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Oestrus and Ovulation Detection in Pasture-Based Dairy Herds: The Role of New Technologies

By

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CONTENTS

ABSTRACT

Automatic milking systems (AMS) are becoming increasingly popular due to the growing cost of labour and reduced labour availability. The voluntary cow traffic and resultant distribution of milkings throughout the day and night affects most aspects of herd and farm management in AMS. The literature review (Chapter 1) highlighted a need to evaluate the effects of milk yield and milking frequency during early lactation on reproductive performance. The analysis of a 5-year historic database from Australia's first AMS research farm (Chapter 2) found no significant association of average milk yield and milking frequency during 100 days in milk with any of the reproductive measures. However, the interval from calving to first oestrus increased gradually within the study period and consequently influenced other reproductive outcomes. As a result, a series of studies were conducted with a multidisciplinary approach (both physiological and technological) to investigate the potential to improve oestrus detection on pasture-based AMS farms. A field study (Chapter 3) was conducted to allow for the development and application of an algorithm to assess the application accuracy of an infrared thermography (IRT) device when used to detect oestrus events or pending oestrus events by detecting the time of ovulation. Vulval and muzzle temperatures were measured by IRT in twenty synchronized cows (using a controlled internal drug release and prostaglandin $F_{2\alpha}$). Whilst the IRT showed some potential as an oestrus detection aid with higher sensitivity than visual observation (67%) and Estrotect activation (67%), the specificity and positive predictive value were lower with the IRT. The vulva and muzzle were the focus areas for the IRT application and some concern was generated with regard to the potential for the IRT data to impacted by faecal contamination, obscuring of the vulva by the tail and time since last drinking (affecting muzzle surface temperature). To address these concerns a further study (Chapter 5) was conducted to test the hypothesis that the specificity of IRT in detecting oestrus (or imminent oestrus) could be improved if other body parts were focused on. In that study (Chapter 5), an additional technology was incorporated to test the hypothesis that the combined activity and rumination data generated by an accelerometer (SCR heat and rumination long distance tags) would provide a more accurate indication of oestrus and/or ovulation than the activity and rumination data alone. Unfortunately the monitoring of eyes and/or ears did not provide the improvement in accuracy of IRT (as an oestrus detection aid) indicating that as an oestrus detection aid there was likely to be limited value in

developing this as an automated stand-alone device. Alerts generated by accelerometer based on a lower activity threshold level had high sensitivity and may be able to detect a high proportion of cows in ovulatory periods in pasture-based system; however, the specificities and positive predictive value were lower than the visual assessment of mounting indicators and would still require the herd's person to filter data to identify the false alerts to ensure that cows are not inseminated unnecessarily. Whilst the use of in-line milk monitoring has already been commercialized for the assessment of milk progesterone, there is potential for other biomarkers to provide further opportunities for the assessment of milk components. Biomarkers of oxidative stress were evaluated in plasma showing that plasma glutathione was lower in ovulated cows compared to those of an-ovulated cows (Chapter 4). Whilst baseline plasma data for oxidative stress biomarkers was a useful starting point, the real value of these biomarkers would be realised if their concentration in milk could be linked with oestrus (and or ovulation). Milk superoxide dismutase activity was shown to be higher in ovulated cows while lipoperoxides, glutathione peroxidase were lower in ovulated cows compared to those in an-ovulated cows (Chapter 6). Further work would be required to determine the accuracy with which these biomarkers could be used to identify oestrus cows but these results are promising and suggest that there may be some potential to develop in-line milk sampling technology to alert the herdsperson to cows that should be inseminated.

In summary, this thesis provides very useful, scientifically based information on potential use of technologies for oestrus and ovulation detection in dairy cows, which should serve as a foundation to develop and upgrade automated on-farm technologies and biosensors for better reproductive management of cows in pasture-based AMS. However, it is noted that the most likely success with automated oestrus detection is to require a combination of different indicators that should be incorporated to truly increase the accuracy of detection beyond that which can be achieved by skilled and devoted herd's people.

PREFACE

All chapters within this thesis have been written in publication style with Australian English as the preferred language. Chapters 2, 3, 4, 5, 7 and 8 have all been published in peer-reviewed journals, whilst chapter 6 has been submitted as a revised manuscript for publication in a peer-reviewed journal; all with S. Talukder as first author as indicated accordingly on the cover page. Sections of chapter 1 are intended for publication with modifications in peer-reviewed journals.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, in either full or in part, for a degree at this or any other institution.

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LIST OF ABBREVIATIONS

The following is a complete list of abbreviations used throughout the thesis, although they are also defined the first time they are used in each separate chapter.

AL	Activity level
AOPP	Advanced oxidation protein products
AmbT	Ambient temperature
AI	Artificial insemination
AMS	Automatic milking system(s)
ADMY	Average daily milk yield
β	Beta
BAP	Biological antioxidant potential
BCS	Body condition score
BW	Body weight
CA	California
CI	Calving interval
Carr U	Carratelli units
CAT	Catalase
CO	Colorado
CI	Confidence interval
CIDR	Controlled intravaginal drug release
CMS	Conventional milking system
Corp.	Corporation
CL	Corpus luteum
r	Correlation coefficient
Cov	Covariate
DIM	Days in milk
DO	Days open
^{o}C	Degree Celsius
dROMs	Derivatives of reactive oxygen metabolites
ELISA	Enzyme linked immuno sorbent assay
equi.	Equivalent
FN	False negative
FP	False positive
FCMY	Fat corrected milk yield
FPR	Fat protein ratio
FSH	follicle stimulating hormone
GLMM	Generalized linear mixed model
GM	Geometric mean
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GnRH	Gonadotrophin releasing hormone
g	Gram(s)
g	Gravity
GR	Grosseto
HR	Hazard ratio
HR	Heat and rumination
HFCMY	High fat corrected milk yield

HFPR	High fat protein ratio
h	Hour(s)
Hum	Humidity
H_2O_2	Hydrogen per oxide
Inc.	Incorporation
IN	Indianapolis
IRT	Infrared thermography
kg	Kilogram(s)
LP	Lactoperoxidase
LSM	Least square mean
LDA	Left displaced abomasum
Ltd.	Limited
LPO	Lipoperoxides
L	Liter(s)
LS	Locomotion score
LD	Long distance
LFCMY	Low fat corrected milk yield
LFPR	Low fat protein ratio
Q1	Lower quartile
LH	Lutenizing hormone
MFCMY	Medium fat corrected milk yield
MFPR	Medium fat protein ratio
m	Meter(s)
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer(s)
µmol	Micromole(s)
mg	Milligram(s)
mL	Milliliter(s)
mm	Millimeter(s)
mmol	Millimole(s)
min	Minute(s)
muzzleT	Muzzle temperature
nanoEq	Nano equivalent
ng	Nano gram
nm	Nanometer
NEB	Negative energy balance
NSW	New South Wales
NC	North carolina
n or no.	Number
OR	Odds ratio
E_2	Oestradiol
OS	Oxidative stress
OSI	Oxidative stress index
%	Percent
PPV	Positive predictive value
P	Probability
P_4	Progesterone
$PGF_{2\alpha}$	Prostaglandin$F_{2\alpha}$
ROS	Reactive oxygen species

ROC	Receiver operator characteristic
REML	Restricted maximum likelihood
RL	Rumination level
s	Second(s)
Se	Sensitivity
Sp	Specificity
SD	Standard deviation
s.e.m.	Standard error of the mean
O_2^-	Superoxide
SOD	Superoxide dismutase
TN	True negative
TP	True positive
UV	Ultraviolet
U	Unit
Umol	Unit mole
UK	United Kingdom
USA	United States of America
Q3	Upper quartile
VIC	Victoria
vulvaT	Vulval temperature
WA	Washington
XO	Xanthine oxidase

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PUBLICATIONS ORIGINATED FROM THIS CANDIDATURE

Publications in peer-reviewed journals

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2014) Factors affecting reproductive performance of dairy cows in a pasture-based, automatic milking system research farm: a retrospective, single-cohort study. *Animal Production Science* **55,** 31-41.

Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P (2014) Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture based system. *Theriogenology* **81,** 925-935.

Talukder S, Kerrisk KL, Ingenhoff L, Gabai G, Garcia SC, Celi P (2014) Changes in plasma oxidative stress biomarkers in dairy cows after oestrus synchronization with controlled internal drug release (CIDRs) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$). *Animal Production Science* **54,** 1490–1496.

