other researchers, with accuracy rates for positive and negative pregnancy tests in cattle of between 81% and 91% (Heersche & Nebel, 1994), 85.1% and 98.8% (Rodtian *et al.*, 1996) and 94.7% and 95.2% (Senger, 1994), being quoted.

Under commercial dairy conditions the accuracy of the milk P4 positive pregnancy test will probably be lower than that recorded under research (artificial) conditions, due to management and facility resources. A high occurrence of inaccuracy of pregnancy diagnosis would be unacceptable to any dairy farmers. This has prompted the opinion that the only information obtained from a milk progesterone pregnancy test, which can completely substitute the clinical pregnancy examination is when progesterone levels are below the discriminatory limit (Waldmann, 1999). Thus it would seem the principle object of the milk pregnancy test is to detect cows that are not pregnant (Båge *et al.*, 1997).

4.5 CONCLUSION

The pregnancy test was accurate provided record-keeping was efficient and up to date. The 90.4% accuracy for pregnancy diagnosis was acceptable when factors affecting this test are taken into consideration. This success rate would have been lower if record-keeping on the farms had been poor. On

numerous occasions when a false positive result was recorded, a check of the records indicated an incorrect sampling time. Such samples were consequently excluded from the analyses. Such experiences support the contention that the value of the test is in the detection of non-pregnant cows, rather than pregnant cows.

CHAPTER 5

CONSTRAINTS OF MILK PROGESTERONE DETERMINATIONS FOR IMPROVING THE EFFICIENCY OF REPRODUCTION IN DAIRY CATTLE

5.1 INTRODUCTION

South Africa is largely dependant on agriculture to feed the nation, with dairy farming making out an integral part of the industry. In general dairy farming is conducted with animals maintained on natural and cultivated pastures and formulated diets which generally utilise maize silage, hay and concentrates in different proportions, according to the nutritional regime and management system implemented. Milk production of dairy cows in South Africa generally ranges between 3000 and 12000 l per lactation, depending on the system used. For dairy cows breeding is throughout the year, to satisfy the continual market demand for dairy products. In the dairy industry the use of AI has increased to over 60% and 85% in cows and heifers respectively, over the last 2 decades (Butterfield *et al.*, 1988).

Generally the most important indicators of fertility in a dairy cattle herd are the first service conception rate, number of services or straws per conception, the intercalving interval and the number of days open. Of the factors that affect reproductive efficiency, failure to detect oestrus is one of the most important (Medina *et al.*, 1994). Oestrous detection problems in dairy cows have been recognised over many years. Barr (1975) was one of the first researchers to evaluate the number of days lost due to undetected oestrous periods and it is still currently the limiting factor of reproductive efficiency in cattle (Heersche & Nebel, 1994). Oestrous detection efficiency as such, is generally defined as the percentage of cows detected in oestrus with relation to those actually in oestrus (Heersche & Nebel, 1994). The use of milk progesterone concentration to monitor the major reproductive events (failure or inappropriate oestrous detection, missed oestrous periods and early embryonic mortality) gives greater accuracy to the determination of the reproductive efficiency in large dairy farms (Cavestany & Foote, 1985).

The objectives of this study were (i) to evaluate/determine the factors involved in successful AI following oestrous detection and conception by means of the analysis of reproductive records and milk progesterone concentrations determined at day 0 (breeding), 10 to 12 and day 22 to 24 after breeding; and (ii) to determine the efficiency of oestrous detection with the aid of milk progesterone values levels obtained on the day of breeding (day 0).

5.2 MATERIALS AND METHODS

5.2.1 Farm, animals and records

The survey was conducted on 5 commercial dairy farms in Gauteng and Mpumalanga South Africa, each with more than 150 cows in milk. Animals that were selected had calved throughout the year and formed part of a population of 556 Holstein cows and 212 Jersey cows (768 cows in total) utilised in this study. Cows varied in age from 30-65 months, with BW ranged from 380 (Jersey) to 670 kg (Holstein). The ODR, PR and CR were recorded over 6 cycles of 21 days.

Nutrition in the herds was based on improved pastures with supplementation of maize silage (20-25kg/cow/day), hay ad lib and concentrates (10-13kg/cow/day) with 17-19% protein content with a relative energy value of 12MJ/kg/day fed in the form of a mixed diet throughout the year. Machine milking was carried out twice daily (6:00, 18:00) on 3 farms and 3 times per day (6:00, 14:00, 22:00) on 2 farms respectively. The oestrous detection system used was based on overt signs of oestrus (cow standing to be mounted) and these observations were carried out twice a day at the time when cows were moved to the milking parlour (6:00, 18:00). The time devoted to observation of the cows for oestrus was approximately 1 hour in the morning and again an hour in the afternoon. AI was used exclusively on the farms with no clean up bulls being used in the breeding system.

The data collected from the herds included calving date, parity (1=first-calf heifers, 2=mature cows), ease of calving (1=normal, 2=simple assistance and 3= major assistance), breeding dates, pregnancy diagnosis, monthly milk production, body weight, body condition score at calving and at each mating and milk progesterone level to monitor the efficiency of oestrous detection. Information was collected in a database designed especially for this purpose. Body weight (BW) and body condition score (BCS) at calving and at AI were only obtained on farm B. The BCS was assessed according to the scale of 1 to 5 as described by Edmonson *et al.* (1989), but for the statistical analysis cows were grouped in low body condition (≤ 2) and moderate (>2), groups which allowed the classing of the cows into 2 respective groups. The milk production of each cow was recorded daily and the total was calculated monthly. Reproductive records were recorded until the cow was certified pregnant or culled. The farm veterinarians performed pregnancy diagnoses every month, starting at 2 months after the beginning of the breeding period or AI.

5.2.2 Milk sampling

Milk samples for progesterone (MP) concentration determinations were collected throughout the breeding period. Milk sampling (6:00) was performed at day 0, 10 and 23 after AI and if the cow returned to oestrus, milk sampling was re-initiated. In this scheme, according to the milk progesterone levels, cows could be classified e.g. as in oestrus (low-low-low milk progesterone levels); bred at the right time and pregnant (low-high-high milk progesterone levels); bred at the right time and open (low-high-low milk progesterone levels); bred at the wrong time (high-low-high- or high-high-low milk progesterone levels); or bred pregnant (high-high-high milk progesterone levels) (Table 5.1 and 5.2).

5.2.3 Milk progesterone analysis

Milk samples were collected in 20 ml plastic vials containing sodium azide (Merck KgaA, Damstadt, Germany) and stored at 4°C at the farm for later transport to the radio-immunoassay (RIA) laboratory. There the milk samples

were centrifuged and the fat free fraction stored at -20°C until analysed for progesterone concentration content with the aid of a solid phase RIA kit. The intra-assay coefficient of variation (CV) for samples with values below 1 nmol/l milk progesterone level was 8.2% and for samples with a P4 value of above 1nmol/l 9.8%. The inter-assay coefficient of variance was 11.7 and 4.5% for samples, with P4 values below or above 1 nmol/l milk progesterone, respectively. Results and concentrations were determined and the standard inter-assay and assay variation is set out in 3.4.7.

Table 5.1 Milk progesterone levels from three milk samples and interpretation in relation to rectal pregnancy diagnosis in dairy cows

Day 0 (day of AI)	Day 9-13	Day 21-24	Rectal palpation results; interpretation
[1]Low	[2]High	High	Pregnant
Low	[3]Intermediate	High	Pregnant; RIA problem, biological variations
Low	High	Low	Non-pregnant; fertilisation failure, early embryonic death, post AI anoestrus
Low	Intermediate	Low	Non-pregnant; fertilisation failure, short luteal phase, RIA problem, biological variation
Intermediate /High	Low/intermediate /High	Low	Non-pregnant; AI at incorrect time, post AI anoestrus
Low	High	High	Non-pregnant; late embryonic death (>16 days) luteal cyst, persistent corpus luteum (CL)
High	High	High	Pregnant; AI on pregnant animal
Low	Intermediate	High	Non-pregnant, RIA problem, biological variation, late embryonic death, persistent CL
Low	High	Intermediate	Non-pregnant, fertilisation failure, late embryonic death RIA problem, biological variation
Low	Low	Intermediate	Non-pregnant, AI in anoestrous cow, RIA problem
Intermediate /High	Low/Intermediate /High	Intermediate /High	Non-pregnant, AI at incorrect time, luteal cyst, persistent CL

[1]Low = <1.0nmol/l [2]High = ≥3.0 nmol/l [3]Intermediate = between 1.0 and 3.0 nmol/l

5.2.4 Oestrous detection rate and pregnancy rate

The oestrous detection rate (ODR) was defined as the percentage of cows bred out of the total number of cows submitted to service in a 21 day period.

Conception rate (CR) again was seen as the percentage of cows pregnant from those bred. Pregnancy rate (PR) is then the product of oestrous detection rate and CR in a period of 21 days (Ferguson *et al.*, 1993). These parameters were determined from the available reproductive records.

Table 5.2 Milk progesterone concentrations on the day of service and on day 9 to 13 with respect to the accuracy of oestrous detection

Day 0 (days of AI)	Day 9-13	Interpretations
[1]Low	[2]High	Milk progesterone concentration within negative range on day 0 and within positive range on day 9-13 indicates an ovulatory cycle-accurate oestrous detection
Low	Low	Milk progesterone concentration within negative range on both days indicates anoestrus, anovulation, or short luteal phase-inaccurate oestrous detection
High	High	Milk progesterone concentration within positive range on both days indicates AI in pregnant animals or in animals with luteal cyst-inaccurate oestrous detection
High	Low	Progesterone concentration within positive range on day 0 and within negative range on day 9-13 indicates that AI was performed during luteal phase-inaccurate oestrous detection

[1]Low = <1.0nmol/l [2]High = ≥3.0 nmol/l [3]Intermediate = between 1.0 and 3.0 nmol/l
Thirty-two (6.7%) services were made in cows with an intermediate level of milk progesterone on day 0, day 9-13 or on both occasions

5.2.5 Inter-oestrous intervals

Another important parameter to determine the efficiency of oestrous detection and to evaluate the embryonic mortality (EM) was by determining the inter-oestrous interval. To calculate this, intervals were classified as: <18 days ("short"), 18 – 24 days ("normal"), 23-35 days ("extended" or EM or incorrect oestrous detection), 36-48 days ("2 x normal", one missed oestrus or EM), and >48 days ("long", two missed oestruses or EM or abortions) (Macmillan & Day, 1982).

5.2.6 Statistical analysis

The statistical analysis of the intervals from calving to first service and from calving to conception, were carried out according to the following general linear model (SAS, 1990):

$$INT_{ijklmnopqrs} = \mu + b_j + c_k + d_l + e_m + f_n + g_o + h_p + i_q + j_r + k_s = \varepsilon_{ijklmnopqrs}$$

Where INT is the interval from calving to first service or to conception; b is the *j*th effect of farm (A, B, C, D and E); c is the *k*th effect of parity (<1 and >2); d is the *l*the effect of ease of calving (1, 2, 3 and 4); e is the *m*th effect of BW (≤500 and > 500kg) f is the *n*th effect of BCS at calving (<1 and >2); g is the *o*th effect of month of calving (1-7); h is the *p*th effect of month of breeding (5-12); I is the *q*th effect of BW at service (≤ 500 and >500 kg); j is the *r*th effect of BCS at service (1 and 2); k is the *s*th effect of milk production at service (5,10,15,20,25,30l) and $\varepsilon_{jklmnopqrs}$ is the error.

Interactions between variables were tested and mean comparison was done using the LSD method. To analyse conception rate at first service and overall pregnancy rate the CATMOD procedure was utilised (SAS, 1990).

5.3 RESULTS

5.3.1 Overall reproductive performance

Of the 768 cows selected only 691 (90%) had at least one service/AI post partum, whereas the remaining 10% were culled or died before the first insemination. From this population, 540 cows (78%) conceived at first service. Table 3 summarises the data for the interval from calving to first service and from calving to conception on the 5 farms. The overall mean interval from calving to first service (ICS) was 101.5 ± 1.9 days and to conception (ICC) 132.4 ± 3.2 days. The first service conception rate was 40.5% and overall conception rate 78.1% with an average of 2.4 services per conception.

Of all the factors analysed, the ICS and ICC were significantly ($P<0.05$) affected only by parity. First-calf heifers had an interval that was 40 days longer than the mature cows (124 versus 84 days). Animals with a BW below 500kg recorded a longer mean interval than heavier (>500kg) cows (118 versus 85 days). Cows calving in a poor (≤ 2) BCS had a mean interval to first breeding of 113 days, compared to cows calving in a moderate BCS (>2), who had an average first service at 88 days after parturition. The intervals to conception followed a similar pattern. BW and BCS at breeding did not affect these parameters, neither did the differences between BW and BCS at first breeding or at calving. There was no significant interaction between parity and BW at calving. The average monthly milk production did also not significantly affect these parameters.

Table 5.3 Effect of parity, body weight and body condition score at calving on the intervals from calving to first service (ICS) and to conception (ICC)

Parameter	Category	n	ICS Mean ± SE	n	ICC Mean ± SE
Parity	Heifers	240	124 ± 2.9^a	188	146 ± 3.7^a
	Cows	451	84 ± 2.2^b	352	113 ± 3.0^b
Body weight	<500kg	348	118 ± 2.6^a	266	140 ± 3.7^a
BW at calving	>500kg	252	85 ± 2.6^b	203	113 ± 3.2^b
BCS	≤ 2	282	113 ± 2.2^a	255	130 ± 3.3^a
At calving	>2	308	88 ± 2.6^b	205	118 ± 3.2^b

a, b Superscripts within columns differ significantly ($P<0.01$)

5.3.2 Accuracy of oestrous detection

Of 1215 milk samples obtained on the day of breeding, 135 (11.1%) had milk progesterone concentrations greater than 3 nmol/l and were considered as luteal levels according to the precision of the RIA test. According to this criterion, cows with milk P4 levels of above 3nmol/l were bred at the wrong time. Only 5 of the total cows were bred pregnant (milk P4 values >3nmol/l on

days 0, 10 and 23) and were thus excluded from this calculation. Farm significantly (P<0.05) affected the incorrect oestrous detection, as is set out in Table 5.4.

Table 5.4 Number and percentage of cows with milk progesterone levels above 3 nmol/l on the day of breeding, relative to the farm

Farm	n	High milk P4 (%)
A	406	7.4[a]
B	116	8.3[a]
C	179	4.5[a]
D	340	10.3[a]
E	174	30.5[b]
Total	1215	
Average		11.1

a = P<0.05
b = P<0.01)

5.3.3 Pregnancy diagnosis

According to the milk P4 values on day 23 following breeding, 564 cows were diagnosed as pregnant, of which 397 were confirmed by rectal palpation at approximately day 60 - representing a 70.4% accuracy rate for milk progesterone determinations. Of the 167 cows pregnant according to RIA determinations and open according to rectal palpation, 154 came into oestrus again during the experimental period. The mean interval from the time of breeding and the following oestrus was 57 days and the median was 48 days.

5.3.4 Oestrous detection success rate (ODR) and pregnancy rate (PR)

Reproductive records were analysed to determine the oestrous detection rate and the pregnant rate following the first service, in cycles of 21 days, from the onset of the breeding observation period (Table 5.5). The overall oestrus detection rate was 37.5% and predicted pregnancy rate was of 15.6 %, due to the low ODR and a conception rate of 41.9%. As cows not bred in the same period, a total of 1424 animals in 6 periods of 21 days were evaluated for the calculation of the ODR and PR.

Table 5.5 Oestrous detection (ODR), pregnancy (PR) and conception rates (CR) recorded in 6 cycles of 21 days (dates in parenthesis)

Period	n	ODR^{NS}(%)	PR^{NS}(%)	CR^{NS}(%)
1(5/20-6/10)	380	42.9	17.6	41.1
2(6/11-7/2)	298	38.3	18.3	47.7
3(7/3-7/24)	222	36.0	14.3	39.7
4(7/25-8/15)	206	38.8	14.5	37.4
5(8/16-9/6)	174	35.1	14.5	42.5
6(9/7-9/28)	144	34.0	14.6	42.9
Total	1424			
Average		37.5	15.6	41.9

^{NS} = Not significant

5.3.5 Inter-oestrous intervals

Another method to calculate the oestrous detection efficiency and embryonic mortality rate was to calculate the intervals between services or the inter-oestrous intervals. A total of 718 intervals were recorded with a mean inter-oestrous interval of 36 days. Only 45.1% of the intervals were within the normal range of 18 to 24 days - 5.8% intervals were "short" (<18 days) and only 1.7% of the intervals were "long", whereas 28.0% were 2x normal (36 to 48 days), meaning one missed oestrus and 19.4% were "prolonged", representing two missed oestrous periods or abortions. Figure 5.1 illustrates the percentage of intervals in the different classes.

5.4 DISCUSSION

5.4.1 Overall reproductive performance

The reproductive parameters recorded were similar to those reported for cows in temperate climates (Esslemont, 1992; Medina *et al.*, 1994; Etherington *et al.*, 1996) or dry tropical regions (Antal *et al.*, 1987). The mean interval recorded from calving to first service of 101.5 days was considerably longer

than the ideal of 65 days as proposed by Van Eederburg *et al.* (1996). A similar long period was reported in the New Zealand dairy systems.

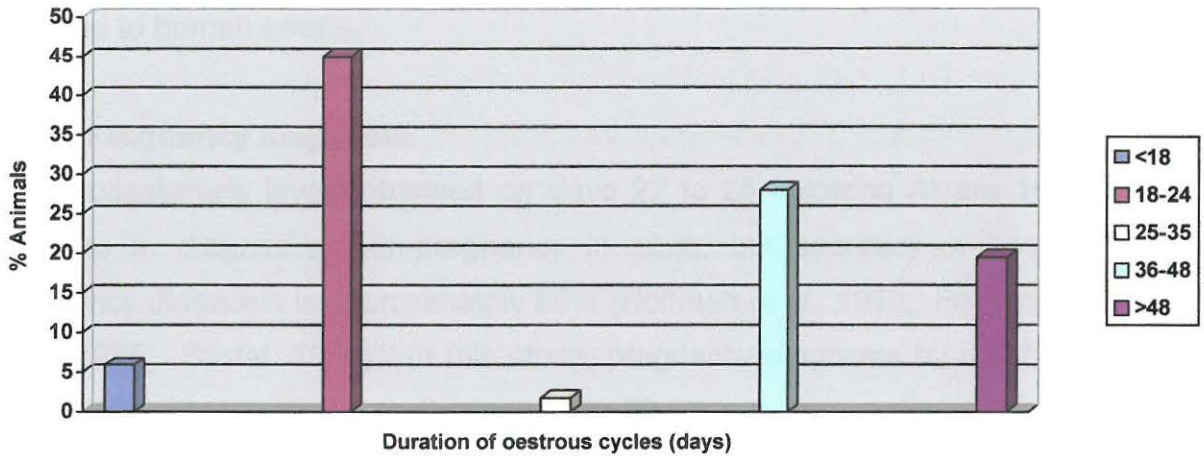


Figure 5.1 Percentage of oestrous cycles of different duration in dairy cows

However with a voluntary resting period of 40 days, the interval was never recorded to be shorter than 70 days (Silva *et al.*, 1992). This could be due to a prolonged post partum anoestrous period or poor oestrous detection. The methodology used in this study was not able to clarify these differences. The interval to first service was also affected by the parity of the cow, being 40 days longer for first-calf heifers (124 days) than for mature cows (84 days). This difference is in agreement with that reported elsewhere (Silva *et al.*, 1992). Body weight and body condition at calving affected this interval (Table 5.3). Wolfenson *et al.* (1994) found cows with a BCS of less than 2.5 at calving to have an interval to service of 80 days, as opposed to 47 days for animals calving with a BCS of greater than 2.5.

5.4.2 Accuracy of oestrous detection

Milk progesterone determination is an important diagnostic tool in large cattle herds (Cavestany & Foote, 1985). In this study 11.1% of th cows were incorrectly detected in oestrus, compared to percentages lower than 10% reported elsewhere (Pennington *et al.*, 1985; Reimers *et al.*, 1985; Rajamahendran *et al.*, 1993). However, errors of approximately 20% have been more commonly quoted (Shemesh *et al.*, 1978; Nebel *et al.*, 1987). The

error in oestrous detection was consistent for different matings, but an important difference between farms was detected - which agrees with Reimers *et al.* (1985), Nebel *et al.* (1987). King (1993) found that one of the main problems in AI programmes to be poor oestrous detection and the cause was due to human error.

5.4.3 Pregnancy diagnosis

Milk progesterone levels obtained on days 22 to 25 following AI are 100% accurate in diagnosing non-pregnancy in cows, but accuracy of positive pregnancy diagnosis is approximately 80% (Hoffman *et al.*, 1976; Pennington *et al.* 1985; Systat, 1996). In this study, pregnancy diagnosis by means of milk P4 on day 23 after breeding was only 70.4% accurate - which is in line with the results of Rajamahendran *et al.* (1993). According to these researchers, early embryonic mortality is another major cause of reproductive failure in cows. Mann *et al.* (1996) recorded an embryonic mortality rate of 10.8% with a period of greater susceptibility for losses between days 31 and 55 of gestation. In this study the mean interval to the next oestrus for cows diagnosed pregnant by means of milk P4 was 57 days - which suggests that this long interval can probably be related to embryonic deaths.

5.4.4 Oestrous detection success rate (ODR) and pregnancy rate (PR)

The oestrous detection success rate was 37.6%, which is similar to the 43% reported by Medina *et al.* (1994), 38% of Antal *et al.* (1987), 38% of Heersche and Nebel (1994) and 48% of Etherington *et al.* (1996). The ODR was lower than the 62.8% reported by Systat (1996), the 52% of Esslemont (1992) and the 74% of Van Eederburg *et al.* (1996). With a conception rate of 42% and a mating efficiency of 37.5%, the reproductive efficiency was only 16%. In dairy systems with a restricted seasonal breeding period, a low pregnancy rate seriously compromises the goal of a 12-month calving interval and partially explains the prolonged period to the first service. This unacceptable low ODR and PR could indicate a serious problem in the dairy industry of South Africa.

5.4.5 Inter-oestrous intervals

The analysis of the inter-oestrous intervals is another way of testing the oestrous detection efficiency (Esslemont, 1992). Evaluated by this method, the mating efficiency in this study (conception rate to first service) was 45.1%, which is greater than the 37.5% recorded previously and considered only at the first service (Heersche & Nebel, 1994). A possible cause of this disparity could be that considering only the cows in anoestrus were included that could not be identified by the methodology used in the study. According to Esslemont (1992) with acceptable oestrous detection efficiency, the ratio between normal inter-oestrous intervals (17-24 days) and abnormal intervals should be 7:1 - much higher than the 2.2:1 ratio found in this study. Expressed in another way, 85% of the inter-oestrous intervals should be within the normal cycle range - different from the 45% reported in this study. This analysis confirms the poor oestrous detection efficiency in dairy herds, but other factors such as high embryonic mortality rates due to pathological causes could possibly have affected the results.

5.5 CONCLUSION

In this study, oestrous detection was satisfactory and very few cows were inseminated wrongly (not in oestrus). On farms with poor conception rates, where oestrous detection inefficiency was suspected, the test has been shown to be of some use. The low progesterone levels around the oestrous period preclude the use of the test to determine the optimum time of insemination. Thus the use of milk progesterone analysis in an artificial breeding program is however also limited.

CHAPTER 6

MILK PROGESTERONE PROFILES OF POST PARTUM DAIRY COWS AS AN AID TO DIAGNOSE PROBLEM COWS

6.1 INTRODUCTION

By monitoring milk/serum progesterone levels of post partum dairy cows at frequent intervals, a continuous assessment of ovarian activity can be made. Information obtained from such profiles can be used to detect abnormal sexual processes, enabling the assignation and subsequent implementation of relevant hormonal treatments (Stevenson & Pursley, 1994). The fertility problems which can be identified using milk progesterone concentrations are those related to progesterone secretion by the corpus luteum and include the occurrence of anoestrus, silent heats, ovarian cysts and irregular oestrous cycles (Butterfield *et al.*, 1988).

Anoestrous cows are those in which the cyclic function of the ovaries is arrested or absent (Macmillan & Burke, 1996), while pospartum or lactation anoestrus has been defined as the absence of cyclic activity (milk progesterone levels of 1 nmol/l or less) for more than 50 days after parturition (Gustafsson *et al.*, 1986; Cavestany & Galina, 2001). These anoestrous conditions may be treated with gonadotrophin releasing hormone (GnRH) or a combination of oestrogen and progesterone therapies (Esslemont & Kossaibati, 1998). Cyclic ovarian function is then normally fully restored in most cases. (Aitken *et al.*, 1995).

Another symptom typical of the problem cow is the occurrence of the phenomenon of silent heat/oestrus. Silent heat can be defined as oestrous symptoms of the cow that were not observed by the herdsman, or identified by the bull, but where milk progesterone concentrations (if determined), reveals a normal cyclic progesterone pattern (Mann & Lamming, 1995). Studies have shown that at least 50% of all cows have one silent heat/oestrus post partum, before showing the first behavioural oestrus (Peters *et al.*, 1992;

Moffitt, 1995; Williams *et al.*, 1997). The number of overt oestruses increase thereafter and the incidence of silent heats/oestruses has been found to be negligible by the end of the fourth month after parturition (Short *et al.*, 1990). However, silent heat has been found to be present in at least 32% of normal post partum oestrous cycles (Claus *et al.*, 1983) and the occurrence is shown by 48% of cows in a herd (Stevenson *et al.*, 1999). The potent luteolytic agent prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and certain of its analogues (e.g. cloprostenol) has been used to induce oestrus in cows with unobserved oestruses (Archbald *et al.*, 1990). Administration of cloprostenol during the luteal phase of the cycle results in a rapid decline in progesterone levels due to luteolysis of the CL. This decline in progesterone generally occurs within 24hours after administration in most cows and is followed by an induced oestrus 36 hours later (Etherington *et al.*, 1994; 1996).

Short (less than 17 days), or irregular oestrous cycles are also associated with problem or sub - fertile cows. These irregular cycle lengths are thought to be caused by an insufficient luteotrophic stimulus, needed to maintain the corpus luteum (CL) for its normal life span. Poor CL function is not effective in maintaining pregnancy and therefore exogenous hormone therapy is often necessary (Etherington *et al.*, 1991). One method of hormonal therapy is the pre-treatment with Norgestomet. Treatment consists of 3 mg of a synthetic progesterone (17 χ- acetoxy - 11β - methyl - 19 - norpre - 4 - enc - 20,dione) in a polymer implant inserted subcutaneously for a few days after the occurrence of oestrus. This has been shown to increase the mean concentration of plasma LH by increasing the frequency of LH pulses (Heuwieser *et al.*, 1997). This in turn causes an increase in oestradiol in the plasma and is associated with ensuring a luteal phase of normal length (Pursley *et al.*, 1997a). Injections of LH during the luteal phase of the cycle would also prolong the functional life span of the CL (Pursley *et al.*, 1997b). The use of this therapy is limited by the lack of sufficient quantities of purified pituitary LH (Pursley *et al.*, 1995). Thus, gonadotrophin preparations such as pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) have been used to support CL function (Fricke *et al.*,

1993).

The occurrence of ovarian cysts is another phenomenon effecting the number of sub-fertile cows. There are 3 types of ovarian cysts, namely follicular, luteal and anovulatory cysts. The first is characterised by low progesterone levels and the latter by high circulating progesterone levels (Macmillan & Burke, 1996). CL's are non-pathological structures and are composed of a cystic type structure in conjunction with a mature CL (Stevenson *et al.*, 1998). Ovarian cysts are normally treated similarly, using luteotrophic agents. Differentiation between luteal and follicular cysts by rectal palpation is often difficult (Risco *et al.*, 1995). However, recent studies have indicated GnRH and hCG therapies to be more effective in cows with follicular cysts, whilst luteal cysts are more responsive to $PGF_{2\alpha}$ (Benmrad & Stevenson, 1986; Bostedt *et al.*, 1980; McClary *et al.*, 1989; Stevenson & Pursley,1994).

Cows in which embryonic mortalities occur are often classified as problem cows. Embryonic mortality refers to reproductive losses during the embryonic period, a period lasting approximately 45 days after AI (Lonergan *et al.*, 1994). After this period, the conceptus is generally called a foetus and reference at this stage is made of foetal mortality and even abortion. However, the conventional terminology of embryonic mortality will be used in this context - referring mainly to embryonic resorption (Martin *et al.*, 1998).

Estimates of embryo mortality range between 9.4 and 21.1%, based on the delay in return to oestrus of the cow - also being affected by the efficiency of oestrous detection (Mann *et al.*, 1998; Lozano *et al.*, 2000). Recently, predictions have been based on (i) sustained production of serum progesterone during the first 28 days following breeding, followed by a sudden decline with a return to oestrus in a 28 to 75 day interval (Mann *et al.*, 1998) or (ii) by the occurrence of oestrus in inseminated cows which is assumed to represent embryonic mortality (Ashworth *et al.*, 1989). Studies using milk progesterone levels as an indication of embryonic mortality rates have reported rates as high as 7.2% (Abecia *et al.*, 1996), 12% (Elrod &

Butler, 1993) and 16.9% (Claus *et al.*, 1983). Normally, the reproductive problems mentioned by these researchers have a low incidence, but collectively they may constitute a major cause of reproductive inefficiency. In the two dairy herds studied, cases with these problems were monitored, enabling early diagnosis and treatment.

6.2 MATERIALS AND METHODS

The two Holstein herds participating in the study were from the Tarlton and Rayton areas of Gauteng. Twenty multiparous mature Holstein cows from each of these Holstein herds were allocated to the study on the basis of either a previous reproductive history of poor conception or reproductive problems (e.g. embryonic reabsorption, abortions, irregular oestrous cycles, cows not showing oestrus, etc.) in their current lactation period, while normal fertile cows (n=20) acted as controls.

Milk samples were collected daily from the morning milking (6:00) as milk strippings in a measuring cylinder. Milk sampling was continued until the cow was diagnosed and confirmed pregnant by rectal palpation. Pregnant cows were removed from analysis and replaced by cows which had recently calved. Any pregnant cows which aborted or resobsorbed were re-assessed in the investigation until pregnancy was confirmed. Milk progesterone levels were measured in 5 cows over two successive open periods. Milk progesterone analysis (RIA) was performed as described in Chapter 3.

6.2.1 Hormone treatments

The 40 cows in the study (n = 40) were treated with reproductive hormones in response to problems encountered during their open period. The occurrence of silent heats/oestruses was overcome by the administration of a single dose of prostaglandin $F_{2\alpha}$ (Estrumate R Schering - Plough) after identified non-pregnant, with cows being inseminated at the induced oestrus. In order to prolong the luteal phase of oestrous cycle and augment serum progesterone levels during the early stage of the cycle (post AI), a Norgestomet capsule of progesterone (Crestar R, Intervet) was inserted. The progesterone capsule

was implanted subcutaneous in the ear 2 days following AI and left in situ for 6 weeks. A PMSG preparation (Chronogest; Intervet) could also be used as a method of supporting luteal function. Treatment consisted of an injection of 150 IU Chronogest, every 2 days, beginning 2 days after AI. Treatment of ovarian cysts consisted of 2ml of Fertagyl R (Intervet) for follicular cysts or 500mg prostaglandin (Estrumate; Schering Plough) in the case of luteal cysts. Cystic CL's were left untreated.

Nutrition in the herds was based on improved pastures with supplementation of maize silage (20-25kg/cow/day), hay ad lib and concentrates (10-13kg/cow/day) with 17-19% protein content with a relative energy value of 12MJ/kg/day administered in the form of a mixed diet throughout the year. Machine milking was carried out twice daily (6:00, 18:00) on both farms.

The oestrous detection system used was based on overt signs (standing to be mounted) and these observations were carried out twice a day at the time when cows were moved to the milking parlour. The time devoted to observation of the cows for oestrus was approximately 1 hour in the morning and again an hour in the afternoon. AI was used exclusively on the farms with no clean up bulls being used in the breeding system.

The data collected included calving rate, parity (1 = first-calf heifers and 2 = mature cows), ease of calving (1 = normal, 2 = simple assistance and 3 = major assistance), breeding dates, pregnancy diagnosis, monthly milk production, body weight, body condition score at calving and at each breeding and milk progesterone level to monitor the efficiency of oestrous detection.

Information was collected in a database designed especially for this purpose. Body weight (BW) and body condition score (BCS) at calving and at AI were recorded. The BCS was assessed according to the scale of 1 to 5 as described by Edmonson *et al.* (1989), but for the statistical analysis cows were grouped in a low body condition (≤2) and moderate (>2) condition group, which allowed the classing of the cows into 2 respective groups. The milk

production of each cow was recorded daily. Reproductive records were recorded until the cow was certified pregnant or culled. The farm veterinarians performed rectal pregnancy diagnoses every month, starting at 2 months after the last breeding period or AI.

6.2.2 Milk sampling

Milk samples for milk progesterone (MP) concentration determinations were collected throughout the breeding period. In this scheme, according to the milk progesterone levels, cows could be classified e.g. as in oestrus (low-low-low milk progesterone levels); bred at the right time and pregnant (low-high-high milk progesterone levels); bred at the right time and open (low-high-low milk progesterone levels); bred at the wrong time (high-low-high- or high-high-low milk progesterone levels); or bred pregnant (high-high-high milk progesterone levels) (Table 6.1 and 6.2).

Table 6.1 Milk progesterone concentrations on the day of service and on day 9 to 13 with respect to the accuracy of oestrous detection

Day 0 (days of AI)	Day 9-13	Interpretations
[1]Low	[2]High	Milk progesterone concentration within negative range on day 0 and within positive range on day 9-13 indicates an ovulatory cycle-accurate oestrous detection
Low	Low	Milk progesterone concentration within negative range on both days indicates anoestrus, anovulation, or short luteal phase-inaccurate oestrous detection
High	High	Milk progesterone concentration within positive range on both days indicates AI in pregnant animals or in animals with luteal cyst-inaccurate oestrous detection
High	Low	Progesterone concentration within positive range on day 0 and within negative range on day 9-13 indicates that AI was performed during luteal phase-inaccurate oestrous detection

[1]Low = <1.0nmol/l [2]High = ≥3.0 nmol/l [3]Intermediate = between 1.0 and 3.0 nmol/l
Thirty-two (6.7%) services were made in cows with an intermediate level of milk progesterone on day 0, day 9-13 or on both occasions

Milk samples were collected daily from the morning milking (6:00) as before as milk strippings in the measuring cylinder. Milk sampling was continued until the cow was diagnosed and confirmed pregnant. Pregnant cows were removed from analysis and replaced by cows which had recently calved. Any pregnant cows which aborted or reabsorbed were re-assessed in the investigation until pregnancy was confirmed. Milk progesterone concentrations were measured in 5 cows over two successive open periods.

Table 6.2 Milk progesterone concentration of three milk samples and interpretation in relation to rectal pregnancy diagnosis in dairy cows

Day 0 (day of AI)	Day 9-13	Day 21-24	Rectal palpation results; interpretation
[1]Low	[2]High	High	Pregnant
Low	[3]Intermediate	High	Pregnant; RIA problem, biological variations
Low	High	Low	Non-pregnant; fertilisation failure, early embryonic death, post AI anoestrus
Low	Intermediate	Low	Non-pregnant; fertilisation failure, short luteal phase, RIA problem, biological variation
Intermediate/ High	Low/intermediate/ High	Low	Non-pregnant; AI at incorrect time, post AI anoestrus
Low	High	High	Non-pregnant; late embryonic death (>16 days) luteal cyst, persistent corpus luteum (CL)
High	High	High	Pregnant; AI on pregnant animal
Low	Intermediate	High	Non-pregnant, RIA problem, biological variation, late embryonic death, persistent CL
Low	High	Intermediate	Non-pregnant, fertilisation failure, late embryonic death RIA problem, biological variation
Low	Low	Intermediate	Non-pregnant, AI in anoestrous cow, RIA problem
Intermediate/ High	Low/Intermediate/ High	Intermediate/ High	Non-pregnant, AI at incorrect time, luteal cyst, persistent CL

[1]Low = <1.0nmol/l [2]High = ≥3.0 nmol/l [3]Intermediate = between 1.0 and 3.0 nmol/l

6.2.3 Milk progesterone analysis

Milk samples were collected in 20 ml plastic vials containing sodium azide (Merck KgaA, Damstadt, Germany) and stored at 4°C at the farm for later transport to the radio-immunoassay (RIA) laboratory. There the milk samples were centrifuged and the fat free fraction stored at -20°C until analysed for progesterone concentration content with the aid of a solid phase RIA kit. The intra-assay coefficient of variation (CV) for samples with levels below 1 nmol/l milk progesterone was 8.2% and for samples with a P4 concentration of above 1nmol/l, 9.8%. The inter-assay coefficient of variance was 11.7 and 4.5% for samples with P4 concentrations below or above 1 nmol/l milk progesterone, respectively.

6.3 RESULTS

6.3.1 Post partum anoestrus

No documented records of post partum anoestrus (lack of cyclic activity for more than 50 days post partum) in the cows evaluated in this study, were recorded and no treatment had previously been given. Of the 40 Holstein cows, 26 (65%) were observed in oestrus. A further 5 cows (12.5%) were cycling, but had not been observed in oestrus. The remaining 9 cows (22.5%) exhibited their first oestrus signs 50 days post partum. Milk sampling from these cows started later than 50 days post partum, thus there were no progesterone profiles to indicate whether these cows had been cyclic at 50 days post partum. The length of the post partum anoestrous period did not seem to be a problem in these herds studied. The problem appeared to lie in silent or unobserved oestrous periods.

6.3.2 Silent heats/oestrous periods

Milk progesterone profiles to determine the number of silent or unobserved oestrous periods occurring in the cows are set out in Table 6.3.