Talukder S, Thomson PC, Kerrisk KL, Clark CEF, Celi P (2015) Evaluation of infrared thermography (IRT) body temperature and collar mounted accelerometer and acoustic technology for predicting time of ovulation of cows in a pasture-based system. *Theriogenology* **83,** 739-748.

Talukder S, Ingenhoff L, Kerrisk KL, Celi P (2014) Plasma oxidative stress biomarkers and progesterone profiles in a dairy cow diagnosed with an ovarian follicular cyst. *Veterinary Quarterly* **34,** 113-117.

Talukder S, Kerrisk KL, Clark CEF, Garcia SC, Celi P (2014) Rumination patterns, locomotion activity and milk yield for a dairy cow diagnosed with a left displaced abomasum. *New Zealand Veterinary Journal* 1-6.

Manuscripts submitted to peer-reviewed journals

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Peer-reviewed conference papers

Talukder S, Kerrisk, KL, Gabai G, Fukutomi A, Celi P (2014) Changes in milk oxidative stress biomarkers in dairy cows with ovulatory and anovulatory oestrous cycles. In 'Animal Production in Australia, Proceeding of the 30th Biennial Conference of the Australian Society of Animal Production'. p. 330. (Canberra, Australia)

Talukder S, Kerrisk, KL, Clark CEF, Celi P (2014) Assessment of neck mounted accelerometer technology for detecting oestrus in pasture-based dairy cows. In 'Animal Production in Australia, Proceeding of the 30th Biennial Conference of the Australian Society of Animal Production'. p. 331. (Canberra, Australia)

Talukder S, Kerrisk KL, Ingenhoff L, Garcia SC, Celi P (2013) Use of digital infrared thermography to measure the skin temperature changes in oestrus synchronized dairy cows. *Journal of Dairy Science* 96, E-Suppl. 1, p. 204.

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2012) Reproductive performance of dairy cows in a pasture based automatic milking system research farm: a retrospective analysis. In 'Animals, Proceedings of the 5th Australasian Dairy Science Symposium 2012'. pp. 302-305. (Melbourne, Australia)

Non peer-reviewed conference papers

Talukder S, Kerrisk KL, Clark CEF, Garcia SC, Celi P (2014) Rumination patterns and locomotion activity in a dairy cow diagnosed with left displaced abomasum (LDA): A case report. In 'Current Topics in Dairy Production, Proceedings of the Dairy Research Foundation Symposium'. pp. 91-95. (Hunter Valley, Australia)

Talukder S, Kerrisk, KL, Gabai G, Fukutomi A, Celi P (2014) Milk oxidant and antioxidant status in dairy cows with ovulatory versus anovulatory oestrous cycles.

In ‘Health and welfare of dairy animals, Proceedings of the 1st Dairycare Conference’. Poster Abstracts. p. 62 (Copenhagen, Denmark)

Talukder S (2014) Infrared thermography (IRT) as a heat detection aid. In ‘Proceedings of the Australian Dairy Conference’. pp. 47-49. (Geelong, Australia)

Talukder S, Kerrisk KL, Ingenhoff L, Garcia SC, Celi P (2013) Can digital infrared thermography help with prediction of ovulation in a pasture-based dairy system? In ‘Current Topics in Dairy Production, Proceedings of the Dairy Research Foundation Symposium’. pp. 145-149. (Kiama, Australia)

Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P (2013) Application of infrared thermography for oestrus detection in dairy cows. In ‘Proceedings of the Veterinary Science Postgraduate Conference’. p. 63. (Camperdown, Sydney)

Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P (2013) Evaluation of infrared thermography for predicting ovulation time in dairy cows. In ‘4th Australian and New Zealand Spatially Enabled Livestock Management Symposium’. p. 43. (Camden, Australia)

Talukder S, Kerrisk KL, Celi P, Garcia SC (2012) Use of digital infrared thermography for oestrus detection in dairy cows. In ‘Improving Smallholder and Industrial Livestock Production for enhancing Food Security, Environment and Human Welfare, Proceedings of the 15th AAAP Animal Science Congress’. p. 74. (Bangkok, Thailand)

Talukder S, Celi P, Kerrisk KL, Garcia SC (2012) Use of thermal infrared scanning technique for oestrus detection in dairy cows. In ‘Current Topics in Dairy Production, Proceedings of the Dairy Research Foundation Symposium’. pp. 107-110. (Camden, Australia)

Talukder S, Celi P, Kerrisk KL, Garcia SC (2012) Factors affecting calving to first oestrus and calving to conception interval in a pasture based, automatic milking system research farm. In ‘Proceedings of the Veterinary Science Postgraduate Conference’. p. 78. (Camperdown, Sydney)

Talukder S, Celi P, Kerrisk KL, Garcia SC (2011) Reproductive performance of dairy cows in an automatic milking system. In ‘Proceedings of the Veterinary Science Postgraduate Conference’. p. 68. (Camperdown, Sydney)

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Dairy herd profitability is highly dependent on reproductive performance, since it directly affects both milk production and the number of pregnant animals available to maintain herd size (Neves 2011). Reproductive performance in dairy cows continues to decline in Australia. The InCalf 2010 analysis commissioned by Dairy Australia identified declines in the 6-week in-calf, three-week submission rate and first-service conception rates whilst the 12-week not-in-calf and 21-week not-in-calf and overall not-in-calf rates continue to increase when compared to the analysis conducted in 2000 (Morton 2011). All major dairying countries have experienced similar declines and it has occurred across the full range of production systems (from pasture-based to total mixed ration systems). However, the reasons are not simple: fertility is recognised as a multi-factorial trait with genetic, nutritional, environmental, and managerial components interacting in a complex network (Walsh *et al.* 2011).

The modern high yielding dairy cow tends to have a lower intensity and shorter duration of oestrus (Kamphuis *et al.* 2012). It is thought that lower blood oestradiol concentrations decrease the intensity of behavioural signs of oestrus (Sangsritavong *et al.* 2002; Wiltbank *et al.* 2006; Aungier *et al.* 2012) which exacerbates the problems associated with oestrus detection. In addition, as the Australian industry continues to consolidate at the farm level, the typical Australian dairy farm now milks more cows, has higher production per cow, employs more labour (but has more cows per labour unit) and operates at a higher stocking rate. Spending less time to monitor cows in oestrus and increased reliance on hired unskilled labour have likely further exacerbated the impacts of the fertility decline of the cow.

Due to the growing costs of labour which is the second largest operating cost on Australian dairy farms (second only to feed), automatic milking systems (AMS) are becoming increasingly popular (Rossing and Hogewerf 1997). The systems were introduced in the early 1990's and since then have been adopted by over 10,000 farmers worldwide (de Koning 2011). Questions have been raised as to whether reproductive performance of cows managed in AMS differ from those managed in conventional system. Weiss *et al.* (2004) reported a neutral effect of milking system (automatic vs conventional) on the interval from calving to conception and resumption of ovarian cyclicity for cows kept in a naturally ventilated, loose housing barn. Dearing *et al.*

(2004) monitored six farms in The Netherlands, from up to 6 months before automatic milking system installation through to 12 months post-installation, and showed an increase in interval from calving to conception as well as reduced conception rate. On the other hand, Löf *et al*. (2007) reported shorter calving intervals and shorter calving to first insemination and conception intervals in AMS herds in Sweden compared with herds managed with conventional milking system (CMS). Of course, it is well known that many factors affect reproductive performance and it is likely that the conflicting results are due to a lack of ability to capture and account for all of those factors. However, no published studies have reported the reproductive performance of dairy cows in pasture-based, AMS herds.

The main characteristic of AMS is that milking-related tasks are automated. Therefore, no opportunity exists for visual observation to detect oestrus behaviour at each milking as is possible in conventional milking systems. Achieving efficient oestrus detection by visual observation depends on the timing, duration and frequency of observation (Roelofs *et al.* 2010). In addition, discrete behavioural signs of oestrus, absence of standing mounts for up to 60% of ovulations and the shorter duration of oestrus in modern, high-yielding dairy cows make visual detection of oestrus more difficult (Holman *et al.* 2011; Saint-Dizier and Chastant-Maillard 2011; Kamphuis *et al.* 2012). This combined with the increase of herd size and the need to improve labour efficiency has increased the reliance on automated technologies to reduce dependence on humans and increase oestrus detection rates.