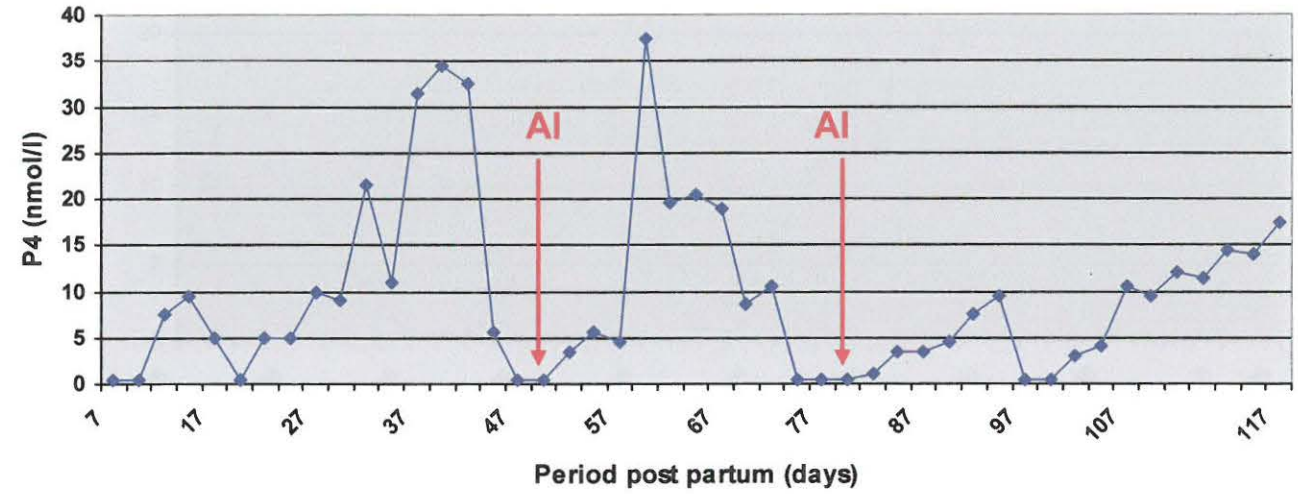
Table 6.3 Occurrence of silent heat/oestrous periods in two dairy herds as determined by milk progesterone profiles

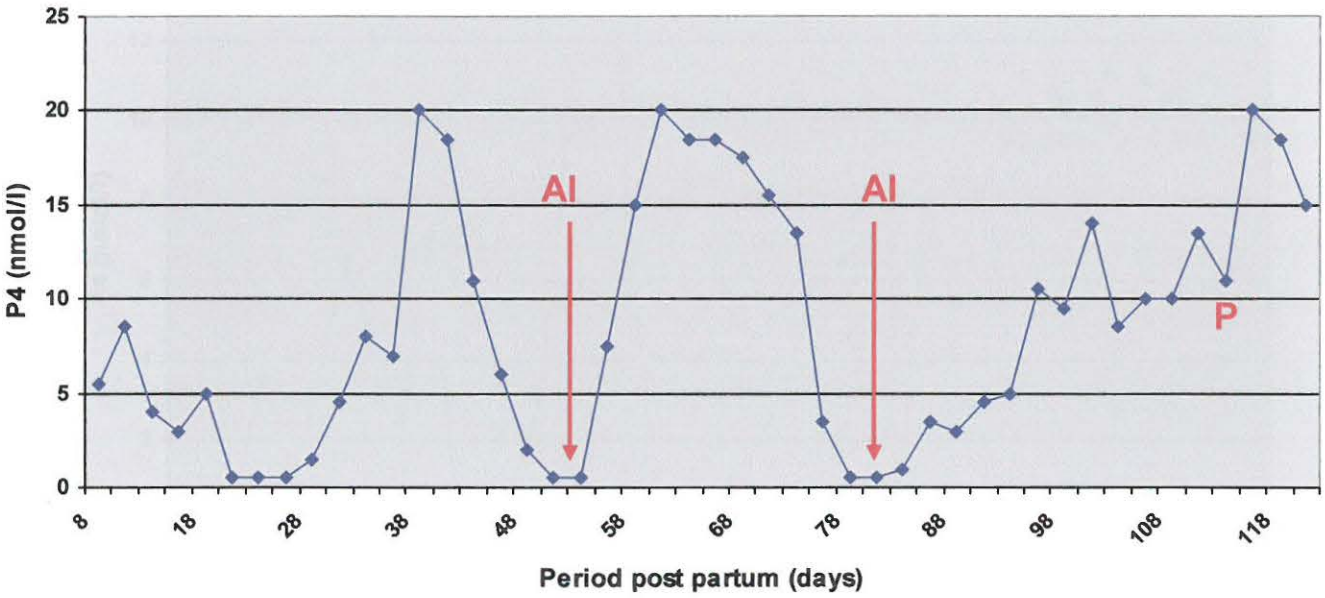
	Cow herd	
	Tarlton	Rayton
Total number of cows with progesterone profiles	16	19
Total number of oestruses	48	65
Number of silent oestruses (%)	12 (25%)	18 (28%)
Number of cows exhibiting silent oestruses (%)	9 (56%)	12 (63%)

Silent, or unobserved oestrous periods were a problem in both dairy herds, being greater in the Rayton herd, but only marginally so. The luteal phase milk progesterone levels were evaluated as a possible sign of silent heats. Examples of the milk progesterone profiles are illustrated in Figure 6.1. A low (3 nmol/l) peak progesterone level before the onset of oestrus was considered indicative of a silent heat, compared to a high (10 nmol/l) peak milk progesterone level. The average peak progesterone levels before silent and observed oestrous periods were 15.2 nmol/l and 16.5 nmol/l respectively. This difference was found not to be significant. Of the 3 cows treated for silent heats, 2 conceived (Figures 6.2). The third cow (Figure 6.3) was inseminated at the observed oestrus, but progesterone concentrations in the subsequent cycle remained low. Although the number of treated cows was small, it appeared that treatment with prostaglandin $F_{2\alpha}$ (Estrumate) was successful in inducing oestrus, and successful conception.

6.3.3 Short oestrous cycles and corpus luteum (CL) lifespan

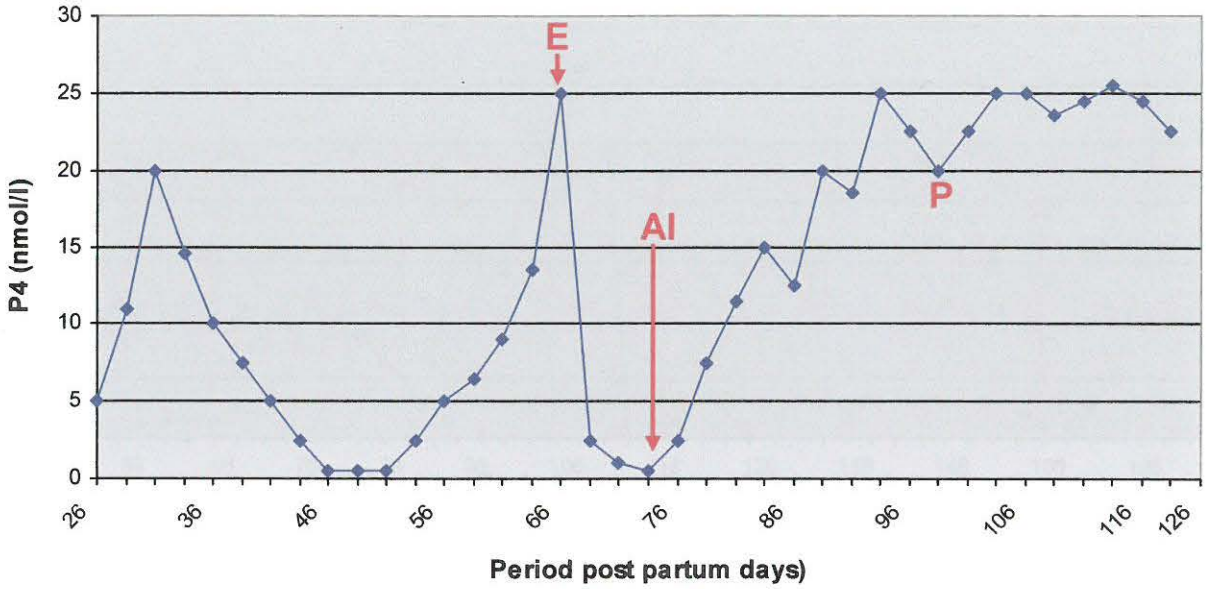
Short oestrous cycles (17 days) were recorded in one cow under investigation (Figure 6.4) and treatment with Norgestomet (progestagen) was advised. Milk progesterone concentrations increased within 2 days of implantation and continued to rise for 8 days. The luteal phase of the cycle was extended to 12 days and peak progesterone levels were increased. The cycle length of 23 days in this cow was considered normal, and although the cow did not conceive, subsequent cycles were of normal length with low progesterone concentrations during oestrus.

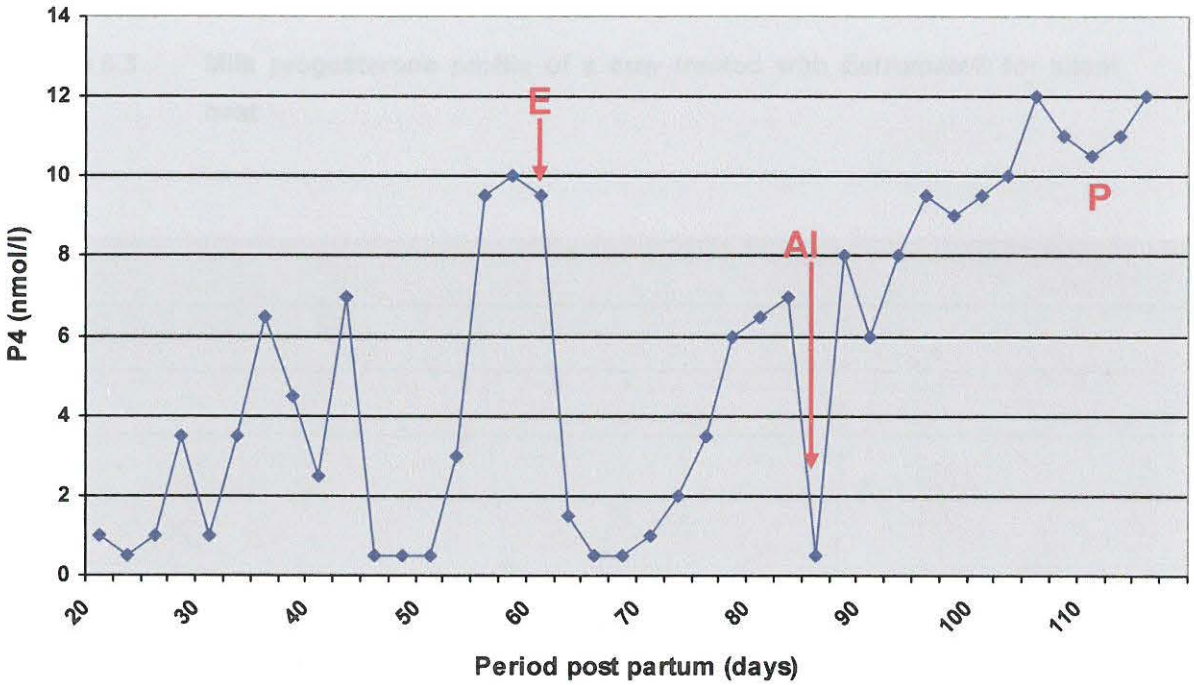




AI = Artificial Insemination
P = Pregnant

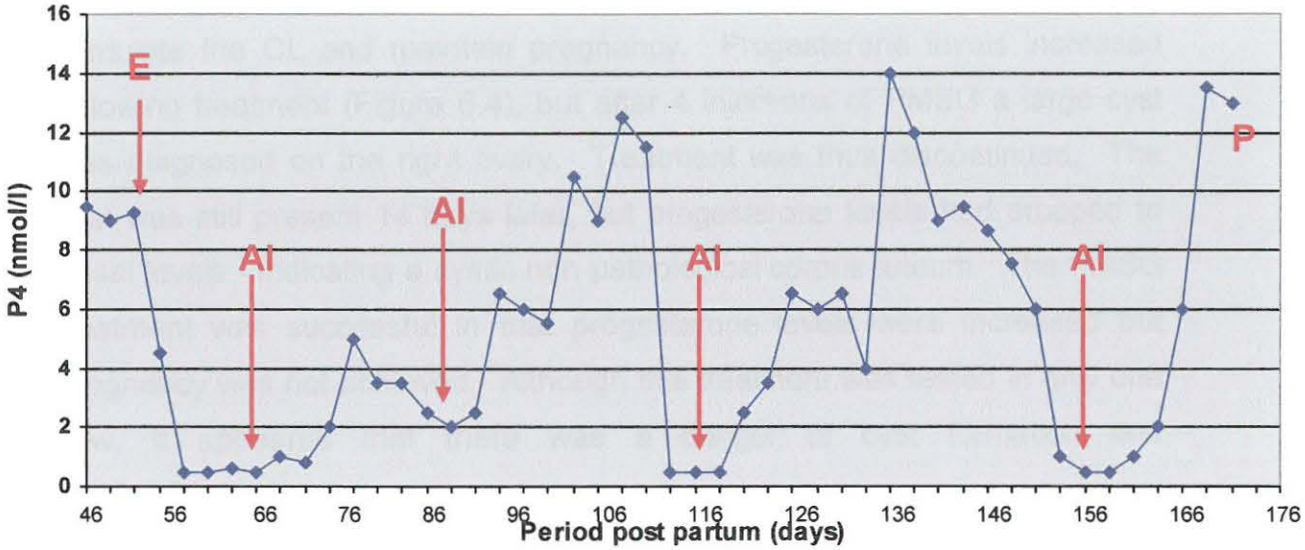
Figure 6.1 Milk progesterone profiles in 2 cows exhibiting silent heats





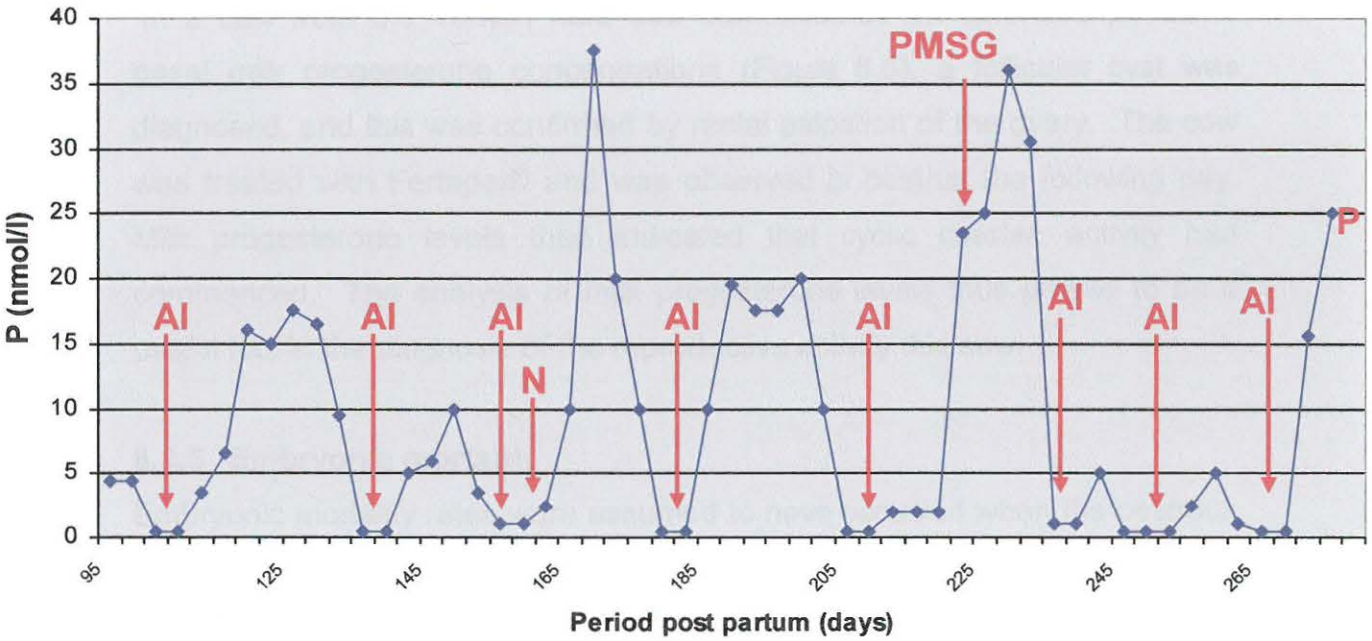
AI = Artificial Insemination
E = Estrumate
P = Pregnant

Figure 6.2 **Milk progesterone profiles of 2 cows treated with Estrumate ® for silent heats**



AI = Artificial Insemination
E = Estrumate
P = Pregnant

Figure 6.3 **Milk progesterone profile of a cow treated with Estrumate® for silent heat**



AI = Artificial Insemination
N = Norgestomet
P = Pregnant
PMSG = Pregnant mare serum gonadotrophin

Figure 6.4 **Milk progesterone profile of cow with short oestrous cycles**

The same cow was treated one cycle later with Chronogest in an attempt to stimulate the CL and maintain pregnancy. Progesterone levels increased following treatment (Figure 6.4), but after 4 injections of PMSG a large cyst was diagnosed on the right ovary. Treatment was thus discontinued. The cyst was still present 14 days later, but progesterone levels had dropped to basal levels - indicating a cystic non-pathological corpus luteum. The PMSG treatment was successful in that progesterone levels were increased but pregnancy was not achieved. Although this treatment was tested in only one cow, it appeared that there was a danger of cyst formation and superovulation.

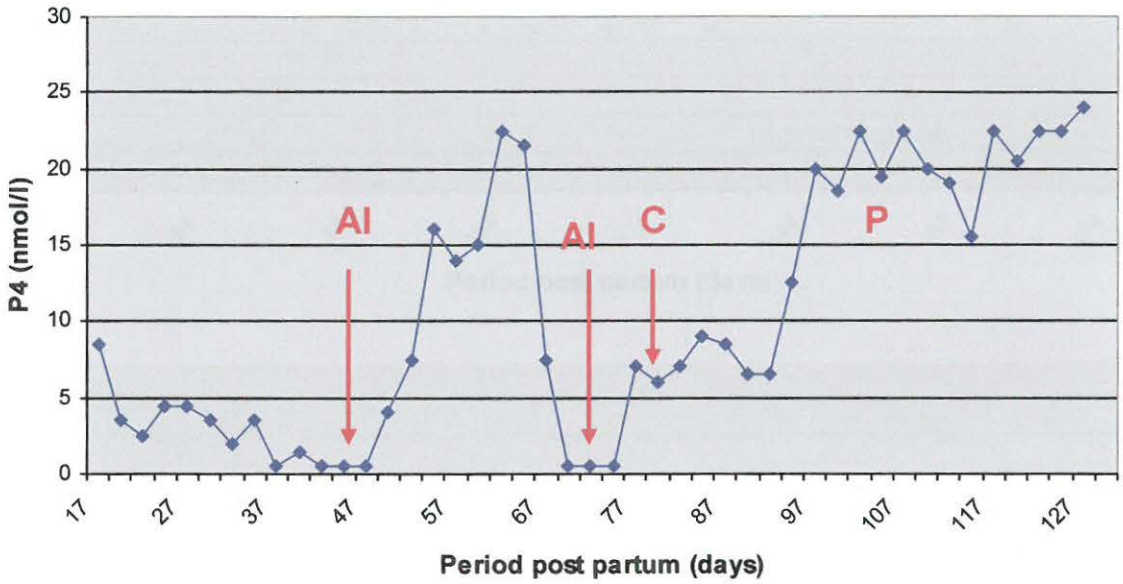
6.3.4 Cystic ovaries

Cystic structures were diagnosed by rectal palpation in 2 cows on the Rayton farm and confirmed by milk progesterone concentrations in one cow in the Tarlton herd. One of the cows at Rayton was subsequently diagnosed pregnant (Figure 6.5), whilst the other cows (Figure 6.4) continued cycling. Cystic CL's were diagnosed in these cows, and no treatment was prescribed. In a cow from the Tarlton herd that demonstrated an extended period of basal milk progesterone concentrations (Figure 6.6), a follicular cyst was diagnosed, and this was confirmed by rectal palpation of the ovary. The cow was treated with Fertagel® and was observed in oestrus the following day. Milk progesterone levels then indicated that cyclic ovarian activity had commenced. The analysis of milk progesterone levels thus proved to be a useful tool in the diagnosis of the reproductive activity this cow.

6.3.5 Embryonic mortality

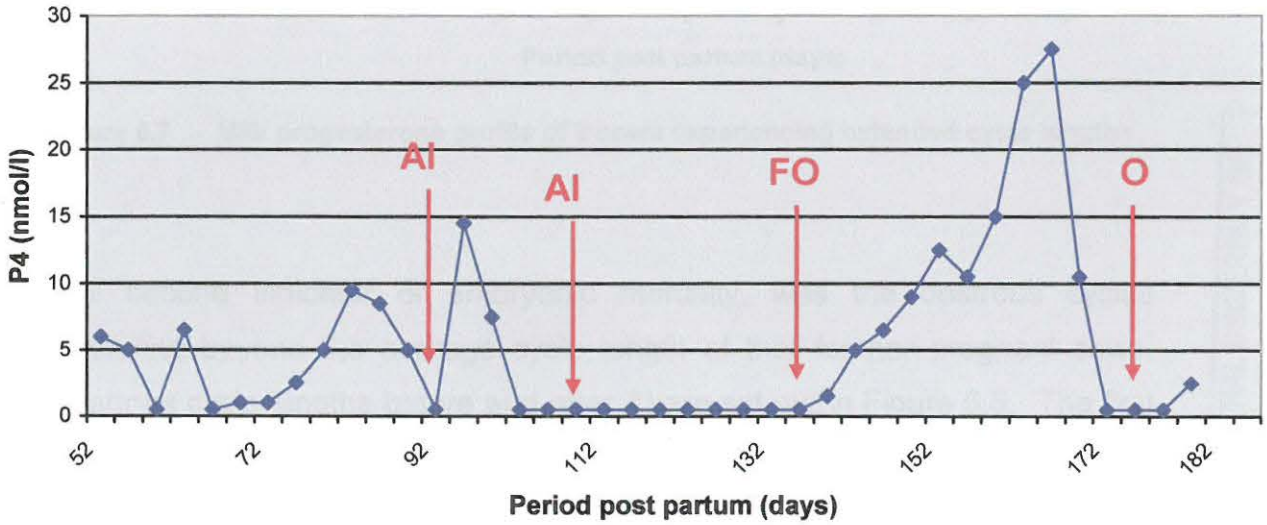
Embryonic mortality rates were assumed to have occurred when the oestrous cycles extended beyond 28 days in length with >3 nmol/P4. The incidence of embryonic mortality in this study, from the 79 oestrous cycle lengths recorded after AI, was 15.2 %. The general milk progesterone profiles of cows experiencing embryonic resorption are illustrated in Figure 6.7. Milk progesterone profiles in these cows were no different to those of cows experiencing normal pregnancy, until the return to oestrus. It also appeared

experiencing normal pregnancy, until the return to oestrus. It also appeared that embryonic losses took place between 28 and 75 days after conception, although this could not be attributed to inadequate luteal support.



AI = Artificial Insemination
C = Chronogest
P = Pregnant

Figure 6.5 Milk progesterone profile of cow with a cystic corpus luteum after AI



AI = Artificial Insemination
FO = Progestagen
O = Oestrus

Figure 6.6 Milk progesterone profile of a cow with a follicular cyst

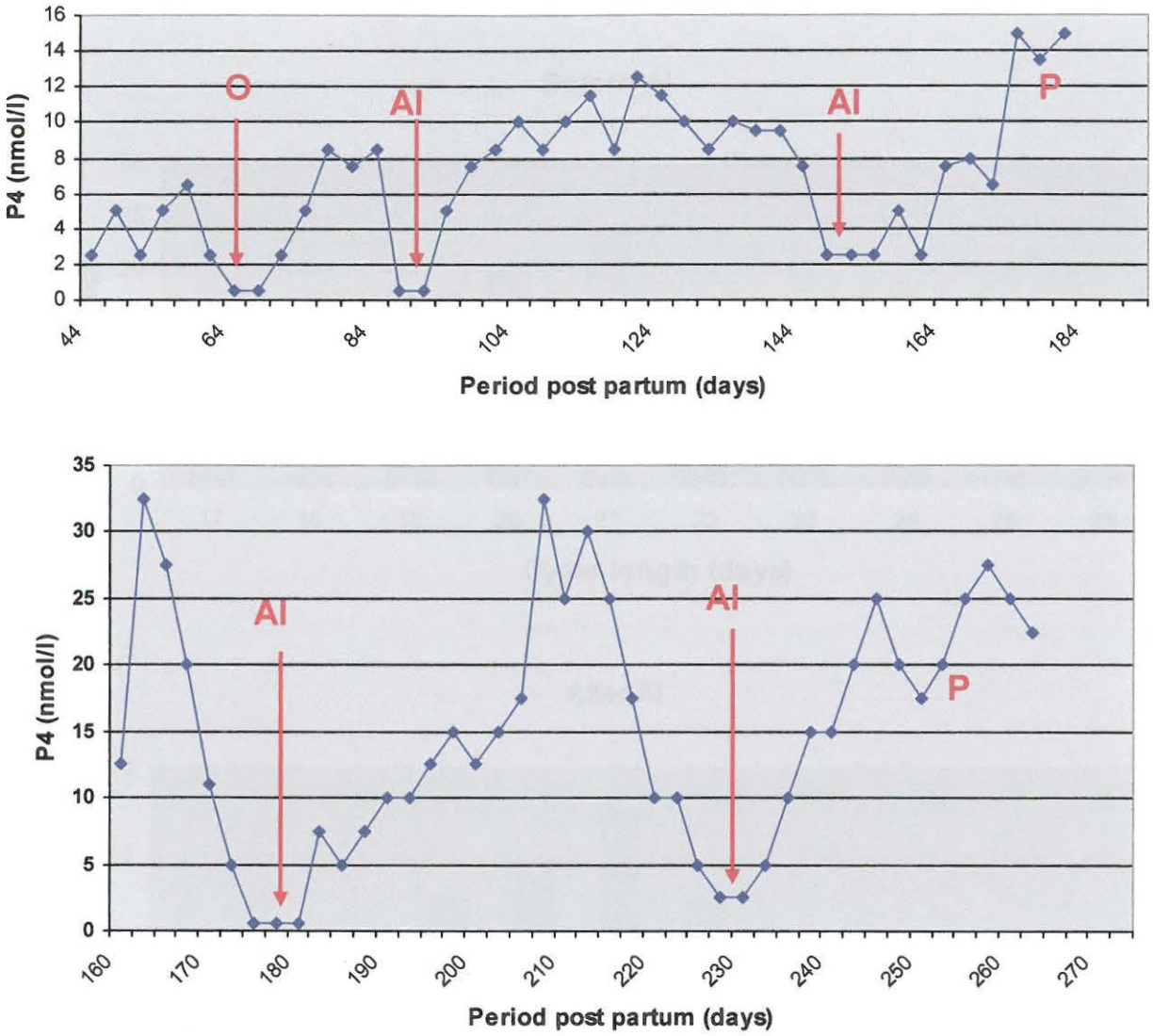


Figure 6.7 Milk progesterone profile of 2 cows experiencing extended cycle lengths

The second indicator of embryonic mortality, was the oestrous cycles extending beyond the average cycle length of that for non-pregnant cows. Oestrous cycle lengths before and after AI are set out in Figure 6.8. The first finding was that 93 % of pre-insemination oestrus cycles were of normal duration (17 - 25 days), compared to only 78.5 % of post-insemination oestrous cycles. The second finding was that the average length of an oestrous cycle for unmated cows was 20.4 days whilst that of inseminated cows was 25.5 days - an increase of just over 5 days. The question is, do these 5 days represent in part embryonic deaths, which were not previously

recognised?

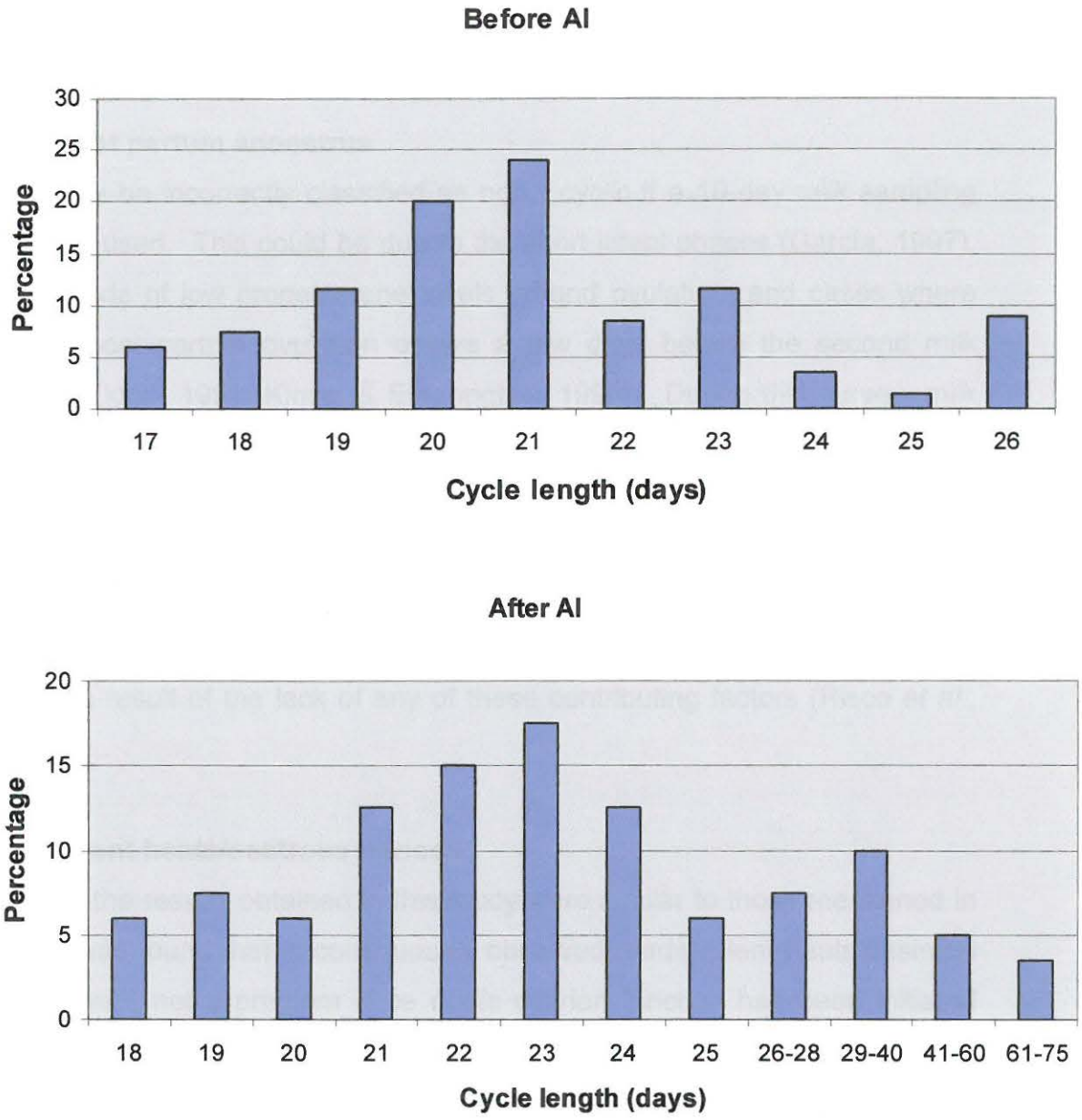


Figure 6.8 Distribution of cycle lengths before and after insemination in dairy cows

A factor which was taken into consideration in this trial was the number of days post partum when the oestrous cycles occurred. It has been demonstrated that the first post partum oestrous cycles are generally shorter than second, and subsequent cycles (Darwash *et al.*, 1997; Woolliams, 1997; Ball & McEwen, 1998; Veerkamp *et al.*, 2000). As few inseminations could be performed at the first oestrus, which may introduce bias results. Oestrous

cycles were thus grouped according to post partum period as well as AI'd or not AI'd.

6.4 DISCUSSION

6.4.1 Post partum anoestrus

Cows may be incorrectly classified as non - cyclic if a 10 day milk sampling interval is used. This could be due to the short luteal phases (García, 1997), long periods of low progesterone levels around ovulation, and cases where the first post partum ovulation occurs a few days before the second milk sample (Eldon, 1991; Kinsel & Etherington, 1998). During this survey, milk samples were collected daily, but analyses were performed on every second sample. There were thus no problems in the diagnosis of the cows. Post partum anoestrus may be due to either the nursing of a calf, poor nutrition, infectious diseases, metabolic disorders, uterine infections or other health problems. The lack of incidence of post partum anoestrus in this study may thus be a result of the lack of any of these contributing factors (Risco *et al.*, 1995).

6.4.2 Silent heats/oestrous periods

Although the results obtained in this study were similar to those mentioned in 5.3.1, it was found that in continuously observed herds, silent heats/oestrous periods were not a problem once cyclic ovarian function had been initiated (Silva *et al.*, 1992). Macmillan and Burke (1996) found the incidence of silent heats to be reduced to a minimum when increasing the number of oestrous observation periods per day. This could point to shortcomings in the oestrous detection routines, rather than reproductive hormone deficiencies. From the milk progesterone profiles (Figure 6.4), it is evident that the milk progesterone levels prior to oestrus vary largely between individual cows and between cycles in an individual single cow. It was thus concluded that milk progesterone profiles could not be used as an indicator to demonstrate a possible cause for the occurrence of silent heats/oestrous periods as the cows do not present different P4 profiles than those showing oestrus.

The possibility exists that some of the silent heats recorded in this study were unobserved due to some flaw in the oestrous detection programme, rather than a silent oestrus. The milk progesterone assay can thus be used to monitor, in retrospect, the efficiency of oestrous detection. This information could then be used, if necessary to improve the oestrous detection routine. The small number of cows receiving prostaglandin in this study made it impossible to quantify the effects of prostaglandin treatment on prolonged oestrous cycles. Williams and Mcleod (1992) and Williams and Esslemont (1993) stated that the induced oestrous period appears in every way to be normal, and fertility at the induced oestrous, and subsequent oestruses are unaffected by treatment. This, and the similar results obtained in this investigation indicated that the use of $PGF_{2\alpha}$ and its analogues to be an effective method of overcoming the problem of silent heats in dairy cattle, provided that the P4 level in milk or serum is >3 nmol/l.

6.4.3 Short oestrous cycles and CL lifespan

Embryonic mortality occurred in a cow exhibiting short oestrous cycles. A possible cause of the short luteal phases could be a reduction in the pituitary responsiveness to GnRH stimulation as a result of high progesterone levels during pregnancy. The pituitary responsiveness to GnRH is usually regained approximately 10 days post partum, but may be delayed in some cows (Stevenson & Call, 1988; Stevenson *et al.*, 1999).

The lack of cow numbers demonstrating short cycles restricts any conclusions from being made. The treatment of this problem with progesterone was successful as exogenous progesterone does not interfere with the functional corpus luteum (Walton *et al.*, 1990) - but it is thought to inhibit the development of a CL in cows which are about to, or have recently, ovulated (Young *et al.*, 1984). This result, along with those of Stevenson *et al.*, (1989) indicated that this inhibition phase of treatment to be limited. Extension of the luteal phase using PMSG was successful in that the luteal phase progesterone concentrations were increased. However, it was hoped that the

luteal support provided would aid conception and prevent early embryonic death. As the cow subsequently cycled, this was not achieved. It was later found that the cow had a blocked right Fallopian tube, and thus conception at that insemination (where ovulation had occurred on the right ovary) was impossible. The possibility of cyst formation and superovulation would act as a deterrent to the further use of PMSG as a method of maintaining the corpus luteum-especially as progesterone treatment has been shown to be more suitable.

6.4.4 Cystic ovaries

From the milk progesterone profiles recorded, diagnosis of an ovarian cyst was possible. These profiles were beneficial and aided in the hormonal therapy, when necessary. Without the knowledge of the progesterone profile that cystic CL's were present, GnRH treatment would have been recommended for 2 cows on the Rayton farm. This would not have been detrimental to both of the cows but would have constituted unnecessary use of exogenous hormones. Diagnosis of the follicular cyst, achieved using milk progesterone levels, was made at an early stage. Thus treatment could be implemented before too much time had been lost. Choice of treatment was also made easier due to the differential diagnosis of the cyst - not easily achieved by rectal palpation of the ovaries. The response of the follicular cyst to treatment was monitored, thus enabling early knowledge of treatment success. Although the cystic structure was palpated 5 days after treatment, the rising milk progesterone levels 5 days after AI indicated that ovulation had occurred and C L activity initiated. Milk progesterone analysis therefore proved to be an invaluable aid to (a) early identification of the presence of a cyst (b) differential diagnosis of cysts and (c) application of appropriate treatment.

6.4.5 Embryonic mortality

The frequency of embryonic mortalities was similar to that reported in other studies (Mann *et al.*, 1998; Smith & Wallace, 1998), using milk progesterone analysis. Milk progesterone levels provide an indication of the embryo

mortality rate which is far more accurate than that obtained using the occurrence of delayed returns to oestrus. A substantial portion of the estimated early embryonic mortalities by delayed returns to oestrus is bias due to undetected oestrous periods (Butler, *et al.*, 1996; Larson *et al.*, 1997).