Numerous studies have evaluated the use of indicators in milk, automated technologies and on-farm biosensors to support feeding management, oestrus detection, and detection of udder infections and abnormal milk (Mottram *et al.* 2007; Hettinga *et al.* 2008). Besides these, the use of biosensors and automated technologies may become a reliable and quick tool to measure physiological traits (e.g., oxidants and antioxidants). Oxidants and antioxidants are involved in the regulation of several reproductive functions such as maintenance of the follicular fluid environment, folliculogenesis, steroidogenesis, corpus luteum function, and luteolysis. These biomarkers are also reported to have a role in the pathogenesis of follicular cysts and repeat breeder syndrome in dairy cows (Rizzo *et al.* 2007; Rizzo *et al.* 2009; Talukder *et al.* 2014) and an association with embryonic loss in dairy cows (Celi *et al.* 2011; Celi *et al.* 2012). Therefore, there is a need to

establish whether oxidants and antioxidants could provide a reasonable index of the reproductive status of dairy cows.

The aim of this thesis was to investigate the factors associated with reproductive performance of dairy cows in pasture-based AMS and evaluate technologies for improving the key identified factors associated with reproduction. The general hypothesis was that factors specific to AMS could be associated with reproductive performance and using new technologies could be able to contribute to improved oestrus detection. Whilst AMS in pasture-based systems is still quite infantile it is important to ensure that strong understanding of the challenges (in this case pertaining to reproductive performance) is captured and addressed. This is to ensure that farmers contemplating or committing to the adoption of AMS have adequate knowledge and support regarding the management of the system thereby providing the greatest potential for success which is important if the Australian dairy industry is to remain sustainable into the future.

THESIS OUTLINE

This thesis takes the novel and multidisciplinary approach whereby the biology, physiology and technology are brought together in various studies ensuring that a comprehensive understanding of the true value of the technological application is developed. It is not simply an investigation of the application potential of various technologies but a methodical development of linking the physiological process of oestrus with potential indicators which might be automated for application in the future.

The thesis comprised of review of literature (Chapter 1), 5 studies arising from a historic data analysis, animal studies and laboratory analyses (Chapter 2 to 6), a case report on oxidative stress biomarkers in a dairy cow diagnosed with an ovarian follicular cyst (Chapter 7); whilst an additional case report on rumination patterns, locomotion activity and milk yield for a dairy cow diagnosed with a left displaced abomasum (Chapter 8) and a general discussion and conclusion (Chapter 9). Each chapter is a stand-alone manuscript, each with its own abstract, introduction, materials and methods, results, discussion, and conclusion.

Chapter 1 is a recent comprehensive review on associated reproductive challenges in AMS, measures for describing herd reproductive performance, the physiology and

pathology of the reproductive system, and technological advances for improving reproductive performance particularly oestrus detection in pasture-based systems. As this review is a stand-alone manuscript with sections intended for publication, it incorporates some key findings from the subsequent chapters of this thesis.

Chapter 2 is a retrospective analysis of the associations of milk yield and milking frequency during early lactation with reproductive performance parameters based on five years' data. A gradual increase in interval from calving to first oestrus was identified as the factor that could have the greatest impact on ongoing reproductive performance, resulting in the culmination of field studies designed to evaluate the potentials of new technologies for oestrus detection.

Chapter 3 is an experimental study that investigated the ability of an infrared thermography (IRT) device to predict cows in oestrus and about to ovulate. It was observed that the sensitivity of the IRT alert was greater than visual observation and Estrotect, however, the specificity and positive predictive value were lower than these two aids. This study formed the basis of designing a further study for improving the specificity and capturing data throughout the entire 21-day reproductive cycles which is reported in Chapter 5.

Chapter 4 focuses on investigations of plasma progesterone concentrations and oxidative stress in cows with ovulatory and an-ovulatory responses after oestrus synchronisation with controlled internal drug release and prostaglandin$F_{2\alpha}$ in Chapter 3. Plasma oxidative stress (OS) biomarkers were changed significantly between ovulated and an-ovulated cows and it was hypothesized that OS biomarkers measured in milk would be able to provide a reliable indication of an ovulation event; this hypothesis was tested in Chapter 6. In Chapter 3, a cow was diagnosed with cystic ovarian disease after oestrus synchrony. Her ovarian follicle diameter, hormonal profiles and OS biomarkers during the entire study period were compared to those of ovulated cows in Chapter 7.

Chapter 5 tested the hypothesis that the specificity of IRT in detecting an ovulation event could be improved if body parts that were less likely to be contaminated by faecal matter or affected by moisture were monitored. In addition, another oestrus detection aid, accelerometer (monitors activity and rumination level) were also evaluated. It was observed that vulval temperature resulted in the greatest (80%) specificity but the

poorest (21%) sensitivity compared to the IRT temperatures of other body areas. A cow was diagnosed with a left displaced abomasum. Her activity, rumination pattern and milk yield were compared to those in her cohort, in a case study reported as Chapter 8.

The thesis concludes with a general discussion and conclusion that focuses on several interpretations and implications from this thesis. Key findings include firstly, irrespective of the different body areas explored for IRT image capture, the accuracy of IRT for oestrus detection was relatively poor. Secondly, alerts generated by accelerometer based on a lower activity threshold level had high sensitivity and may be able to detect a high proportion of cows in ovulatory periods in pasture-based system, however, low activity threshold level also resulted to increased false positive cases resulting low specificity and positive predictive value of accelerometer. Further field studies are warranted to reduce the number of false positives. Thirdly, OS status may have important physiological role in facilitating the ovulation process in dairy cows.

REFERENCES

Aungier SPM, Roche JF, Sheehy M, Crowe MA (2012) Effects of management and health on the use of activity monitoring for estrus detection in dairy cows. *Journal of Dairy Science* **95,** 2452-2466.

Celi P, Merlo M, Barbato O, Gabai G (2012) Relationship between oxidative stress and the success of artificial insemination in dairy cows in a pasture-based system. *The Veterinary Journal* **193,** 498-502.

Celi P, Merlo M, Da Dalt L, Stefani A, Barbato O, Gabai G (2011) Relationship between late embryonic mortality and the increase in plasma advanced oxidised protein products (AOPP) in dairy cows. *Reproduction, Fertility and Development* **23,** 527-533.

Dairy Australia (2011) Improvement of the reproductive performance of Victorian dairy herds.

de Koning K (2011) Automatic Milking: Common practice on over 10,000 dairy farms worldwide. In 'Dairy Research Foundation Symposium 2011.' pp. 14-31. (The University of Sydney: Camden, Australia)

Dearing J, Hilerton JE, Poelarends JJ, Neijenhuis F, Sampimon OC, Fossing C (2004) Effects of automatic milking on body condition score and fertility of dairy cows. In 'A better understanding automatic milking.' (Eds. A Meijering, H Hogeveen, CJAM de Koning) pp. 135–140. (Wageningen Academic Publishers: Wageningen, The Netherlands)

Hettinga KA, van Valenberg HJF, Lam TJGM, van Hooijdonk ACM (2008) Detection of mastitis pathogens by analysis of volatile bacterial metabolites. *Journal of Dairy Science* **91,** 3834-3839.

Holman A, Thompson J, Routly JE, Cameron J, Jones DN, Grove-White D, Smith RF, Dobson H (2011) Comparison of oestrus detection methods in dairy cattle. *Veterinary Record* **169,** p. 47.

Kamphuis C, DelaRue B, Burke CR, Jago J (2012) Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. *Journal of Dairy Science* **95,** 3045-3056.

Löf E, Gustafsson H, Emanuelson U (2007) Associations between herd characteristics and reproductive efficiency in dairy herds. *Journal of Dairy Science* **90,** 4897-4907.

Morton J (2011). 'Report on InCalf Fertility Data Project, 2011.' (Dairy Australia: Southbank, Vic.)

Mottram T, Rudnitskaya A, Legin A, Fitzpatrick JL, Eckersall PD (2007) Evaluation of a novel chemical sensor system to detect clinical mastitis in bovine milk. *Biosensors and Bioelectronics* **22,** 2689-2693.

Neves RCDF (2011) 'Investigation of automated activity monitoring systems for reproduction in dairy cattle.' MSc thesis, The University of Guelph, Canada.

Rizzo A, Minoia G, Trisolini C, Manca R, Sciorsci RL (2007) Concentrations of free radicals and beta-endorphins in repeat breeder cows. *Animal Reproduction Science* **100,** 257-263.

Rizzo A, Minoia G, Trisolini C, Mutinati M, Spedicato M, Jirillo F, Sciorsci RL (2009) Reactive Oxygen Species (ROS): Involvement in bovine follicular cysts etiopathogenesis. *Immunopharmacology and Immunotoxicology* **31,** 631-635.

Roelofs J, López-Gatius F, Hunter RHF, van Eerdenburg FJCM, Hanzen C (2010) When is a cow in estrus? Clinical and practical aspects. *Theriogenology* **74,** 327-344.