Progesterone profiles in cows experiencing early embryonic mortalities were not able to demonstrate the cause of embryonic death, and milk progesterone levels were of no use in predicting the cows in which embryo mortality may occur. Similar results were found by Lamming and Bulman, (1976) and Lamming and Darwash (1998). Studies have shown that the major portion of embryonic losses to occur well before day 15 after service (Sturman *et al.*, 2000; Qureshi *et al.*, 2000). Such cows will therefore return to oestrus after the same interval as in non-pregnant cows, despite being pregnant (Scipioni & Foote, 1999). This may be the cause why cycle lengths are slightly extended. Neither delayed returns to oestrus nor milk progesterone analysis is capable of detecting early embryonic deaths. Although embryonic mortality rates predicted in this manner (cycles longer than 21 days) was higher than in the normal cycle, the economic consequences are more or less the same, as failure of fertilization in that cycle is not excessively long and fertility at the subsequent oestrus is not affected. However, the high proportion of this "fertilization failure" indicated that this phenomenon is more of a problem than is currently realised (Reist *et al.*, 2000). Waldmann *et al.* (1999) found 90% of pre-insemination cycles were of normal length (17 to 25 days), compared with only 43.5 % post insemination cycles. Cows exhibiting extended cycles (over 21 days) after insemination could thus be classified as problem cows. Milk progesterone measurements played a large part in this analysis in that estimates of embryo mortality were unbiased as returns to oestrus were monitored, and cycle lengths could be measured accurately without relying on oestrous detection efficiency.

6.5 CONCLUSION

The identification of the "problem" cows was relatively ineffective with the aid of milk progesterone determinations. Problem cows were chosen on the

basis of their previous or current reproductive status. Cows which were classified as "repeat breeders" in their previous lactation were selected for the study and conceived at the first insemination during their current lactation or seemed to conceive at the subsequent insemination. Thus, during the second phase of the study, cows which had recently calved were randomly chosen and monitored from calving to conception. These cows provided more information. From these observations it was concluded that to make efficient use of milk progesterone analysis, it would be necessary to monitor the entire herd. At the moment though, this would prove to be too expensive.

CHAPTER 7

THE RELATIONSHIP BETWEEN PLASMA (PUN), MILK (MUN) UREA NITROGEN AND PLASMA PROGESTERONE CONCENTRATIONS AT THE TIME OF AI

7.1 INTRODUCTION

As the genetic potential of milk production in dairy cows has increased in the past, so the conception rate has correspondingly decreased (Butler & Smith, 1989). Similarly high protein intake in cows has been found to stimulate milk production which confounds the problem, as this increase in dietary protein is also associated with lower fertility in the herd (Jordan & Swanson, 1979a; Kaim *et al.*, 1983; Canfield *et al.*, 1990; Grings *et al.*, 1991). So e.g. cows fed high levels of dietary protein have recorded an increase in uterine pH and reduced conception rates (Jordan *et al.*, 1983; Elrod & Butler, 1993; Elrod *et al.*, 1993).

Both an excess and shortage of MUN and PUN concentrations could have a negative effect on fertility. Plasma progesterone concentrations (associated with pregnancy) have also been reported to be higher in cows fed high dietary protein levels (Jordan & Swanson, 1979b; Sonderman & Larson, 1989). Regarding dietary protein degradability and its relationship with reproductive performance, Ferguson *et al.* (1988) reported plasma urea nitrogen (PUN) concentration exceeding 20mg/dl to be associated with low conception rates in lactating cows. PUN and milk urea nitrogen (MUN) as such have also been found to be indicative of the protein metabolism and the nutritional status of the cow and has thus been found to be advantageous for practical monitoring purposes (Roseler *et al.*, 1993). The objectives of this study were to determine the relationship between plasma and milk urea nitrogen concentrations with that of plasma progesterone concentrations at day 21-24 or at pregnancy diagnosis in high producing dairy cows (35 to 55 l/day), fed complete diets.

7.2 MATERIALS AND METHODS

7.2.1 Trial 1: Relationship between PUN and plasma progesterone (P4) concentrations at AI and the affects on conception rate in first AI of dairy cows

Multiparous lactating Holstein cows (total herd n = 160), between the age of 4 and 6 years, weighing between 450 and 650 kg and between 1 and 250 days in lactation were used in this trial. The herd was located at the Cees Legemaat Dairy Herd in Rayton, Mpumalanga. All cows were fed a total mixed dairy diet (50% forage and 50% concentrate). formulated to provide at least 1.62 Mcal/kg ME and 17.5 to 19% crude protein. On the day of AI (first oestrus ±60d post partum), blood (10 ml) was sampled from the jugular vein into heparinized tubes from a group (n = 51) of cows. Blood sampling commenced at AI and continued daily for 5 days. Following collection, blood samples were centrifuged (1200G for 20 minutes), the plasma harvested and plasma aliquots (1 ml) stored (-20°C) for later plasma urea nitrogen and P4 concentration analyses. The PUN assays were performed using the automated diacetylmonol x amine method (Auto-method, Technicon Industrial Method 339-01, U.P., Gauteng).

Plasma samples collected during the 5 day period after AI were also analysed for plasma P4 concentration (as set out in 3.4) with the aid of a RIA method, with an intra-assay coefficient of variation of 7%. Cows were later certified pregnant 60 days following AI, by rectal palpation. To compare the pregnancy rates with the PUN levels, the chi-square analysis (Number Cruncher Statistical Systems) was used. Pregnancy rates in cows were related to PUN concentrations greater than or less than the mean of the sample population. To further clarify the relationship between the PUN level and the reproductive performance, cow data were categorized into incremental ranges of 3 mg/dl PUN for the determination of the pregnancy rate likelihood ratios (Ferguson *et al.*, 1993). Only data of the first AI on each cow was taken into account to avoid the confounding effects of repeated measures and repeat breeding. A repeated measure ANOVA was used to compare the effect of the PUN concentration categories (above or below the

mean), pregnancy status and days following AI on milk progesterone concentration.

7.2.2 Trial 2: Relationship between Plasma Urea Nitrogen (PUN) and Milk Urea Nitrogen (MUN) concentrations

Blood samples (10 ml) were randomly collected from 22 multiparous Holstein cows out of a herd of 160 cows, aged between 4 to 6 years (bodyweight of 450 to 650 kg) and between 0 and 250 days in lactation. The location was the Cees Legemaat Dairy Herd in Rayton, Mpumalanga. Cows were maintained on diets as described in 7.2.1. Milk samples (30 ml/cow) were obtained from the individual milk measuring cylinders at the end of each milking from the 22 cows at AI and 5 days after AI. Additional milk stripping samples were taken from the same quarter of the udder before and after milking from 10 cows. A preservative (Broad Spectrum Microtab, D & F Control Systems) was added to half (n = 11) of the milk samples of all cows, with the rest acting as controls. Within a 30 to 45 minute period following milking, blood (10 ml) was sampled from the jugular vein of all 22 cows, into heparinized tubes. The plasma was harvested as set out in 7.2.1 for later urea nitrogen determinations. Milk samples (30 ml) were centrifuged at 1000G for 20 minutes and the fat layer aspirated. Aliquots (3 ml) of the supernatant were stored (-20°C) until the analysis for urea nitrogen. The PUN an MUN samples were analysed with the aid of the Auto-method as described in 7.2.1. In preliminary trials, defatting (skimming) was found essentially to prolong the lifespan of the osmotic membrane, integral to the Auto-instrument used for the urea nitrogen determination. The milk samples were also analysed for urea nitrogen, either using a manual urease/Berthelot assay (Sigma, No. 640, Sigma Diagnostics) or a dipstick urease/pH assay (Azotest, Comagnie Chimique Aquitaine, La lande de Pomerol, France). In the Azotest, the assay was performed utilizing whole milk samples as prescribed by the manufacturer. Using specifications by the manufacturer, results of the Azostest were converted to rank and range values that allowed a comparison of the results with that of the automated procedure (Table 7.1). The comparison between the Azotest and the Auto-methods was repeated on all

22 cow samples. In evaluating the tests used for determining urea nitrogen, the Auto-method was used as the basis for standardization of the assay. The equation describing the relationship between the MUN (y) and PUN (x) concentrations was y = 0.76 (x) + 6.3 (R^2 = 0.69) (Heap *et al.*, 1973; Gustafsson & Palmquist, 1993).

Table 7.1 Azotest rank of colour and urea concentration (MU) relating to the milk urea nitrogen (MUN) concentration in dairy cows

Rank	Colour	MU (g/l)	MUN (mg/dl)
1	Orange	0.10	4.7
2	Very light green	0.20	9.4
3	Light green	0.30	14.1
4	Green	0.35	16.5
5	Dark green	0.40	18.8
6	Very dark green	0.50	23.5

MUN (mg/dl) = MU (g/l) x 47 (urea = 47% nitrogen on a weight basis)

A paired t-test was used to statistically compare the PUN and MUN concentrations in the same cows. The Sigma-manual and Auto-method were implemented to compare MUN values in the same milk samples and the effect of the preservative on the assay. The GLM and ANOVA tests were used to compare the concentrations obtained in the Azotest and Auto-method across test ranks. Where the interactions were significant, a t-test performed within each rank. Differences were significant at the $P<0.05$ and $P<0.01$ level of confidence (Shelton *et al.*, 1990; Roseler *et al.*, 1993).

7.2.3 Trial 3: Relationship between milk urea nitrogen concentration on day of AI with pregnancy rate in dairy cows

155 Holstein cows (105 multiparous and 50 primiparous) from the Cees Legemaat herd (total herd of n = 160) were allocated to this trial in Rayton, Mpumalanga. Cows were maintained on diets as set out in 7.2.1. Milk samples (30 ml) were collected on the day of AI at first demonstration of oestrus after 60 days post partum. From a group of 23 cows, milk was continued to be sampled at each milking (twice daily) for a 5 day period

following AI. All milk samples were prepared for MUN determination as set out in 7.1.1, using the Auto-method.

All cows (n = 155) were pregnancy diagnosed via rectal palpation of the reproductive tract 40, to 50 days following AI. To relate the MUN concentrations with the pregnancy diagnosis, cows were categorized into incremental ranges of 3mg/dl MUN for determination of the pregnancy ratios (7.1.1).

The chi-square test was used to compare the differences in pregnancy rate with MUN concentrations >19mg/dl. Only data of the first AI was used in the analysis (7.1.1). The MUN concentrations at AI for the pregnant and non-pregnant cows were compared with the aid of the Student's t-test. The variation in MUN levels over 10 milkings relative to the pregnancy status of the animals was determined using repeated measures ANOVA and further analysed in terms of regression procedures (Ferguson *et al.*, 1993). MUN concentrations of 44 samples were determined with the aid of the Auto-method and categorized into ranks for a comparison with the A20 test (Table 7.2).

Table 7.2 The comparison between the Auto- and Azotest methods for determination of milk urea nitrogen (MUN, mf/dl) in dairy cows (n = 44)

	Rank[a]			
Method	**3**	**4**	**5**	**6**
n	5	10	15	14
Auto[b]	14.7	16.5	19.3	24
Azotest[c]	16.5	18.2	22.6	22.6
CV, %[d]	0	14	8	7

a Rank for Azotest based on Auto MUN values
b Mean MUN for samples classified within a rank
c MUN values determined from Azotest
d CV = coefficient of variation between the Azotest results

7.3 RESULTS

7.3.1 Trial 1

The mean PUN concentrations for lactating Holstein cows (n = 160) on the day of AI was 18.9 ± 3mg/dl. The pregnancy rate in cows was higher for those with PUN concentrations higher than the recorded mean, compared to cows with a PUN concentration lower that the mean (18%) – see Figure 7.1. The relationship between the PUN levels and the predicted pregnancy rate was further emphasized with the ratio tests as based on the 3 mg/dl increments of PUN concentrations (Table 7.3). As the PUN increased to levels of more than 19 mg/dl, the rate at first AI for pregnancy decreased markedly. The rate (Table 7.3) seemed to decrease for the highest PUN category, but here only a few determinations were available. The rate seemed to increase again for the highest PUN category (Figure 7.1).

Table 7.3 The pregnancy rate (PR) in dairy cows with different plasma urea nitrogen (PUN) concentrations on the day of AI

PUN Category (mg/dl)	Cows n	PR %	Classification		Pregnancy ratio*
			Not pregnant %	Pregnant %	
<16	51	49	29.5	34.7	1.18
16-18.9	44	57	21.6	34.7	1.61
19-21.9	40	32	30.7	18.1	0.59
22-24.9	17	29	13.6	6.9	0.51
>25	8	50	4.5	5.6	1.24

* Cows pregnant cows/not pregnant

For the group of 51 cows from which blood was sampled for 5 days following AI, the plasma progesterone concentrations increased gradually after oestrus and AI. Neither the profile of the mean daily progesterone concentration nor the level of PUN concentration differed between pregnant and non-pregnant cows. The PUN concentrations recorded varied below and above the mean plasma progesterone concentrations. When the cows were compared

relative to their subsequent pregnancy status the plasma progesterone concentration was higher (P<0.05) in the pregnant cows on day 4 and 5 following AI (Figure 7.2). The mean PUN concentrations recorded on the day of AI were 18.7 ± 0.6 and 20.7 ± 0.6 mg/dl for cows diagnosed as pregnant and non-pregnant respectively (P<0.05). As PUN values increased to more than 19 mg/dl, the ratio for pregnancy markedly decreased.

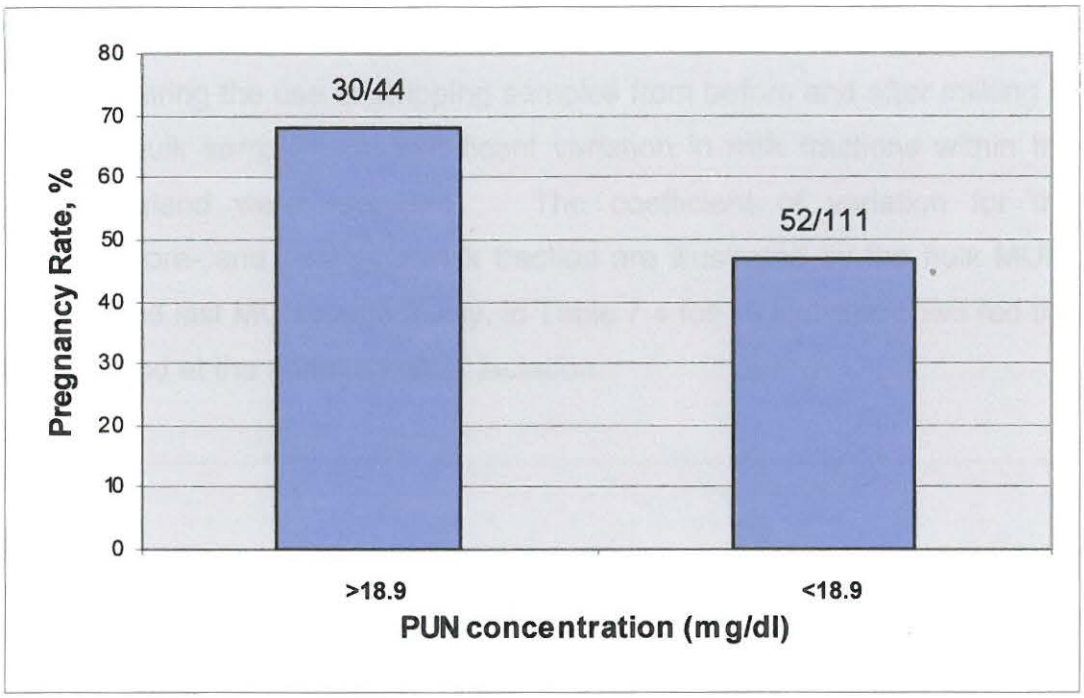


Figure 7.1 The relationship between PUN concentration and pregnancy rate at first AI

7.3.2 Trial 2

The pregnancy rate was lower (P<0.05) in cows with a MUN concentration of 19.0mg/dl. Figure 7.3 illustrates the number of cows pregnant following AI within each MUN category.

The pregnancy rate was reduced (P<0.05) in cows with a MUN level of 19.0mg/dl, with the number of cows becoming pregnant to AI, indicated within each MUN category. The PUN and MUN levels recorded in the same cows (n = 160) were not significantly different, but the two concentrations (n = 22) were significantly (P<0.01) correlated (r = 0.82) with mean values of 20.9 ± 0.7 and 22.2 ± 0.6 mg/dl respectively. Results recorded following the Sigma and Auto-method of analysis for MUN concentrations in defatted (skimmed)

samples (n = 22) were similar, in the Sigma Azotest and Auto-method recording values of 17.2 ± 0.9 and 16.9 ± 0.8 mg/dl respectively and a correlation of r = 0.93 (Table 7.2). The coefficient of variation within each rank for the Azotest was less than 15% - proof of the acceptable repeatability of the Azotest within each rank. The MUN concentrations recorded following the Auto-method were not affected by the preservative used (7.9 ± 0.7 vs 17.19 ± 0.7 mg/dl for the preserved and control samples (n = 22) respectively). The preservative also had no significant effect on the other techniques used. When comparing the use of stripping samples from before and after milking of composite bulk samples, no significant variation in milk fractions within the mammary gland were recorded. The coefficient of variation for the composite, fore- and post-strip milk fraction are illustrated by the bulk MUN, first MUN and last MUN respectively, in Table 7.4 for 10 Holstein cows fed the same diet and at the same stage of lactation.

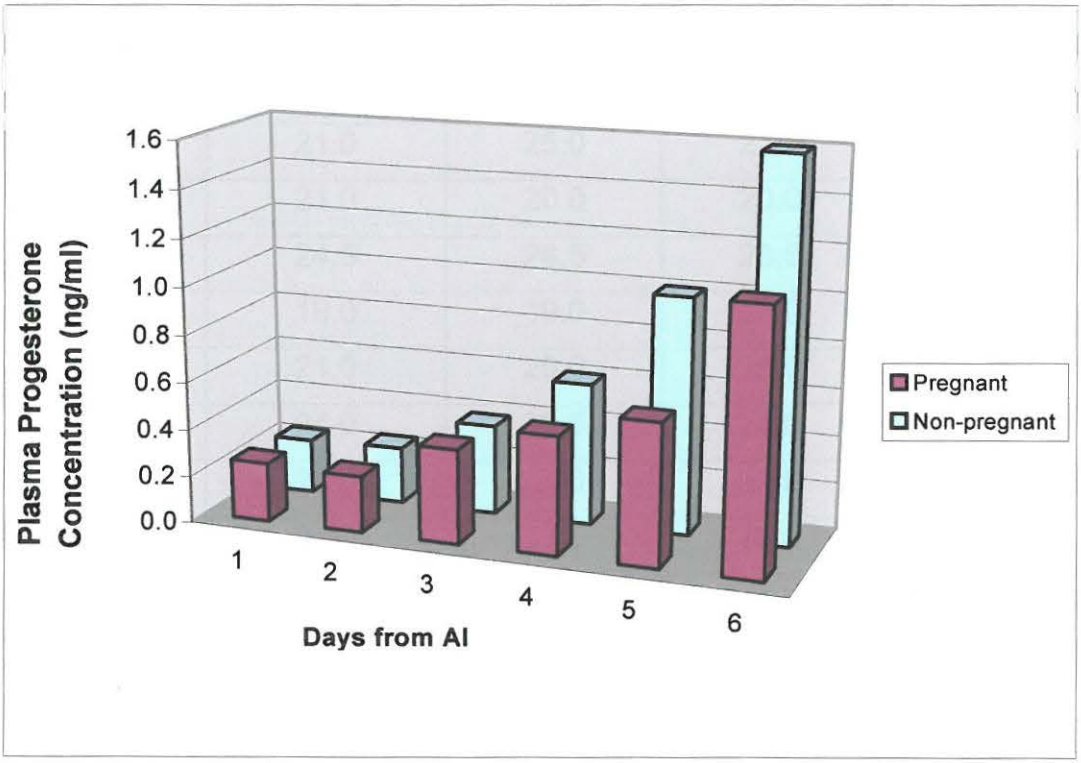


Figure 7.2 **Differences in rate of increase of plasma progesterone concentrations after AI of cows later diagnosed as pregnant (n = 29) or non-pregnant (n = 22)**

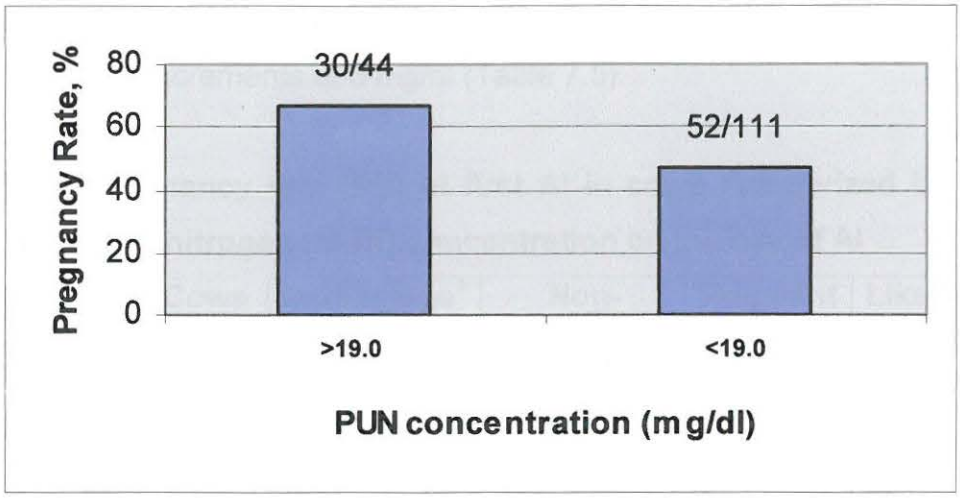


Figure 7.3 **The relationship between MUN concentration and pregnancy rate at first AI in lactating dairy cows**

Table 7.4 **Mean concentration (mg/dl) and coefficient of variation (CV) for milk urea nitrogen (MUN) for milk samples collected during different stages of milking**

Cow Number	Bulk MUN	First MUN	Last MUN	CV %
5237	24.0	24.5	24.0	1.0
5209	21.0	25.0	21.0	1.0
5214	21.0	20.0	20.0	2.3
5068	24.5	24.5	23.5	1.9
5278	19.0	19.0	19.0	0
4931	21.0	20.0	25.0	2.0
5156	24.0	23.5	23.0	1.7
4602	23.0	22.0	22.0	2.1
5250	24.0	23.0	23.5	1.7
4799	21.0	21.0	22.0	2.2
Mean	22.3	21.8	21.9	1.6
CV %	8.1	8.6	7.3	-

7.3.3 Trial 3

For cows later diagnosed as pregnant (n = 82) and non-pregnant (n = 73) a significant difference ($P<0.05$) in MUN concentration was recorded (21.3 ± 0.4 vs 22.8 ± 0.5 mg/dl respectively). The overall relationship between MUN

level and the pregnancy rate in cows was determined using likelihood ratio tests, based on increments of 3 mg/dl (Table 7.5).

Table 7.5 Pregnancy rate (PR) at first AI in cows categorized by milk urea nitrogen (MUN) concentration on the day of AI

MUN Category mg/dl	Cows n	Percentage[a] %	Non-pregnant	Pregnant	Likelihood ratio[b]
<16	16	75	5.5	14.6	2.65
16-18.9	28	64	13.7	22.0	1.61
19-21.9	46	48	32.9	26.0	0.81
22-24.9	36	47	20.7	26.0	0.80
>25	29	45	15.9	21.9	0.73

a Percentage of total cows within each MUN category
b Percentage cows pregnant divided by the percentage cows not pregnant

MUN values were higher ($P<0.01$) over time in the non-pregnant cows than the pregnant cows (24.9 ± 0.9 vs 21.1 ± 0.9 mg/dl respectively). A significant ($P<0.05$) interaction was recorded between the pregnancy status of the cows and the MUN concentration over time. From the regressions (Figure 7.4), it is evident that the MUN concentrations were lower at AI and decreased ($P<0.01$) subsequently in cows diagnosed pregnant. In contrast, MUN concentrations were consistently higher in non-pregnant cows. From regression analyses, the equation for the regression line in pregnant cows (blue; n=11) was $Y=-.30X+22.6$ ($P<0.01$, $R^2=0.59$, SE=0.6); for non pregnant cows (yellow;n=12) the slope was not significantly different from zero (Figure 7.4).

7.4 DISCUSSION

7.4.1 Trial 1

High dietary protein levels result in high concentrations of urea nitrogen in the plasma and milk of dairy cows and this has been associated with resultant decreased fertility (Jordan *et al.*, 1983; Ropstad & Refsdal, 1987; Canfield *et al.*, 1990; Elrod & Butler, 1993).

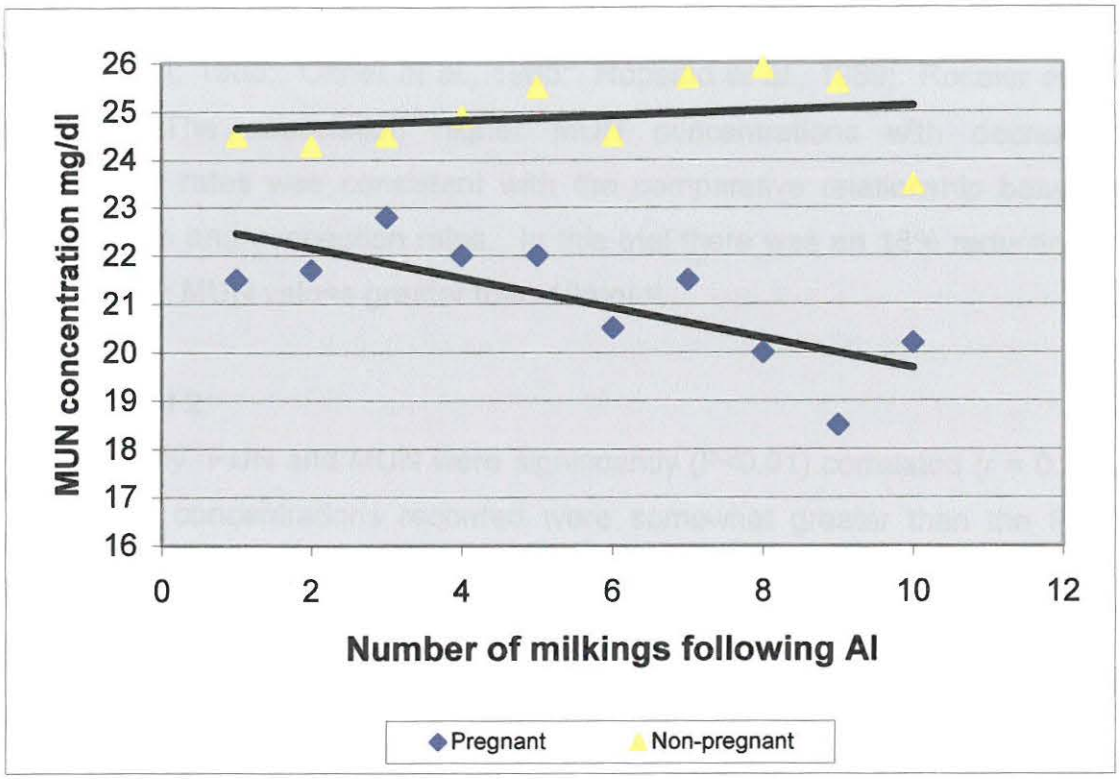


Figure 7.4 **Mean MUN concentrations for 10 consecutive milkings in pregnant and non-pregnant Holstein cows starting from the day of AI**

In the present study, the PUN concentrations in cows higher than 19 mg/dl was associated with a decrease in pregnancy rate (18%). As cows were fed typical balanced diets for lactating cows, the recorded urea concentrations should be indicative of the normal values in a dairy population. Previously, Ferguson *et al.* (1988; 1993) reported a similar range of blood urea concentrations in cows and also reported serum urea nitrogen values greater than 20 mg/dl to result in decreased conception rates. The magnitude of the associated decrease in pregnancy rate of high blood urea nitrogen seems to be related to the underlying reproductive performance between dairy herds (Ferguson *et al.*, 1993). Carroll *et al.* (1988) however found no detrimental effect of high dietary protein on reproductive performance. These authors suggested that the decreased fertility, associated with high protein intake, to be possibly attributed to differences in reproductive management, rather than in protein intake or urea production as such.

As urea equilibrates within the body fluids, MUN levels should be very similar to the PUN concentrations and should be an indicator of the urea nitrogen status of the dairy cow and can be conveniently monitored (Oltner & Wiktorsson, 1983; Oltner *et al.*, 1985; Ropstad *et al.*, 1989; Roseler *et al.*, 1993). The association higher MUN concentrations with decreased pregnancy rates was consistent with the comparative relationship between PUN levels and conception rates. In this trial there was an 18% reduction in fertility with MUN values greater than 19mg/dl.

7.4.2 Trial 2

In this study, PUN and MUN were significantly ($P<0.01$) correlated ($r = 0.82$). The MUN concentrations recorded were somewhat greater than the PUN concentrations when milk samples were collected 4 to 5 h after feeding and the blood was sampled after milking. Elrod and Butler (1993) and Gustafsson and Palmquist (1993) reported PUN concentrations to fluctuate throughout the day. Generally, the minimum PUN concentrations are recorded before feeding and the maximum levels recorded approximately 4 to 6 h after feeding - as there is a lag period of approximately 1 to 2 h between the PUN and MUN concentration peaks, depending on the sampling times relative to the time of feeding dietary forages and concentrates separately or as a total mixed diet (Gustafsson & Palmquist, 1993). The MUN concentrations were somewhat higher than the PUN values. This difference was probably due to the differential timing of milk and blood sampling relative to the time of feeding. In a recent study of Frajblat and Butler (unpublished data) for milk and blood sampled simultaneously, the MUN level was found not significantly different from that of the PUN concentration in dairy cows.

As nitrogen urea equilibrates across the mammary epithelium (Gustafsson & Palmquist, 1993), little variation in MUN concentration in the different milk fractions collected was recorded during milking - as also noted by Carlsson and Bergstrom (1994). Thus, it should make little difference whether a milk sample intended for MUN analysis comes from a composite whole milk sample or from only a quarter strip sample, before or after milking (Carlsson & Bergstrom, 1994). In addition to the small difference in urea nitrogen found

between milk fractions, the coefficient of variation in MUN concentration between cows fed the same diet or across sequential milkings was low (8%). It is important to note that this small variation in MUN levels possibly reflects the feeding of total mixed diets to cows and which results in rather small variation in blood urea during the day (Elrod *et al.*, 1993). In turn, the small variation recorded could indicate that MUN analyses from several cows could be representative of a population (i.e. cows receiving the same diet and at a similar stage of lactation). Previously urea concentrations in a bulk milk tank have been used to predict the protein quality of the milk (Refsdal *et al.*, 1985; Ropstad *et al.*, 1989) and demonstrate fertility differences between herds (Ropstad & Refsdal, 1987).

No significant difference was recorded between the Auto- and Sigma laboratory methods of MUN concentration analysis in skimmed milk. Selection of the Sigma or the Auto- method is based on the convenience and availability of equipment. The addition of preservatives to the milk samples had no effect on MUN analyses. This was to be expected, as the preservative does not contain chemicals that should interfere with these analyses or change the composition of the milk. Being able to use a milk preservative is beneficial in practical dairy situations, where a relatively long interval between milk samplings and the MUN analysis as such may occur. Field trials or on-farm MUN analyses in the dairy herd would benefit from such an easy and reliable evaluation system. Although a positive correlation ($r = 0.6$) between the Azotest and Auto-method, the two methods yielded significantly different results. The Azotest as such consistently over-estimated the values obtained by the Auto values by approximately 2 mg/dl - except in the highest ranking, where the Azotest value was lower than the Auto-method concentration. This is because the 23.5 mg/dl concentration is the upper limit sensitivity designed for the Azotest, and any samples with higher concentrations will lead to an underestimation by this method. Although the Azotest tended to overestimate the concentration compared to the Auto-method, the variation within each rank of the Azotest values was within an acceptable range. This would indicate that the overestimation of the MUN levels are consistent and that the

Azotest has value as a semi-quantative measure of MUN concentration in dairy cows.

7.4.3 Trial 3

Plasma progesterone concentrations during mid-diestrous period of the cycle were reported to be approximately 30 % lower in cows with a high PUN concentration, due to the nutrition of high-protein diets (Jordan & Swanson, 1979b; Sonderman & Larson, 1989). In the present trial plasma progesterone concentrations did not differ significantly during the 5 day period following AI (early diestrous) when cows were categorized into high and low PUN groups. The differences in PUN concentration between these groups were however not as large as in previous reports (Williams & Esslemont, 1993). However, when categorized according to the pregnancy status of the animal, the rate of increase in plasma progesterone was significantly greater by day 4 following AI in pregnant than in non-pregnant cows. This early increase in progesterone in pregnant cows is in agreement with several other researchers (Randel *et al.*, 1971; Erb *et al.*, 1976; Lee *et al.*, 1985). Shelton *et al.* (1990) concluded the increase in the post-ovulatory peripheral progesterone concentrations to be delayed and occur more gradually in sub-fertile cows, compared to e.g. heifers. It has been suggested that luteal inadequacy, due to diminished responsiveness to luteotrophic hormones, may contribute to embryonic mortalities in sub-fertile cows. In contrast, the presence of a viable embryo may indicate a luteotrophic effect of an early embryo and possible blood progesterone concentration (Maurer & Echternkamp, 1982). Thus, the differential increase of plasma progesterone concentration for pregnant and non-pregnant cows in the present study could reflect either differences in luteal function or luteotrophic stimulation by the embryo or a combination of the two. Regardless, it would seem that greater blood progesterone availability 3 to 4 days after ovulation is conducive to an increase in the survival rate of early embryos, and consequently results in higher pregnancy rates.

Early embryonic development requires an appropriate oviductal and uterine environment and Jordan *et al.* (1983) quoted alterations in uterine secretions

of high-producing cows fed diets high in crude protein, to result in high PUN concentrations. When heifers were fed high protein diets, PUN concentrations increased, uterine pH decreased, and pregnancy rates decreased (Elrod *et al.*, 1993). Uterine pH is significantly affected when the PUN concentrations exceed 19 mg/dl. In sheep fed excess rumen degradable proteins, PUN concentrations greater than 18 mg/dl were found to be detrimental to the early development and survival of embryos (Bishonga *et al.*, 1994). Excess rumen degradable protein has also been reported to be detrimental to embryonic development in lactating cows (Blanchard *et al.*, 1990), but not in non-lactating cows (Garcia-Bojalil *et al.*, 1994). Little variation was found between cows regarding the MUN levels over a period of time after AI. An interaction was recorded between the pregnancy status following AI and the MUN levels. Non-pregnant cows maintained higher MUN concentrations over time. The MUN values were low at oestrus and the concentrations tended to decrease over the subsequent 5 days following AI in cows that were later palpated and diagnosed as pregnant.

7.5 CONCLUSION

Urea nitrogen concentrations higher than 19 mg/dl whether in the plasma or milk can be associated with a decreased pregnancy rate in dairy cattle. Therefore, it may be beneficial to dairy producers to monitor urea concentrations in their herds in an effort to improve the reproductive efficiency of the herd. If chemical tests are not available, MUN concentrations can be determined quickly, easily and reliable with the Azotest. The Azotest can indicate whether the milk sample warrants more precise and expensive chemical procedures. Monitoring MUN in a dairy herd implies the formulation and modification of the protein composition of the diet in such a way that it optimises nitrogen utilization for milk production and avoid possible negative effects on herd fertility. With the high correlation between MUN and PUN levels, milk is easier to sample and stress-free to the animal and thus the obvious choice to determine the urea nitrogen of the animal.

CHAPTER 8

ECONOMICAL IMPLICATIONS OF PREGNANCY DIAGNOSIS IN DAIRY CATTLE

8.1 INTRODUCTION

Pregnancy diagnosis is regarded as an important and essential component in a complete reproduction management program (Kourletaki *et al.*, 1995). Although generally regarded as economically beneficial, no cost-benefit analysis has been set out in the literature and it is generally assumed that the economic merits of such an input in a reproduction program are based principally on theoretical grounds (Armitage & Berry, 1994). Rectal uterine palpation has been used and accepted for many years and presently an alternative option namely, the milk progesterone test, has become available. A detailed description of several of the commercially available milk progesterone tests has been set out by Royal *et al.* (1999). In a simulation study, Williams and Esslemont (1993) evaluated the use of the milk progesterone test as a means of detecting non-pregnant cows and limiting insemination errors – here it was concluded that the use of this technology in pregnancy diagnosis to be profitable and viable for use in the management program of dairy cows in terms of the time saved.

The early identification of serviced cows that did not conceive provides an opportunity to implement prompt measures that could improve the reproductive efficiency of a dairy herd. Among the available options currently, are oestrous observations with the aid of pressure sensitive mounting detectors and the occurrence of abortions. It is also reasonable to assume that economic benefits resulting from an early pregnancy diagnosis depend on several factors such as the time post-service when it was performed, the accuracy of the technique, its effect on possible embryonic losses and the efficiency of oestrous detection (Rajamahendran *et al.*, 1993).

The objectives of this study were to relate the effect of pregnancy diagnosis on the reproductive efficiency of cows in order to determine the economic

benefits and to evaluate (using simulation analysis), how this economic feasibility is affected by various managerial and biological factors.

8.2 MATERIALS AND METHODS

The following 4 alternative methods of pregnancy diagnosis (PD) in 96 multiparous Holstein dairy cows between 40 and 180 days in lactation were evaluated: 1) Milk progesterone was determined on day 19 post-service (MPPS) (n = 24); 2) Rectal uterine palpation was performed on day 35 post-service (UPL1) (n = 24); 3) Rectal uterine palpation was performed on day 50 following service (UPL2) (n = 24); 4) Rectal uterine palpation was performed on day 65 post-service (UPL3) (n = 24). These PD predictions were compared to a control herd (n = 24), characterized by an identical reproduction management program, but without the use of pregnancy diagnosis. Kamar heat detectors and $PGF_{2\alpha}$ (prostaglandin) were only used to detect oestrus and in addition to induce oestrus in cows not showing oestrus.