Rossing W, Hogewerf PH (1997) State of the art of automatic milking systems. *Computers and Electronics in Agriculture* **17,** 1-17.

Saint-Dizier M, Chastant-Maillard S (2011) Towards an automated detection of oestrus in dairy cattle. *Reproduction in Domestic Animals* **47,** 1056–1061.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC (2002) High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17[beta] in dairy cattle. *Journal of Dairy Science* **85,** 2831-2842.

Talukder S, Ingenhoff L, Kerrisk KL, Celi P (2014) Plasma oxidative stress biomarkers and progesterone profiles in a dairy cow diagnosed with an ovarian follicular cyst. *Veterinary Quarterly* **34,** 113-117.

Walsh SW, Williams EJ, Evans ACO (2011) A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science* **123,** 127-138.

Weiss D, Reist M, Bruckmaier RM (2004) The acyclic period postpartum in automatic and conventional milking. *Journal of Veterinary Medicine Series A* **51,** 268-272.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A (2006) Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology* **65,** 17-29.

CHAPTER 1

Review of Literature

INTRODUCTION

Reproduction is one of the key factors influencing the profitability of a dairy farm. It is an essential management practice for a dairy herd in order to initiate lactation in cows, produce replacement stock, and control the production system. Reproductive performance in dairy cows continues to decline in Australia. The InCalf 2010 analysis commissioned by Dairy Australia (2011) identified declines in the 6-week in-calf rate, three-week submission rate and first-service conception rate whilst the 12-week not-in-calf rate the 21-week not-in-calf rate and the overall not-in-calf rate continue to increase when compared to the original analysis conducted in 2000 (Morton 2011). The trends of declining fertility seen in Australia have been reported in most dairy countries, including the USA, Netherlands, Ireland and New Zealand (Walsh *et al.* 2011). While it is difficult to determine the exact reason for the observed decline in fertility (Walsh *et al.* 2011), it seems that the impact of management factors including the trend towards larger herd sizes, intensified nutritional management of herds and increased reliance on hired unskilled labour have all contributed to the observed decline in fertility of the modern dairy cow.

Labour is the second largest operating cost on Australian dairy farms (second only to feed) and is therefore a logical factor to target in improving on-farm productivity. In an age of precision agriculture, on-farm automation, 'working smarter, not harder' and robotic technologies, the opportunity exists for research to investigate the potential to improve productivity of the dairy farming operation. This might be achieved by increasing the information made available to the farmer to aid in timely management decisions and/or by freeing up time to allow a shift in focus from labour intensive tasks like milk harvesting, to higher impact management tasks like reproduction and nutrition.

Due to the growing costs of labour in many dairy countries, automatic milking systems (AMS) are becoming increasingly popular (Rossing and Hogewerf 1997). Switching from a conventional milking parlour to automatic milking results in big changes for both the herdsman and the cows. The impact of AMS on reproductive performance is a matter of debate and is likely impacted by associated changes in milking frequency and milk yield. Whilst some studies (Kruip *et al.* 2000; Dearing *et al.* 2004) have been conducted to investigate the impact of AMS on fertility of cows housed indoors; the

findings are inconsistent. Regardless, there have been no significant studies relating to the reproductive performance of pasture-based cows managed with AMS.

Automatic milking systems are often described as a completely new way of farming since the voluntary cow traffic and resultant distribution of milkings throughout the day and night impact on most aspects of herd and farm management. For example, when cows are managed with a conventional milking system they are herded (which may in itself prompt the display of oestrus behaviour) by farm staff who can then take the opportunity to identify individuals displaying oestrus behaviour at each milking session. Since milking occurs largely without human intervention in AMS, traditional oestrus detection methods and/or routines require reconsideration. Development and adaptation of automated technologies to aid oestrus detection may have a valuable application in AMS farms.

Besides adopting automated technologies, support software is required to provide the farmer with data or information to aid decision making regarding the reproductive status of individual cows (Ordolff 2001; Brandt *et al.* 2010). Cow activity, moving/trafficking behaviour, and physiological changes can be used to monitor their health and fertility (Brandt *et al.* 2010). In addition, numerous studies have evaluated the use of indicators in milk to support feeding management, oestrus detection, and the detection of udder infections and abnormal milk (Chagunda *et al.* 2006; Mottram *et al.* 2007; Hettinga *et al.* 2008). Sensors for on-farm analysis of milk composition or physiological status have been developed either to replace or aid visual inspection or for monitoring alterations in milk indicators (Brandt *et al.* 2010). Some examples of sensors include (but are not limited to) milk electrical conductivity (Hogeveen *et al.* 2010) to detect mastitis, accelerometer for measuring gait for lameness (Pastell *et al.* 2009) and oestrus (Kamphuis *et al.* 2012) and ruminal pH sensors (AlZahal *et al.* 2011) for the detection of sub-clinical ruminal acidosis. Biosensors are another powerful tool for measuring milk constituents, which consist of a biological component (e.g., an enzyme, antibody, or microorganism) and new technology has been developed to measure some of these milk constituents assisting farmers in nutritional management and to monitor milk quality. Biosensors measuring milk urea nitrogen, lactate dehydrogenase, lactose and progesterone (P_4) have also been reportedly used to monitor reproductive status (Brandt *et al.* 2010). Infrared thermography (IRT; used to measure body surface temperature) is

another example of advanced technology although investigations into cattle applications have predominantly focused on the early diagnosis of disease (mastitis, lameness) and in thermophysiological studies. Though IRT has been used to measure the vulval temperature changes during oestrus in sows, gilts (Scolari *et al.* 2011; Sykes *et al.* 2012) and cows (Hellebrand *et al.* 2003; Jones *et al.* 2005), there have been no published studies which evaluated the potential of IRT for oestrus detection and prediction of ovulation in dairy cows managed in pasture-based conditions.

Oxidants and antioxidants are key signalling molecules involved in various physiological processes in the female reproductive tract such as, steroidogenesis, folliculogenesis, ovulation, pregnancy but they are also involved in pathological processes (Agarwal *et al.* 2006). Biosensors and automated technologies have the potential to become reliable and user-friendly tools, which give real-time measurements of an individual cows oxidant and antioxidant levels thereby providing an automated indication of oestrus. An opportunity exists for clinicians to explore their effects on reproductive outcomes particularly in dairy species. Oxidative stress (OS) is associated with embryonic losses in dairy cows (Celi *et al.* 2011; Celi *et al.* 2012) and has also been shown to have a role in the pathogenesis of follicular cysts and repeat breeder syndrome in dairy cows (Rizzo *et al.* 2007; Rizzo *et al.* 2009; Talukder *et al.* 2014*b*). However, to the best of our knowledge, there are no published studies measuring the differences in concentration of oxidative biomarkers between ovulatory and anovulatory cycles in dairy cows.

Oxidative stress can be measured in a number of ways, however measuring individual oxidants in the laboratory can be time-consuming, labour intensive, and costly (Celi 2011b). The development of a cow-side automated test measuring the oxidative status of individual cows would prove to be an invaluable tool, which would allow on farm and non-invasive monitoring of reproductive events such as ovulation and the common reproductive disorders (embryo mortality, and cystic ovarian disease).

Developing a strong understanding of AMS management and challenges associated with reproductive management in these systems is core to determining potential solutions.

AUTOMATIC MILKING SYSTEMS

The introduction of AMS is one of the most significant technological changes in the dairy industry. In Europe, AMS have been commercially available since 1992. Thereafter, the number of AMS installed throughout the world has increased steadily in the last decade. In late 1997, more than 100 farms were using AMS and by early 2004, the figure had increased to over 2,200 farms and more recently it has been reported that more than 10,000 farms globally are using AMS (de Koning and Rodenburg 2004; de Koning 2010). Milking cows is one of the most time consuming activities on dairy farms that requires about 25-35% of the annual labour demand (de Koning 2010). A detailed survey of Australian farmers in 2004 found that 70% of farms spend five or more hours a day milk harvesting (Jago *et al.* 2007). Automatic milking systems provide a viable alternative to the intensive labour required for milk harvesting in conventional parlours. Besides dramatically reducing the labour requirement for milk harvesting it also has the ability to result in significant improvements in the hours worked and flexibility of routines on farm (Molfino 2014).