Additionally in the MPPS pregnancy test, all cows (n=10) with a low milk progesterone value were bred when detected in oestrus or treated with 2ml prostaglandin $PGF_{2\alpha}$ (Estrumate,Shering Plough) after 10 days if not observed in oestrus. Those cows not bred (not responding to treatment or not detected in oestrus) were treated again 13 days after the initial PGF injection, and bred at the detected oestrus.

In the UPL1, UPL2, and UPL3 pregnancy diagnosis groups, a pressure-sensitive mounting device (Kamar) was applied to all cows diagnosed as not pregnant prior to the next oestrus to facilitate and improve oestrous detection in these groups.

Fertility problems in the herd were structured as a sequence of events, and a decision tree model used to describe the process and to quantify the uncertainties associated with various managerial events (nodes) was set up. This tree diagram was constructed using 4 nodes, namely a service node

(SERV), a pregnancy diagnosis node (PRGT), a prostaglandin treatment node ($PGF_{2\alpha}$) and a new cycle node treatment node (NCYC). Diagrams of the first 3 nodes describing possible outcomes (following events) with the associated probabilities and times of occurrence are set out in Figures 8.1, 8.2, and 8.3. The NCYC node only resulted in two outcomes, namely a SERV or NCYC node. The tree diagram is initiated at the onset of lactation, when service first occurred. Each branch is terminated when pregnancy is confirmed, or at day 210 of lactation, or after 5 subsequent services when an open cow was classified as a reproductive cull and scheduled for replacement. For each branch, the final probability of the associated sequence of events, the day of conception, number of services, number of pregnancy diagnoses, and number of treatments were recorded. This information was subsequently used to calculate the expected days open, number of services and proportion of cows to be culled due to their failure to conceive (Ding *et al.*, 1985).

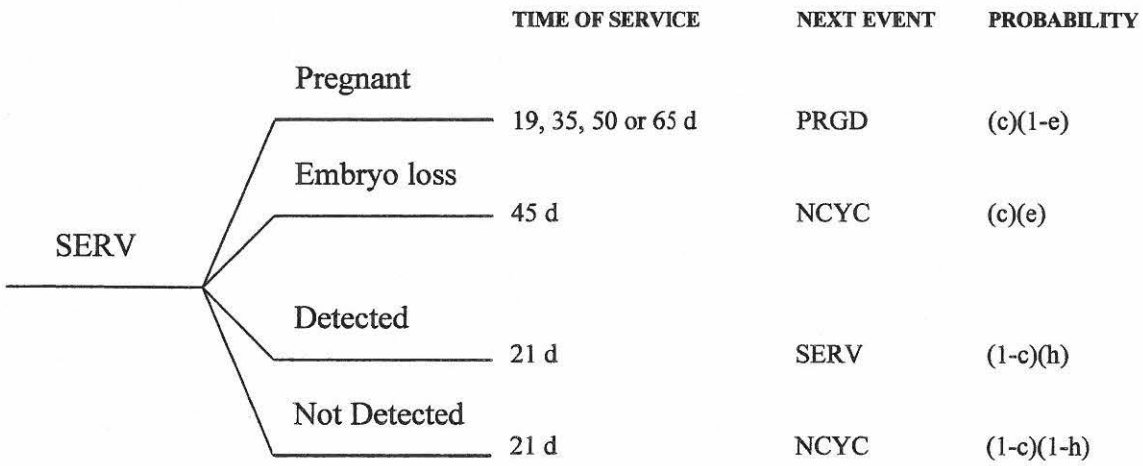


Figure 8.1 **A diagram of the service node (SERV) describing the next events and their probabilities (Ding *et al.*, 1985)**

The pregnancy diagnosis techniques considered were evaluated using the following baseline set of parameters: The average day of first service post partum, d + 80; the probability of conception, c = 0.50; the probability of embryonic mortality, e = 0.10; probability of errors in the pregnancy diagnosis, t = 0; probability of oestrous detection and percentage increase in probability of estrous detection among cows diagnosed as non-pregnant, hb =

30%. A sensitivity analysis of the economic response associated with these strategies with respect to the value of the parameters d, h, hb, t and e was also performed (Ding *et al.*, 1985).

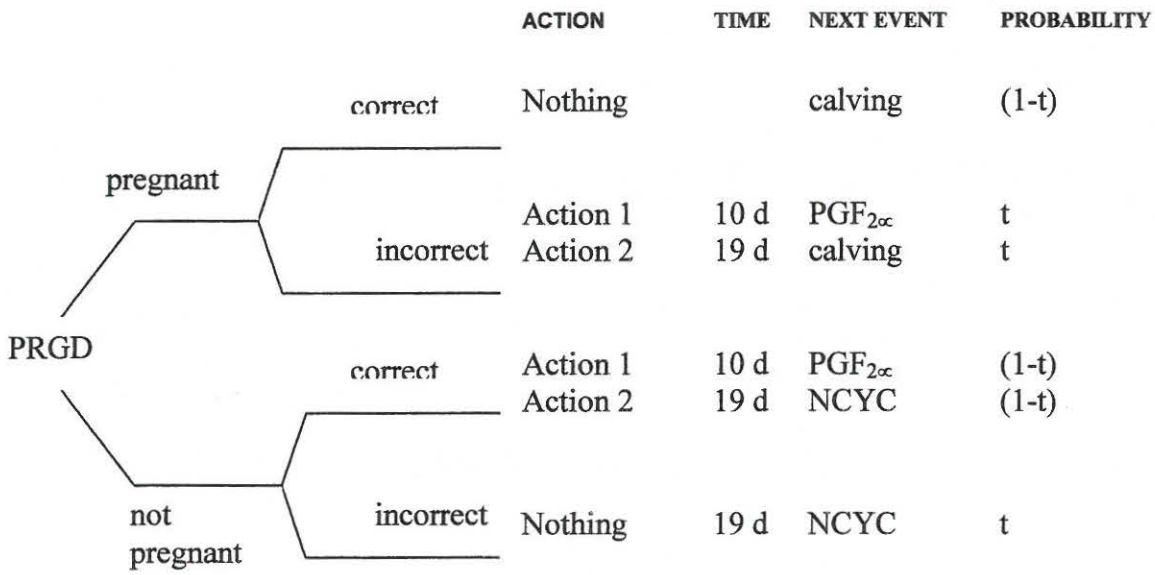
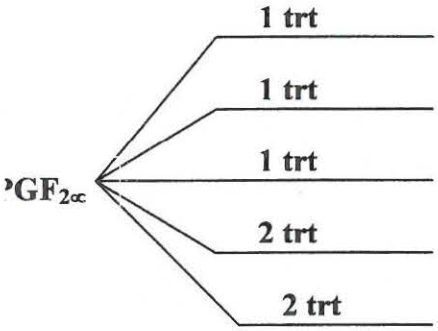


Figure 8.2 **The diagram of pregnancy diagnosis node (PRGD) describing the next events and their probabilities (Ding *et al.*, 1985)**

The following costs were utilized in the model: service cost (semen): R35; milk progesterone test cost: R17.50; prostaglandin ($PGF_{2\alpha}$) treatment: R17.50; pressure-sensitive mounting devices: R3.50; cost of rectal palpation: R7.00. For cows confirmed pregnant, the cost associated with time of conception was calculated using loss figures losses of R175, R262.50, R5.25, R8.75 and R14.00 for each day in the delay of conception for the intervals 50 to 80; 81 to 110; 111 to 140; 141 to 170 and >170 d in lactation respectively. For cows culled due to the failure to conceive, a replacement cost of R2450.00 (the difference between the cost of a pregnant heifer and the salvage value of a culled cow) was used (Oltenacu *et al.*, 1986).



TIME	NEXT EVENT	PROBABILITY
+2 d	SERV	(r1)(hb)
+4 d	SERV	(r2)(hb)
+7 d	SERV	(r3)(hb)
+15 d	SERV	{1-hb(r1+r2+r3)}(hb)
+36 d	NCYC	{1-hb(r1=r2=r3)}(1-hb)

r1,r2,r3 * Proportion of cows responding with an ovulation after the first treatment

1-r1-r2-r3 * Proportion of cows not responding to the first treatment

hb * Enhanced probability of estrus detection for cows diagnosed not pregnant

Figure 8.3 The diagram of the prostaglandin treatment node ($PGF_{2\alpha}$) describing the next events and their probabilities (Ding *et al.*, 1985)

8.3 RESULTS AND DISCUSSION

Nebel (1988) reviewed the aspect regarding the use of milk progesterone for early pregnancy diagnosis in cattle by on-farm milk P4 tests and concluded that the accuracy of identifying pregnant cows, varied between 60 to 96%. This was consistently lower than the test for non-pregnant cows, which varied between 81 and 100%. The lower accuracy in identifying pregnancy in cows could be due to embryonic mortality, a prolonged luteal lifespan, luteal cysts, or other pathological conditions resulting in high milk progesterone concentrations, suggestive of pregnancy, and not of accuracy in detecting higher levels of progesterone. Only the errors associated with the progesterone test failing to detect the true progesterone concentration were considered. Errors in diagnosis were allowed to vary from 0 to 5% and were assumed equally likely - i.e. a 5% error rate would indicate that 5% of the cows labeled as high in milk progesterone concentration and diagnosed

pregnant, in fact, had low progesterone levels and were not pregnant, and vice versa.

Accuracies in pregnancy diagnosis following uterine palpation of 93 and 91% at 60 day post service have been reported by Reimers *et al.*, (1985), for cows diagnosed pregnant and non-pregnant, respectively. Errors in pregnancy diagnosis are naturally occurring embryonic mortalities and induced embryonic mortality caused by the rectal palpation. Paisley *et al.* (1978) found that on average, 96.4% of cows diagnosed pregnant following uterine palpation subsequently calved, while 94% calved if the palpations were performed earlier than day 50 following service. According to Vaillancourt *et al.* (1979) 92.8 and 96.8% of the cows diagnosed as pregnant by uterine palpation at less than 50 days and more than 50 days following service respectively, subsequently calved. The lower calving rate for earlier uterine rectal diagnosis could be due to lower diagnostic accuracy, but also because of a higher embryonic mortality rate, as diagnosis of pregnancy occurs at an earlier stage of gestation and thus the possibility of palpation damage is higher. Errors in the diagnosis of the pregnancy status using rectal uterine palpations were assumed similar in both pregnant and non-pregnant cows and varied between 0 and 5%. In the scheme using rectal uterine palpation at day 35 post-service, an increase in embryonic mortality due to palpation was detected and allowed to vary between 0 and 5% above the normal rate of embryonic mortality of 10% (Cai *et al.*, 1997).

Many trials using luteolytic agents have been conducted and it was concluded that the conception rate in cows bred at a observed oestrus, after treatment with prostaglandin - was 10% higher than at the naturally occurring oestrus. It was hypothesized that the increase in conception rate was due to a higher efficiency and accuracy of oestrous detection. The efficiency of the detection of oestrus in cows identified as non-pregnant at pregnancy diagnosis tended to increase as the percentage of detection efficiency in the herd increased (Macmillan & Day, 1982).

Following a prostaglandin $F_{2\alpha}$ injection, 10, 65 and 10% of the cows were assumed to be in oestrus 2, 4, and 7 days later according to Shi and Wu (1987) and Kourletaki *et al.* (1995) and AI'd when detected in oestrus.

8.3.1 Evaluation with parameters at baseline values

The reproductive status of a cow was simulated for each pregnancy diagnosis technique used and for the control breeding program group of cows. The average days open, number of services per pregnancy and the number of pregnancy tests per cow, probability of culling for failure to conceive, total cost per cow, and the net gain associated with each pregnancy diagnosis scheme relative to the control program are set out in Table 8.1.

Table 8.1 Reproductive and economic performance measures for various pregnancy diagnosis methods simulated for a baseline herd

[1]Diagnosis scheme	Number of tests per cow	Culling rate (%)	Services per cow	Days open	[2]Cost per cow (R)	[3]Net gain (R)
Control	0	11.79	1.91	108.31	527.13	0
MPPS	1.80	9.82	1.96	103.92	490.52	36.61
UPL1	1.34	10.80	1.93	107.62	509.25	17.88
UPL2	1.11	11.04	1.93	108.41	518.45	8.68
UPL3	0.96	11.42	1.92	108.49	526.64	0.49

1 Control = No pregnancy diagnosis; MPPS = milk progesterone test on day 19 after service, followed by prostaglandin treatment of cows diagnosed not pregnant; UPL1, UPL2, UPL3 = uterine palpation per rectum on days 35, 50, and 65 after service, followed by a device on cows diagnosed not-pregnant
2 Includes the cost of pregnancy diagnosis, treatment cost, service cost and costs associated with cow's reproductive performance
3 The difference in average cost per cow relative to control method

In this regard the most profitable technique was MPPS (milk progesterone test), which reduced the number of days open by 4.4 days, reproductive culling by 2% relative to the control group and returned a premium of R36.75 per cow above the cost of the treatment program. Ruiz *et al.* (1989) reported a similar increase of R45.50 in the net return per cow following the use of on-farm progesterone test kits for the diagnosing of non-pregnant cows. The UPL1 and UPL2 methods were also financially viable, with a return of R17.85

and R8.75 per cow above the costs of the treatment programs. The UPL3 diagnosis was not profitable and could possibly be attributed to a certain extent by the time lost (65 days post service).

8.3.2 Sensitivity analysis

The profitability of each pregnancy diagnosis method was assessed using the change in the total cost per animal relative to the control group. The sensitivity of this economic feasibility analysis with regard to the value of the parameters considered, was performed changing one parameter at a time - with all other factors being kept constant.

The effects of h (probability of oestrous detection) and hb (detection of oestrus in cows diagnosed non-pregnant) on the financial returns due to the pregnancy diagnosis method utilized, is set out in Table 8.2. The profitability of the pregnancy diagnosis was inversely related to h and directly related to hb in all the other schemes. A change in the efficiency of oestrus detection in the herd from 0.40 to 0.60 caused the financial return per cow related to the MPPS diagnosis to decrease by R32.20, while for the UPL1, UPL2, and UPL3 techniques, the decreases were R8.75, R10.85 and R9.80 respectively. The inverse relationship between the financial returns from using the on-farm milk progesterone pregnancy test to identify non-pregnant cows and the efficiency of oestrous detection in the herd had also been reported by Ruiz *et al.* (1989). An increase in efficiency of oestrus detected in cows as diagnosed non-pregnant (as a percentage of the herd oestrous detection rate) from 10 to 50% increased the financial returns per cow by R61.60 for the MPPS diagnosis while for the UPL1, UPL2 and UPL3 techniques, the financial increases were R40.95, R25.20 and R10.85 respectively. Without an increase in the efficiency of oestrous detection in cows diagnosed as non-pregnant, none of the pregnancy diagnosis methods evaluated were profitable. For the MPPS technique to break even, a 10% increase in efficiency of oestrous detection is required - while for the UPL1, UPL2, and UPL3 techniques, increases of 15 to 30% in detection are essential (Williams & Esslemont, 1993).

Table 8.2 Sensitivity analysis of the economic return (R) per cow[1] for the different pregnancy diagnosis methods with respect to the probability of detection of oestrous in the herd

Pregnancy diagnosis method	Oestrous detection		Efficiency	
	For the herd		After diagnosis	
	h = .40	h = .60	hb =10%	HB = 50%
MPPS	14.60	5.40	0.20	17.80
UPL1	6.10	3.50	-0.70	11.00
UPL2	4.00	0.90	-1.20	6.00
UPL3	1.10	-0.70	-1.40	1.70

[1] The differences, relative to control, in total cost per cow for each method

The sensitivity of the financial returns from pregnancy diagnosis with respect to d (day at first service) and t (errors in pregnancy diagnosis) are set out in Table 8.3. The economic returns of the pregnancy diagnosis was directly related to d and inversely related to t for all techniques studies. A change in the day of first service from 60 to 100 days resulted in an decrease in the return per cow from R28.70 to R7.70 for the MPPS test and UPL1 technique, while for the UPL2 and UPL3 methods the financial returns were basically unchanged. The profitability of the MPPS scheme was more sensitive to the probability of an error in the pregnancy diagnosis than the methods based on rectal uterine palpation evaluation. The return per cow for the MPPS diagnosis scheme decreased by R16.45 and R32.90 when the probability of errors in the diagnosis (t), increased from 0 to .025 and 0.05, respectively. For the same changes in t, the return per cow decreased by R0.70 and R35.00 for the UPL1, UPL2, or UPL3 diagnosis. The larger financial implication associated with diagnostic errors in the MPPS test was due to abortions caused by the prostaglandin treatment of pregnant cows erroneously classified as not pregnant after the progesterone test.

Table 8.3 Sensitivity analysis of the economic return (R) per cow[1] for various pregnancy diagnosis methods[2] with respect to day of first service post partum and probability of errors in pregnancy diagnosis

	Time to first service post partum (days)		Error rate in pregnancy diagnosis	
Pregnancy diagnosis method	60.00	100.00	0.03	0.10
MPPS	7.50	16.00	5.80	1.10
UPL1	4.90	7.10	4.90	4.70
UPL2	3.20	3.20	2.30	2.20
UPL3	0.60	0.30	0.10	0.00

[1] The differences, relative to control, in total cost per cow for each scheme
[2] For definition of pregnancy diagnosis schemes see Table 8.1

The MPPS method of pregnancy diagnosis was the diagnosis of choice for most of the circumstances considered, but the increase in efficiency of detection of oestrus among cows diagnosed as non-pregnant was critical for its economic viability. An increase in efficiency of more than 20% above the prevailing oestrous detection practices in a herd is needed to ensure its profitability (Kourletaki *et al.*, 1995). As the time of oestrus is predicted more reliably in a cow with low progesterone concentrations on day 19 after service and for a cow treated with prostaglandin, this increase in detection efficiency could easily be achieved. Another critical parameter was probable errors in the diagnosis of pregnancy. With a 3% error, the test lost all its economic superiority over the UPL1 assessment method. If the probability of an error in pregnancy diagnosis cannot be kept below 2%, other alternatives to avoid wrongly treating pregnant cows, incorrectly diagnosed as non-pregnant with prostaglandin, should be considered. One option may be the use of rectal uterine palpation prior to treatment time to confirm non-pregnancy (Esslemont *et al.*, 1985). The financial returns associated with the MPPS test was sensitive to service cost or the cost of prostaglandin treatment. The financial return of R36.75 (Table 8.1) decreased to R35.70 and R34.65 if the semen cost increased to R52.50 and R70.00 and increased to R37.80, R39.20 and

R40.60 if prostaglandin treatment was decreased to R14.00, R10.50 and R7.00 respectively.

The UPL1 method was the second most suitable test with a net gain of R17.50 to R21.00 per cow above the cost of the breeding program. This method had a lower profitability than the MPPS pregnancy test, but was less sensitive to the efficiency of oestrous detection in the herd (Table 8.2) or to the error rate in pregnancy diagnosis (Table 8.3). Like in the MPPS test, its profitability was dependent on the percentage increase in oestrous detection efficiency in cows diagnosed as non-pregnant.