Reproductive performance of dairy cows in AMS

Questions have been raised as to whether reproductive performance of cows managed in an AMS differ from those managed in CMS. In intensive indoor AMS, a number of studies have been conducted to evaluate reproductive performance, but findings are inconclusive due to short data collection periods and use of limited measures to quantify reproductive performance. Under research conditions, Dearing *et al.* (2004) monitored six farms in The Netherlands for up to six months prior to AMS installation through until 12 months post installation and noted an increase in calving to conception interval as well as reduced conception rate. Whilst that study (Dearing *et al.* 2004) suggests an initial decline in reproductive performance during the first 12 months of operation (a period generally agreed as an adaptation period; for both cows and people), it does not allow us to speculate on the long term impact of the operating an AMS on reproductive performance. In another study, analysing the data of 415 lactations, Kruip *et al.* (2000) reported that higher milk production levels were associated with a delay in commencement of ovarian activity and oestrus expression resulting in a significant increase in the interval from calving to first insemination. The lactations evaluated in the study were predominantly from herds that were within the first 12 months of operation

with AMS and therefore cannot be deemed 'established' herds. However, Weiss *et al.* (2004) suggested that even with the 415 lactations involved in the Kruip *et al.* (2000) study, there is a need for even larger datasets to be collected and evaluated to determine the true effects of automatic milking on fertility (Weiss *et al.* 2004) which is a valid point as larger datasets may have an impact on epidemiological studies. On the other hand, Löf *et al.* (2007) reported shorter calving intervals and shorter calving to first insemination and conception intervals in AMS herds in Sweden compared with herds managed with CMS. It is possible that the combined effects of individual cow performance, management policies (for example voluntary waiting period, the method of oestrus detection) and herd size are all factors contributing to discrepancies in findings across the studies (Lof *et al.* 2012). It is also possible that as time has passed since the time of the first AMS installation, the study of Löf *et al.* (2012) is a reflection of herds being managed in an environment of improved industry knowledge around AMS. To the best of our knowledge, there are no reported studies that have evaluated the reproductive performance of dairy cows in AMS herds within pasture-based systems.

Washburn *et al.* (2002*b*) evaluated the reproductive performance of dairy cows in confinement vs pasture-based feeding systems. Whilst cows in pasture-based systems had consistently lower average body condition scores and the numerical advantage for overall pregnancy rate (71.7% vs 64.2%), the reproductive performance did not differ significantly in that study (Washburn *et al.* 2002*b*). On the other hand, Bela *et al.* (1995) observed that pasture-based dairy cows required fewer services per conception and had shorter calving interval compared to confined cows. This may be explained (at least in part) by the space allowed for cow-to-cow interaction (to display oestrus behaviour) which is significantly greater in pasture-based management systems compared to confined systems (Diskin and Sreenan 2000). In addition, cows in confinement are more prone to feet and leg problems which may restrict their mounting behaviour (White 2000). As a result, operators are more likely to rely on automatic oestrus detection tools and/or to detect oestrus by other means if overall herd reproductive performance is to be maintained at acceptable levels.

Besides housing, nutrition plays an important role in oestrus expression and associated reproductive outcomes (Roelofs *et al.* 2010). Energy stores during late gestation,

calving, and early lactation affect the interval from calving to first oestrus and the likelihood of a successful pregnancy (Beam and Butler 1998; Chagas *et al.* 2006; Roche *et al.* 2007). In a study of Armstrong *et al.* (2001), heifers were evaluated for the effect of either low or high levels of energy intake and dietary crude protein on follicular dynamics and oocyte quality and reported a reduction in oocyte quality when dietary protein was increased, consistent with the reduction in conception rate with increased dietary protein in dairy cows (Canfield *et al.* 1990). In a meta analysis with 21 studies to evaluate the effects of dietary intervention of crude protein on fertility, Lean *et al.* (2012) concluded that increased concentration of crude protein reduced the conception rate in dairy cows. Nevertheless, in pasture-based production systems, where the availability of dietary protein can be twice as much as required, conception rates are generally better than those seen in systems in which balanced diets are offered (Royal *et al.* 2002; Harris 2005; Chagas *et al.* 2007). However, it is unlikely that any of these specific dietary components will provide simple solutions to the problem of sub fertility associated with lactation in high-yielding dairy cows (Chagas *et al.* 2007). Low dry matter intake of cows relative to their potential intake is a problem in pasture-based system (Roche *et al.* 2011). Kolver and Macmillan (1994) reported that greater than 60% of the milk production difference between pastured cows and those fed a total mixed ration was dry mater intake. Estimations of pasture mass can be made using physical cuts of pasture, electronic density measures, plate meters or geo-spatial methods. However, the precision of these methods is variable with farmers often losing confidence in the absolute figures, becoming frustrated with the time required to capture data regularly and finding it difficult to justify the time required in relation to the perceived value. Moreover, cows kept in pasture spend more energy for walking (both between the paddock and the dairy and during grazing) than cows kept in confinement (Washburn *et al.* 2002*b*). Some grazing paddocks were up to 1200 m from the milking area, which could have had an impact on the cow's energy balance (Washburn *et al.* 2002*b*). The consequences of severe negative energy balance (NEB) are an increased risk of metabolic diseases that largely occur within the first month of lactation, reduced immune function and a reduction in subsequent fertility (Roche *et al.* 2009; Walsh *et al.* 2011).

DESCRIBING REPRODUCTIVE PERFORMANCE

Reproductive outcomes should be evaluated in a dairy herd to identify the associated risk factors of poor reproductive performance. Evaluating and quantifying the factors associated with reproductive performance requires accurate data and appropriate analytical techniques. According to different calving management strategies, several reproductive measures can be used to describe reproductive performance of dairy cows (Table 1). Some of these are discussed below.

Calving interval

Mean calving interval (CI) has been used widely to determine the reproductive performance in dairy cows. Calving interval is the amount of time (days or months) between two subsequent calving events (for the same dam). A 13-month CI corresponds to approximately 115 days open (DO; based on 280-day gestation length) in Holstein Friesian cattle (Meadows 2005*b*). The accuracy of this approach depends on analytical strategies, herd size and normal distribution of these values (Morton 2004). It is recognised that inaccuracies due to normal variation, bias, momentum and lag effects might limit the use of this indicator in modern dairy herd. Moreover, average CI does not take into account cows that fail to recalve i.e. failure of conception (Esslemont 1992). This is also largely determined by retrospective rather than present reproductive indices (Ferguson and Galligan 2000). Projected calving intervals (PCI) are an alternative (more timely) indicator for monitoring overall herd reproductive performance because it uses current year data rather than the previous years' CI (Meadows 2005*b*). The projected CI can be defined as the projected minimum days open plus a standard 280-day gestation (Fetrow *et al.* 1990).

Table 1. Definitions, targets and triggers of reproductive measures used in different calving herds to describe the reproductive performance [Source: (InCalf 2007)].

Calving herds	Reproductive measures	Definitions	Targets[a]	Triggers[b]
Year round	100-day in-calf rate	Percentage of cows pregnant by 100 days after calving	58%	<45%
	200-day not in-calf rate	Percentage of cows not pregnant by 200 days after calving	13%	>19%
	80-day submission rate	Percentage of cows that were inseminated by 80 days after calving	73%	<61%
	Conception rate	Percentage of cows detected in oestrus and inseminated that became pregnant	51%	<43%
Seasonal /split	6-week in-calf rate	Percentage of cows pregnant in the first 6 weeks of mating	71%	<60%
	Not-in-calf rate	Percentage of cows not pregnant after the end of mating	Dependent on the length of mating (Table 2)	
	Not-in-calf rate after two mating periods (split calving herds only)	Percentage of cows not pregnant after two successive mating periods	Depend on the length of mating (Table 2)	
	10-day submission rate	Percentage of cows inseminated or served in the first 10 days of the mating period	41%	<36%
	3-week submission rate	Percentage of cows inseminated or served in the first 3 weeks of the mating period	86%	<75%
	Conception rate	Percentage of cows detected in oestrus and inseminated that became pregnant	53%	<49%

[a]The targets describe the results achieved by the top 25% of farmers during the InCalf research project, based on 6 week or 100-day in-calf rates.

[b]The triggers indicate when the farmer ought to seek help from experts and to evaluate the factors associated with the respective reproductive measures.

Table 2. Targets and triggers of not-in-calf rates for mating periods of different lengths [Source: (InCalf 2007)].

Length of mating	Targets[a]	Triggers[b]
6 weeks	29%	>40%
9 weeks	20%	>28%
12 weeks	13%	>21%
15 weeks	10%	>17%
18 weeks	9%	>13%
21 weeks	8%	>11%

[a]The targets describe the results achieved by the top 25% of farmers during the InCalf research project, based on 6 week or 100-day in-calf rates.

[b]The triggers indicate when the farmer ought to seek help from experts and to evaluate the factors associated with the respective reproductive measures.

Days open

Days open (DO) is the time (days) between calving and conception. Cows with shorter DO enhance herd profitability as they have an increased proportion of lactational days (during a cows' lifetime) that are attributed to high milk production during early lactation (Louca and Legates 1968). Cows with shorter DO also produce a higher number of replacement stock during their lifetime. Stevenson and Lean (1998) conducted an epidemiological study of data from 1992 to 1994 in New South Wales, Australia and reported that average DO was 101 days while Moss *et al.* (2002*b*) evaluated the data of 10 commercial dairy herds in the Camden region of New South Wales from 1995 to 1996 and reported that DO was 118 days. In a recent study of AMS herd, Talukder *et al.* (2014*a*) observed longer DO (128 days) than the findings of the previous Australian researchers. However, the declining trend of reproductive performance during the last 10 years (Morton 2011) supports and could explain, at least in part, the relative reduction of the reproductive outcomes with time. The disadvantage of average DO is that it does not account for cows that fail to conceive (Esslemont 1992). It is also largely a retrospective determinant of reproductive performance (Ferguson and Galligan 2000).

Conception rate and pregnancy rate

Reproductive performance can also be evaluated by calculating conception rate (i.e. proportion of cows detected in oestrus and inseminated that became pregnant) and pregnancy rate (proportion of cows becoming pregnant per unit of time; literally, the speed at which cows become pregnant; Meadows 2005*a*). Pregnancy is usually confirmed by rectal palpation between 28 and 50 days post insemination (Leblanc 2010). Numerous studies in Australia (Stevenson and Lean 1998; Moss *et al.* 2002*a*), in Europe (Pinedo *et al.* 2011; Walsh *et al.* 2011) and in USA (Washburn *et al.* 2002*a*) have used this reproductive parameter to quantify reproductive performance.

Proportion of cows pregnant by specified periods after calving can also be used to define reproductive performance (Morton 2004). Different time periods after calving were used in different studies including 80 days (Uchida *et al.* 2001), 100 (Lucy 2001), 115 (Ferguson 1996), 150 (Westwood *et al.* 2000; Raizman and Santos 2002), 210 (Ferguson 1996) and 320 days (Raizman and Santos 2002). Proportion of cows pregnant in respect to a specific time after the start of the breeding season is also used in some studies such as 42 (Walsh *et al.* 2008) or 150 days (Coleman *et al.* 2009). The major limitation of this approach is that it overestimates pregnancy rate by overestimating the actual proportion of cows becoming pregnant but it is easy to measure and is less biased than calving interval, which requires the data of two consequent calvings (Leblanc 2010).

The most commonly used methods in Australia rely on non-return to insemination and rectal pregnancy detection (Brightling *et al.* 1990; Morton 2004). In an Australian study, Williamson *et al.* (1978) reported 11% higher conception rate based on non-return to insemination relative to calculations based on rectal pregnancy diagnosis. Non-return to insemination relies on occurrence and accurate detection and recording of all oestrus events subsequent to the service of interest (Morton 2004). In general, measures based on non-return to insemination data overestimate herd reproductive performance (Morton 2004).

Measures of submission rate

Submission rate is another parameter used to measure herd reproductive performance. It describes the proportion of eligible cows that were inseminated by a specified time after calving such as 65 (Eddy 1980), 80 (InCalf 2007) or 90 days (Henry 1986). This method reduces the difficulties associated with skewed data for calving to first insemination interval whilst also accounting for non-inseminated cows (Morton 2004). Ferguson (1996) used 24-day submission rate (calculated based on insemination within the first 24 day of the breeding season irrespective of calving date) along with other measures of reproductive traits. In Australia, Brightling *et al.* (1990) analysed the data from 1984-1986 and reported a 30 day submission rate of 77% while in the same study analysing the data from 1985 to 1987, 30 day submission rate was reported of 81%. Evaluating the data in 2009, the In Calf Fertility Data Project (2011) reported the 21-day submission rate as 72%. Submission rate for first service has been used as an indicator of oestrus detection rate (Morton 2004). However, it is affected by the occurrence of anoestrus (Morton 2004).

Time to first insemination/service

Time to first insemination is the average days in milk at first insemination. This value could be highly confounded by oestrus detection rate and management decisions about voluntary waiting period (days after calving during which cows are not bred, even if they are seen in oestrus). It is reported that over the past 30 to 50 years, the percent of cows displaying standing oestrus has been reduced from 80 to 50% and the duration of standing oestrus has shortened from 15 to 5 hour (Dobson *et al.* 2008). In commercial herds, detecting oestrus based on visual signs and or monitoring of mounting using tail paint or heat mount detectors, it is not practical to determine the exact time of onset of standing oestrus (Hockey *et al.* 2010*b*). In addition, managers of large pasture-based herds face particular problems with cow identification, record keeping, oestrus detection, and drafting cows for artificial insemination (Verkerk 2003; Morton 2004). Previous work even suggests that conception rates are reduced if insemination is delayed more than 14–16 hours after onset of oestrus behaviour (Maatje *et al.* 1997; Dransfield *et al.* 1998; Morton 2004). Early Australasian work demonstrated that conception rates were highest when insemination was delayed for 9-15 hours after cows were first recorded as being in oestrus (Arnott 1961; Macmillan and Watson 1975*a*;

Macmillan and Watson 1975*b*), even where signs of oestrus were only recorded twice daily (Macmillan and Watson 1975*a*; Macmillan and Watson 1975*b*). If automated oestrus detection technologies are to have widespread uptake they need to detect and report oestrus events in a timely manner with a high level of accuracy to ensure that herd submission and conception rates are not compromised.

Numerous methods can be used to describe the reproductive measures. The choice of methods affects the statistics describing the reproductive performance. Therefore, the methods used needs to be considered carefully when making comparisons of reproductive performance among studies conducted in different herds under different management systems. The interval between calving to conception and two subsequent calvings are widely used measures in year-round calving herds while measures that describe the percentage of the herd pregnant by specified times after mating start date are used in seasonal calving herds.

Calving interval is a general measure of reproductive performance; conception rate is an indicator of things like technique of the inseminator, the quality of the semen, the timing of insemination and accuracy of oestrus detection. Submission rate is an indicator of how well the cows are cycling and how effective or accurate the farm staff are at detecting oestrus. If everything is done perfectly, e.g., semen quality is good, cows are cycling, people are accurately detecting oestrus etc, and then the key determinant of reproductive performance is determined by the physiological state of the cow. To understand this it is important to comprehend the reproductive biology of lactating dairy cows.

REPRODUCTIVE BIOLOGY OF LACTATING DAIRY COWS

When describing the reproductive performance in dairy cows, it is important to consider how these may be mediated in biological terms. The reproductive biology of lactating dairy cow is briefly discussed below.

Oestrus cycle

The oestrous cycle represents the cyclical trend of ovarian follicular activity that enhances the female to be sexually receptive for mating and facilitates the establishment of pregnancy (Forde *et al.* 2011). The normal duration of an oestrous cycle in cattle is

18–24 days and it can be divided into four phases: prooestrus, oestrus, metoestrus and dioestrus. The first two phases are called the follicular phase (4-6 day duration) and the second two phases are the luteal phase (14-18 day durations; Figure 1). The follicular phase is characterized by two (Knopf *et al.* 1989) or three (Ireland and Roche 1987) consecutive follicular waves per oestrous cycle, selection of a dominant follicle that continues to grow and matures to the preovulatory stage and ovulates allowing the oocyte to be released into the oviduct creating the potential for fertilization. Following ovulation during the luteal phase, the corpus luteum (CL) is formed (metoestrus) and luteolysis of CL occurs (dioestrus).