Those results pertaining to the effect of the UPL1 pregnancy test up to this point are not in agreement to values quoted by White *et al.* (1989) who reported an average ICP of 377 and 378 days in groups of cows diagnosed pregnant by rectal palpation at less than 50, 51 to 56 days after service respectively. It was concluded that the optimum time for rectal uterine palpation for pregnancy diagnosis in Holstein dairy cows to be 51 to 56 days post service and early palpation was not effective in reducing the ICP - probably due to higher embryonic mortality rate, a lower pregnancy diagnosis accuracy, or both. To determine the effect of embryonic mortality profitability, the cost per cow was determined with a baseline value of 10% for embryonic mortality, as well as an increased embryonic mortality of 12 and 15%. The net return per cow decreased from R17.85 (baseline, Table 8.1) to R16.83 and R41.05, respectively. Even a small increase in embryonic mortalities due to early palpation made the UPL1 scheme not financially viable.

8.4 CONCLUSION

Routine pregnancy diagnosis in dairy cows can be a profitable intervention if the identification of non-pregnant cows is made relatively early after service and if effective measures are implemented to increase the probability of conception in these cows. Among the alternatives considered, the most profitable PD method evaluated was on milk progesterone levels on day 19 following service - followed by a prostaglandin treatment of the non-pregnant cows not seen in estrus within 10 days after test with a net return of R36.75

per cow. If rectal uterine palpation, followed by use of pressure sensitive mounting devices on non-pregnant cows was used, only the techniques performing pregnancy diagnosis on day 35 or 50 post-service were profitable with a net return of R17.85 and R8.75 per cow, respectively. For all tests evaluated, an increase in the efficiency of oestrous detection in cows diagnosed as not pregnant of more than 20% in the herd is needed to ensure profitability and viability of the techniques. To ensure profitability in the milk progesterone/prostaglandin treatment, it was essential to keep the probability of inducing abortion in a cow wrongly diagnosed as open, below 2%. This could be achieved by reducing diagnostic errors and using other techniques to verify that the cow was not pregnant prior to prostaglandin treatment. The techniques using rectal uterine palpation were profitable only if embryonic mortality rates were not increased as a result of rectal palpation.

CHAPTER 9

GENERAL CONCLUSIONS

From the trials undertaken to evaluate the implementation of a milk progesterone test to improve artificial insemination efficiency and related aspects in dairy cattle, the following conclusions were drawn:

1. The milk progesterone radioimmunoassay which was tested, was accurate and reliable and could thus be implemented commercially.

   The requirements of a commercial progesterone RIA test in dairy cattle has the ability to process large numbers of samples fast with precision, repeatability and reliability. The assay implemented for this study was based on the large-scale technique of the IAEA (1996), which meets all these requirements. Although the number of milk samples processed in this investigation (approximately 3000 in duplicate) was relatively small, this assay was more rapid and the results indicated precision and reliability to be acceptable. Thus, it appeared as if the assay could easily be adapted and is acceptable for commercial use under South African conditions.

2. The pregnancy test was accurate provided record-keeping was efficient and up to date.

   The 90.4% accuracy for pregnancy diagnosis was acceptable when factors affecting this test are taken into consideration. This success rate would have been lower if record-keeping on the farms had been poor. On numerous occasions when a false positive result was recorded, a check of the records indicated an incorrect sampling time. Such samples were consequently excluded from the analyses. Such experiences support the contention that the value of the test is in the detection of non-pregnant cows, rather than pregnant cows. The

identification of these cows early post partum allows the administration of cost effective treatment.

3. Testing the time of insemination was helpful in cases of luteal phase inseminations only.

In this study, oestrous detection was satisfactory and very few cows were inseminated wrongly (not in oestrus). On farms with poor conception rates, where oestrous detection inefficiency was suspected, the test has shown to be of some use. The low progesterone levels around the oestrous period preclude the use of the test to determine the optimum time of insemination. Thus the use of milk progesterone analysis in this field is limited.

4. The choice of "problem" cows was relatively ineffective.

Problem cows were chosen on the basis of their previous or current reproductive status. Cows which were classified "repeat breeders" in their previous lactation were selected for the study and would conceive at the first insemination during their current lactation or seemed to conceive at the subsequent insemination. Thus, during the second phase of the study, cows which had recently calved were randomly chosen and monitored from calving to conception. These cows provided more information.

From these observations it was concluded that to make efficient use of milk progesterone analysis, it would be necessary to monitor the entire herd. At the moment though, this would prove to be too expensive.

5. Milk progesterone profiles were useful in the diagnosis and treatment of some specific fertility problems only.

The results of this study indicate that anoestrus, poor luteal function (short luteal phases), irregular cycles, embryo mortality and cysts are identifiable from the milk progesterone profiles. Treatment of these problems, using exogenous hormones, could be monitored and rectified. In such cases, milk progesterone determinations proved to be invaluable. In contrast to this, some cows had irregular patterns of progesterone secretion, yet conceived whilst other appeared to have normal progesterone profiles but did not conceive. Such cases demonstrate the limitations of the analyses.

6. Progesterone profiles alone did not provide complete evidence of certain reproductive disorders, but used in conjunction with rectal palpation, a correct diagnosis was achieved.

These conclusions encompass some of the advantages and disadvantages of milk progesterone analysis as an indicator of the reproductive status of the dairy herd.

Progesterone levels in milk provided a convenient and effective method of monitoring certain reproductive activities such as cyclic and acyclic patterns, cystic ovaries and early pregnancy diagnosis. However, in the areas of oestrous detection and the diagnosis of early embryonic mortality, more specific tests regarding the occurrence of oestrus and pregnancy would provide valuable additional information.

Despite these limitations, milk progesterone analyses have been shown to provide useful information in the field of dairy reproduction management - with the accuracy of the information obtained being related to the efficiency of the management. The future of milk progesterone assays as a means of monitoring reproductive efficiency of cows lies in the overall cost benefit and practicability of the programme. The ideal system would involve milk sampling in the National Dairy Improvement Scheme.

An investigation into the practical application of the radioimmunoassay (RIA) test of milk progesterone to improve artificial insemination (AI) management in dairy cattle

by

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ABSTRACT

The aim of the study was to investigate and improve the success of AI services of cattle in South Africa via milk progesterone determinations and increase the reproduction efficiency by the identification of causes of reproductive inefficiency and to implement appropriate changes to rectify these problems.

These objectives were expected to be achieved by:

- Improving the efficiency of oestrous detection, resulting in an increased number of cows being inseminated;
- Improving the timing of insemination, resulting in increased conception rates;

- Identifying anoestrous cows at the time of expected breeding, allowing the adoption of appropriate remedial measures;
- Identifying cows which should return to oestrus after AI, focusing the attention of farmers on these animals;
- Have cows bred in time.

The standardized methodology and uniform approach to data recording and analyses used in this study resulted in the generation of an unique international and national data set of the current status of artificial insemination (AI) in dairy cattle. Determination of progesterone by radioimmunoassay (RIA) in milk samples collected at specific times in relation to AI, combined with the use of the computer database AIDA, proved to be a powerful tool for calculating reproductive indices and identifying factors which affect these factors.

The methodology and approach provided a better understanding of the integrated factors influencing AI programmes. This study resulted in the first reliable assessment of the success rate of AI and the efficiency of the reproductive management programmes by dairy farmers in South Africa. It shifted the research emphasis to a more problem solving approach. The results are based on over 3000 services on 1850 cows on 5 farms and have permitted a clear understanding of the major constraints and factors contributing to the inefficiency of AI in the cattle industry. The results have highlighted the need for closer monitoring of field results by AI service providers and for better education of AI technicians and farmers.

The 90.4% accuracy for pregnancy diagnosis and 98.3% accuracy for non-pregnant diagnosis by milk P4 tests, was acceptable in this study - taking into consideration factors playing a role. The main causes of low fertility experienced in the herds were oestrous detection failure, inseminations at inappropriate times, embryo mortality, reproductive abnormalities and factors related to management on the individual farms. Of the 768 cows selected only 691 (90%) had at least one successful breeding, whereas the remaining

10% were culled or died before the first insemination. From this dairy population, 540 cows (78.1%) conceived. The first service conception rate recorded was 40.5% and overall conception rate 78.1% with an average of 2.4 services/straw per conception. Of all the factors analysed, the interval from calving to first service (ICS) and interval from calving to conception (ICC) were affected only by parity. First-calf heifers had an interval 40 days longer than mature cows (124 versus 84 days). Animals with a BW of below 500 kg recorded a longer intercalving interval, compared to heavier (>500 kg) cows (118 versus 85 days).

Of the 1215 milk samples obtained on the day of breeding, 135 (11.1%) had milk progesterone values greater than 3nmol/l and were considered as luteal levels - according to the precision of the RIA test. According to this criterion, cows with a milk P4 level of above 3nmol/l were bred at the wrong time.

Reproductive records were analysed to determine the oestrous detection success rate and the pregnancy rate following the first service, in cycles of 21 days. The overall oestrous detection rate (ODR) was 37.5% and predicted pregnancy rate (PR) was 15.6% - due to the low ODR and conception rate of 41.9%. As cows were not bred at the same time, a total of 1424 animals in 6 periods of 21 days were evaluated for the calculation of the ODR and PR.

Another method to calculate the oestrous detection efficiency and embryonic mortality rate was to calculate the interval between service or the inter-oestrous intervals. A total of 718 intervals were recorded with a mean inter-oestrous interval of 36 days. Only 45.1% of the intervals were within the normal range of 18 to 24 days - 5.8% intervals were "short" (<18 days) while only 1.7% of the intervals were "long"; 28.0% were 2 x normal (36 to 48 days), meaning one missed oestrus and 19.4% were "prolonged" periods, representing two missed oestrous periods/abortions.

Milk or plasma (MUN or PUN) urea nitrogen concentrations were higher than 19mg/dl and the plasma or milk urea concentration could be associated with a

decrease in pregnancy rate of dairy cattle. It may be beneficial for dairy producers to monitor urea concentrations in their herds in an effort to improve the reproductive efficiency of the herd. With the high correlation between MUN and PUN levels, milk is the more practical to sample and stress free to the animal and thus the obvious choice to determine the urea nitrogen status of the animal. If a representative milk sample is used, the nutritional management of the farm can be monitored regularly.

Routine pregnancy diagnosis in dairy cows can be a profitable intervention if the identification of non-pregnant cows is made relatively early after service/AI. Effective measures have to be implemented to increase the probability of conception in these cows. Of the alternatives considered, the most profitable PD method evaluated is the milk progesterone levels on day 19 following service/AI – followed by a prostaglandin treatment of the non-pregnant cows not seen in oestrus within 10 days after the test - with a net return of R36.75 per cow. If rectal uterine palpation, followed by use of pressure sensitive mounting devices on non-pregnant cows was used, only the techniques predicting pregnancy diagnosis on day 35 on 50 post-service were profitable - with a net return of R17.85 and R8.75 per cow, respectively. For all tests evaluated, an increase in the efficiency of oestrous detection in cows diagnosed as not pregnant of more than 20% in the herd is needed to ensure profitability and viability of these techniques. To ensure profitability in the milk progesterone/prostaglandin treatment, it was essential to keep the probability of inducing abortions in a cow wrongly diagnosed as open, below 2%. This can only be achieved by reducing the diagnostic errors and using other techniques to verify the cow as not pregnant prior to prostaglandin treatment. The techniques using rectal uterine-palpation were profitable only if embryonic mortality rates were not increased as a result of rectal palpation.

'n Ondersoek na die praktiese aanwending van radioimmunologiesebepalings - (RIA) van melkprogesteroon om die bestuur van kunsmatige inseminasie (KI) in melkbeeste te verbeter

deur

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OPSOMMING

Die doel van die studie is om die kwaliteit van KI dienste by beeste in Suid-Afrika te ondersoek, te verbeter en ook om die reproduksiedoeltreffendheid te verbeter deur reproduktiewe probleme te identifiseer en op te los d.m.v. melk progesteroon bepalings.

Hierdie doelwitte kon verkry word deur:

- Doeltreffendheid van estrusbepalings te verbeter om sodoende meer koeie te insemineer;
- Op die regte tyd te insemineer vir verhoogde bevrugting;
- Anestrus koeie betyds te identifiseer en sodoende geskikte behandelings toe te pas;

- Koeie in estrus na KI te identifiseer en die aandag van die boere op dié diere te vestig sodat teling betyds kan plaasvind;
- Teling binne die minimum tydperk te laat plaasvind.

Die gestandaardiseerde metodologie en ooreenkomstige benadering tot die rekordhouding en analisering van data gebruik in die studie, het 'n unieke internasionale en nasionale datastel om die huidige status van KI by beeste te bepaal, geskep. Die bepaling van progesteroon deur radio-immunologiebepalings (RIA) in melkmonsters, gekollekteer op 'n spesifieke tyd na KI, gekombineer met die gebruik van 'n rekenaardatastel AIDA, is gewys om 'n magtige instrument te wees om tekens van reproduksie steurnisse te identiseer, asook faktore wat hierdie faktore affekteer.

Hierdie metodologie en benadering het 'n beter begrip van faktore wat KI programme beïnvloed tot gevolg gehad. Tydens hierdie studie is die eerste betroubare skatting van die sukses van KI en die effektiwiteit van reproduksiebestuursprogramme by melkboere in Suid-Afrika bepaal. Dit het die navorsingsklem na 'n meer probleemoplossingsbenadering verander. Die resultate is gebaseer op meer as 3000 inseminasies op 1850 koeie op 5 plase en 'n beter begrip van die beperkings en faktore wat bydra tot die oneffektiwiteit van KI in die beesbedryf. Die resultate het die behoefte aan beter monitering van veldresultate deur insemineerders en vir beter opleiding van insemineerders en boere uitgewys.

Die akkuraatheid van die diagnose vir dragtigheid (90.4%) en nie-dragtig (98.3%) is aanvaarbaar vir die studie, as die faktore wat die uitslag beïnvloed in aanmerking geneem word. Die hoofoorsake van die lae vrugbaarheidsyfer in die kudde is estrus wat nie waargeneem word nie, inseminasies op verkeerde tye, swak semen gehalte, embrio mortaliteit, reproduksie abnormaliteite en swak bestuur op die verskillende plase. Van die 768 koeie geselekteer het slegs 691 (90%) ten minste een suksesvolle konsepsie gehad, terwyl die oorblywende 10% uitgeskot of dood is voor die eerste inseminasie. Uit hierdie koeie het 540 koeie (78%) beset geraak. Die eerste

konsepsiesyfer waargeneem was 40.5% en die algemene konsepsyfer 78.1% - met 'n gemiddeld van 2.4 strooitjies per konsepsie. Uit al die faktore wat geanaliseer is, is die ICS en ICC deur pariteit geaffekteer. Eerste-kalf verse het 'n 40 dae langer interval as ou koeie (124 versus 84 dae) gedemonstreer. By koeie met 'n liggaamsgewig van minder as 500 kg is 'n langer kalf interval aangeteken, vergeleke met swaarder (>500 kg) koeie (118 versus 85 dae).

Uit die 1215 melkmonsters geneem op die dag van KI, het 135 (11.1%) melkprogesteroon waardes groter as 3nmol/l gehad en is oorweeg as luteale vlakke volgens die akkuraatheid van die RIA toets. Volgens hierdie kriteria is koeie met melk P4 vlakke van 3nmol/l op die verkeerde tyd geteel.

Reproduksierekords is ontleed om die suksesvolle estruswaarnemings- en dragtigheids-tempo vas te stel na die eerste besetting in siklusse van 21 dae - vanaf die eerste observasies tydens die teelperiode. Die algehele estruswaarnemingstempo was 37.5% en die bepaalde dragtigheidstempo 15.6% - as gevolg van die lae ODR en 'n konsepsiesyfer van 41.9%. Aangesien koeie nie op dieselfde tyd geteel is nie, is 'n totaal van 1424 diere in 6 periodes van 21 dae elk estrus waarnemingstempo, geëvalueer om die ODR en RG te bereken.

Nog 'n metode om estrus waarnemingsdoeltreffendheid en embrioniese mortaliteit te bepaal, was om die periodes tussen KI of tussen estrus intervalle te bepaal. Met die totaal van 718 intervalle wat aangeteken is, is 'n gemiddelde estrus interval van 36 dae vasgestel. Slegs 45.1% van die intervalle was binne die normale bestek van 18 tot 24 dae – 5.8% intervalle was "kort" (<18 dae), terwyl slegs 1.7% van die intervalle 'lank" was; 28.0% was 2 x normaal (36 tot 48 dae), menende dat dit een nie-observeerde estrus ingesluit het en 19.4% was verlengde periodes en verteenwoordig 2 of meer nie-waargeneemde estrus periodes of aborsies.

Melk of plasma (MUN of PUN) ureum stikstofkonsentrasies was hoër as 19 mg/dl en die plasma of melk konsentrasies kon met 'n verlaging in

dragtigheidstempo in melkbeeste geassosieër word. Dit kan voordelig vir melkboere wees om ureumkonsentrasies te monitor om sodoende die reproduksiedoeltreffendheid van die kudde te verbeter. Met die hoë korrelasie tussen MPN en PUN vlakke is dit meer prakties om 'n melkmonster te neem om die ureum stikstof status van die dier te bepaal.

Roetine dragtigheidsdiagnose in melkbeeste kan voordelig wees omdat nie-dragtige koeie gou na KI geïdentifiseer kan word. Effektiewe maatstawwe moet implementeer word om alle konsepsiemoontlikhede in hierdie koeie te verhoog. Van die moontlikhede wat oorweeg is, is die mees betalende dragtigheidsdiagnose om die melkprogesteroonvlakke op dag 19 na KI te meet, gevolg met 'n prostaglandien behandeling van nie-dragtige koeie waar estrus nie waargeneem is binne 10 dae na die toets - met 'n netto bedrag van R36.75 per koei. As rektale palpasie, gevolg deur die gebruik van sensitiewe druk toerusting op nie-dragtige koeie gebruik word, was slegs die tegnieke om dragtigheidsdiagnose uit te voer op dag 35 en 50 na paring bekostigbaar, met 'n netto inkomste van R17.85 en R8.75 per koei, respektiewelik. Uit al die toetse wat geëvalueer is, is 'n verhoging in die sukses van estrus waargeneem van meer as 20% in koeie gediagnoseer as nie dragtig van 'n kudde nodig om die winsgewendheid van die tegniek te verseker. Om winsgewendheid in die melk progesteroon/prostaglandien behandeling te verseker, is dit noodsaaklik om die moontlikheid van geïnduseerde aborsies in 'n koei foutief gediagnoseer as nie-dragtig, tot laer as 2% te beperk. Dit kan bereik word deur diagnose foute te verminder en ander metodes te gebuik om vas te stel of die koei nie dragtig was voor die prostaglandin behandeling nie. Hierdie metodes waar gebruik gemaak is van baarmoeder-palpasie was winsgewend slegs as embrioniese mortaliteite nie verhoog as gevolg van die rektale palpasie nie.

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