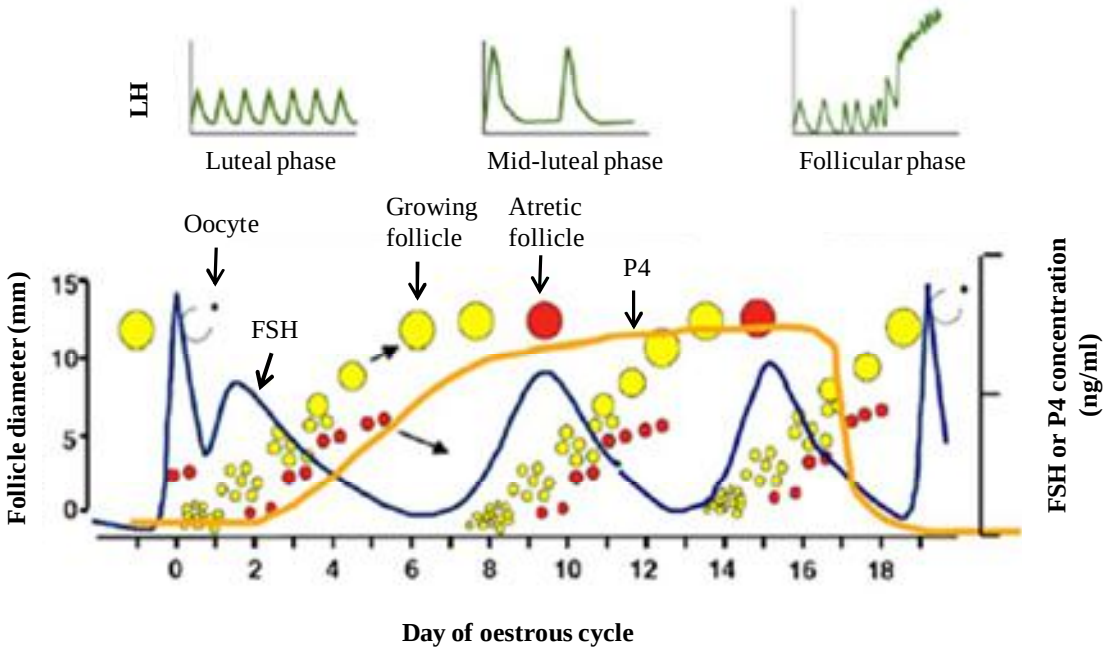

Figure 1. Schematic depiction of the pattern of secretion of follicle-stimulating hormone (FSH), luteinising hormone (LH), and progesterone (P_4); and the pattern of growth of ovarian follicles during the oestrous cycle in cattle. Each wave of follicular growth is preceded by a transient rise in FSH concentrations. A surge in LH and FSH concentrations occurs at the onset of oestrus and induces ovulation. The pattern of secretion of LH pulses during an 8-h window early in the luteal phase (higher frequency, lower amplitude), the mid-luteal phase (lower frequency, higher amplitude), and the follicular phase (high frequency, building to the LH surge) as indicated in the inserts in the top panel (adapted from Forde *et al.* 2011).

Endocrine control of oestrus and ovulation

The oestrous cycle of cattle is governed by the positive and negative feedback mechanism of different hormones produced by various organs and tissues (Forde *et al.* 2011). Gonadotrophin releasing hormone (GnRH), a decapeptide, secreted from the hypothalamus into the pituitary portal vasculature in pulsatile fashion, regulates the follicle stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and secretion (Bernard *et al.* 2010). In female mammals, FSH stimulates ovarian follicle growth and maturation, as well as oestradiol (E_2) synthesis by granulosa cells, whereas LH stimulates androgen production by theca cells and ovulation of the dominant follicle(s) (Bernard *et al.* 2010). At the ovarian level, high concentrations of E_2 are secreted from the preovulatory graffian follicles (at oestrus) that stimulate uterine growth and the production of prostaglandins by the uterus (Hafez *et al.* 2000). This increase in E_2 concentration in conjunction with inhibin gives negative feedback to reduce the FSH concentration to basal levels (Forde *et al.* 2011). The preovulatory surge of LH and FSH, a second type of LH and FSH release, occurs before ovulation and lasts for 6-12 hours. An increase in the circulating oestrogen concentration has a positive feedback effect on the hypothalamus, inducing a sudden surge of GnRH release, which combines with the preovulatory surge of LH and FSH (Hafez *et al.* 2000). Luteinising hormone initiates a cascade of processes by binding to tissues of a preovulatory follicle that terminates in release of an oocyte into the oviduct during ovulation (Roelofs *et al.* 2010; Figure 2). Ovulation occurs 10–14 h after oestrus followed by the luteal phase of the oestrous cycle.

Signs of oestrus

The primary behavioural sign of oestrus is that the cow stands to be mounted by another cow or a bull (Ball and Peters 2004). The period between the first and last time the cow stands to be mounted is known as standing oestrus. Hurnik *et al.* (1975) used the following definition of standing oestrus: “the interval during which the cow makes no effort to escape when mounted by others (standing oestrus)”. There are also a number of notable behavioural changes around the time of oestrus. These can include aggressiveness, bellowing, chin resting, ano-genital licking and sniffing (van Eerdenburg *et al.* 1996), restlessness (Ball and Peters 2004), increase in agonistic interactions [e.g. head to head fights (Kerbrat and Disenhaus 2004)], sniffing of the vagina of herd-mates,

flehmen reaction (wrinkling of the nose and curling of the lip) and increased activity (Neves 2011).

Numerous studies employ oestrus detection by visual observation of oestrus signs. Oestrus detection efficiency by visual observation of the standing heat varied a lot according to published reports, from 96% to 58% (Roelofs *et al.* 2005*b*) depending on the timing of observation on the day, time spent on oestrus detection, and frequency of observation.

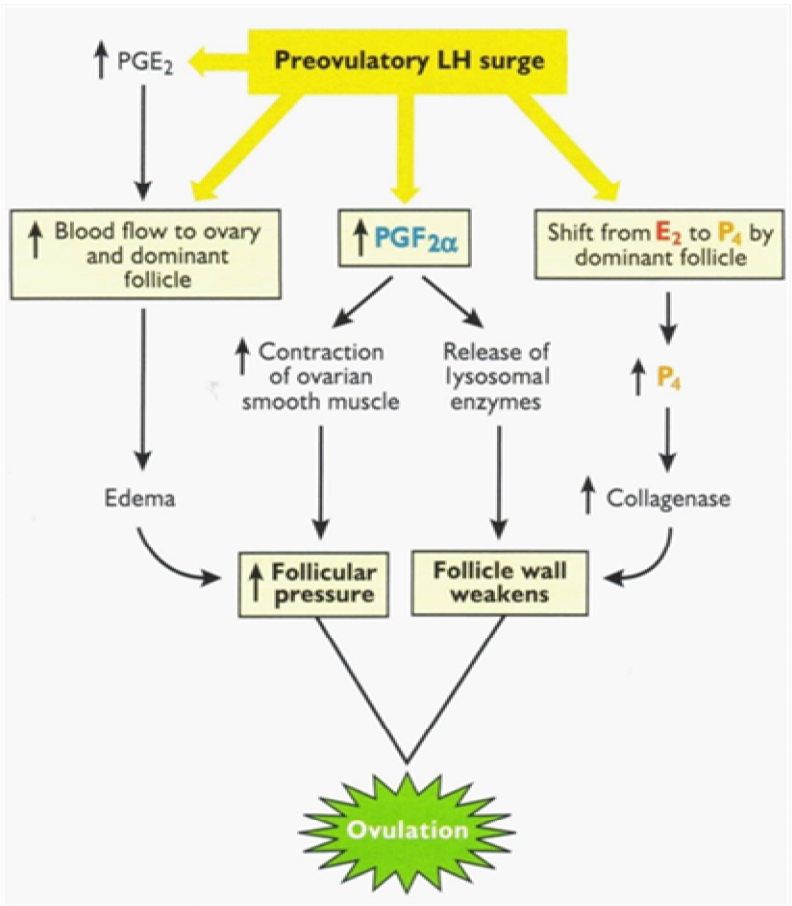

Figure 2. Ovulation events, adapted from Senger (1999).

OESTRUS DETECTION

Efficient oestrus detection by visual observation is time-consuming and requires diligent attention (Firk *et al.* 2002). An ideal automated system for detecting oestrus should have the following characteristics (Senger, 1999):

1. continuous surveillance of the cow
2. accurate and automatic identification of the cow in oestrus
3. operation for the productive lifetime of the cow
4. minimal labour requirements and
5. high accuracy and efficiency (95%) for identifying the appropriate physiological events that correlate with oestrus or ovulation or both.

Aids used for oestrus detection

Besides visual observation of behavioural signs, various types of oestrus detection aids like P_4 measurement, activity meters, mount detection devices, pressure sensing radio telemetry, video cameras, tail painting, milk and body temperature measurements, and milk conductivity measurement have been developed to improve oestrus detection and/or aid visual observation. Table 3 summarises different methods used for oestrus detection in terms of which of the essential characteristics (as per above) they possess. Each of oestrus detection aids has its own advantages and disadvantages, which are discussed below.

Table 3. Comparisons of different methods used for oestrus detection in terms of characteristics.

Characteristics of an ideal oestrus detection method	Methods used for oestrus detection			
	P_4 test	Pressure activated heat mount detectors	Devices for monitoring the activity	Body temperature measurement
Continuous surveillance	√	√	√	√
Accurate and automatic identification			√	
Operation for the productive lifetime			√	√
Minimal labour requirements		√	√	
High accuracy and efficiency	√			

Progesterone test

Progesterone measurement is used as the traditional way of determining the period from calving to resumption of luteal activity (Petersson *et al.* 2007; Lovendahl and Chagunda 2010). Luteal activity is defined as the time when P_4 concentration first exceeds a set threshold of, for example, 3 ng/mL (Bulman and Lamming 1978). Measurements of P_4 concentration generally provide the highest degree of accuracy (Ball and Peters 2004) and act as the gold standard for reproductive status measurement (Friggens and Chagunda 2005). However, the need for sampling of blood or milk, sample processing in laboratories as well as high costs have traditionally limited the use of P_4 measurement as an on-farm tool (Friggens NC, 2005). Recent advances in biosensor technology (Herd Navigator®, HN; Lattec I/S, Hillerød, Denmark) have overcome these drawbacks by introducing in-line automated milk sampling and processing systems (Mottram *et al.* 2002). This system measures milk P_4 content and generates individual cow P_4 concentration curves to monitor the oestrus cycle, and detect non-cycling cows, pregnancy, and abortion. The associated software triggers an oestrus alert as soon as the P_4 value drops to less than 4 ng/ml. This system has benefits in terms of improved pregnancy rates (due to having less insemination/cow), less veterinarian visits and reducing the time needed for management and observation by the herdsman (Rodenburg 2014). However, the availability (in some countries) and compatibility with different milk harvesting systems is a limiting factor. The sensitivity and specificity of any applied sensors have to be the main factors in any evaluation, in addition to calibration and maintenance requirements, operating cycle duration, costs, and robustness (Brandt *et al.* 2010).

The relatively high cost and extra labour for manual collection and labelling of milk samples limits the practical use of progesterone test for oestrus detection on farm. The HN system is the only known fully automated milk progesterone monitoring system available, which overcomes this limitation. However, the HN system calls for a major initial investment of 40,000 Euros (AU$ 61,017) for a herd of 120 dairy cows and an additional average of 50 Euros (AU$ 75) per cow-year for assay reagents (Saint-Dizier and Chastant-Maillard 2011) which may limit its uptake although a full cost/benefit analysis is justified.

Pressure activated heat mount detectors

Pressure activated heat mount detectors are attached to the tail head to aid in detection of cows, which have been mounted by other cows. They commonly consist of cylindrical plastic container of red dye inside a clear plastic capsule, which is obscured by an opaque cover. The pressure of a mounting animal causes the capsule to burst and the red dye becomes visible (Ball and Peters 2004). The oestrus detection rate achieved with the use of this device varies from 50% to 85% across different studies (Ball and Peters 2004). The relatively low oestrus detection efficiency and time consuming task of applying this device to the tail head is probably its greatest limitation. Instead of intermittent observation, cows in oestrus may be detected by Heat watch® (Heat-Watch II, CowChips, Manalapan, NJ, USA) which is an example of an electronic modification of the pressure activated heat mount detectors which analyses the mounting profile of each cow (Saint-Dizier and Chastant-Maillard 2011). This system involves a miniaturized radio wave transmitter linked to a pressure sensor on the cows tail head which is activated by the weight of a mounting herd mate for a minimum of 2 seconds (Diskin and Sreenan 2000; Saint-Dizier and Chastant-Maillard 2011). Mounting activity (cow identification, time, date of mount, and duration of the sensor activation) are transmitted to a receiver. The computerised support software allows the herdsperson to retrieve the alerts at a convenient time. The efficiency of oestrus detection of this system in a free stall housed barn was reported at 86.8% compared to the visual observation of 54.4% (At-Taras and Spahr 2001). The gold standard was categorised as any event in which at least two detection methods (Heat Watch/activity device/visual observation) were positive. On the other hand, Cavalieri *et al.* (2003) reported the efficiency of Heat watch and visual observation in pastured cows, as 90 to 100% and 95 to 100% respectively. In this study milk P_4 concentrations were the gold standard for oestrus. The discrepancy between the two studies is likely attributed to the gold standard method and the vigilance of the visual observers.

Mounting activity can be observed continuously using the Heat watch® system, however, as with any oestrus detection system, it is not without its challenges. Firstly, the housing and the type of flooring have a dramatic effect on mounting activity as reported in the previous studies (At-Taras and Spahr 2001; Cavalieri *et al.* 2003; Saint-Dizier and Chastant-Maillard 2011). Secondly, the patch that holds the transmitter is

time consuming to attach to the cows and is not particularly well suited to re-use if it is rubbed off the rump. If the patch is rubbed free of rump, the transmitter is at risk of being lost (Rae 2010) although this can be significantly minimised if care is taken to attach the tail strap securely. Thirdly, as with any mechanised system, the receiver, buffer, computer, wiring, and software require a level of maintenance and upkeep. As with any oestrus detection aid a full cost-benefit analysis should be conducted prior to investment. An adaptation of Heat watch® system, the Heat Watch Xpress® is available in market which does not need a computer or software to process and display the data. The system monitors the mounting activity continuously via a small battery powered radio transmitter and sending mount data to a radio receiver and then to a buffer in the farm office from which the data can be printed (Diskin and Sreenan 2000; Rae 2010). Future studies need to be conducted in terms of its accuracy and cost compared to other oestrus detection aids.

Devices for monitoring individual cow activity

Increased movement of the cow is a well known behavioural change associated with oestrus (Lamming and Darwash 1998). Cows that are in oestrus have been reported to walk 2–4 times more than non-oestrus cows (Diskin and Sreenan 2000). Activity meters are commonly attached to the leg or neck of the cow to measure the amount of activity over a unit time span (Diskin and Sreenan 2000). These devices are commonly grouped into one of the three types: (i) leg activity metres recording the number of steps made by the cow per time unit (pedometers); (ii) neck mounted activity metres recording the movements of neck in three dimensions; and (iii) activity-metres attached to the leg that comprehensively assess the activity of dairy cows by measuring the number of steps and quantifying lying and standing behaviours using 3d-accelerometer technology (Saint-Dizier and Chastant-Maillard 2011). Activity devices continuously monitor the individual cow activity in time blocks, with data downloaded automatically to the support software on a computer commonly located at the dairy. A base unit receiver located above the entrance of the milking parlour retrieves the data (via radio communication). From this receiver, the data are automatically forwarded to support software in a central computer and/or to a cell phone (Saint-Dizier and Chastant-Maillard 2011). Deviations in activity during oestrus are detected automatically by

comparing the activity value of the day of oestrus with activity values of a reference period (Firk *et al.* 2002).

Numerous studies have investigated the relationships between increased activity, time of ovulation and fertility (López-Gatius *et al.* 2005; Roelofs *et al.* 2005*a*; Hockey *et al.* 2010*a*). Ovulation takes place on average 29–33 h after the onset of increased activity and 17–19 h after the end of increased activity in lactating Holstein cows (Roelofs *et al.* 2005*a*; Hockey *et al.* 2010*a*). The interval from onset of increased cow activity to ovulation was positively correlated with interval from onset of increased cow activity to AI and interval from AI to ovulation (Hockey *et al.* 2010*a*).

The efficiency of oestrus detection using pedometers and activity meters is generally more than 80% but is likely impacted by threshold set by the manufacturer and the reference period of previous activity that are used to define the increase in activity (de Mol 2000; At-Taras and Spahr 2001; Firk *et al.* 2002; Cavalieri *et al.* 2003; Hockey *et al.* 2010*a*; Lovendahl and Chagunda 2010; Saint-Dizier and Chastant-Maillard 2011). The specificity of pedometers and activity meters has been reported between 90% and 100% but their accuracy depends on the algorithms used by the software (de Mol 2000; At-Taras and Spahr 2001; Firk *et al.* 2002; Cavalieri *et al.* 2003; Hockey *et al.* 2010*a*; Lovendahl and Chagunda 2010; Saint-Dizier and Chastant-Maillard 2011). Moreover, the specificity of activity meter can be affected by walking distances (between paddocks and dairy) and distances covered during grazing (significantly covered by pre-grazing biomass; Hockey *et al.* 2010*a*; Saint-Dizier and Chastant-Maillard 2011). Whilst effective incorporation of activity meters into indoor voluntary milking systems (Stevenson *et al.* 2014) and pasture-based conventional milking systems has been shown (Kamphuis *et al.* 2012) no such studies have reported effective incorporation into pasture based voluntary milking systems (AMS). It is possible that cows managed within pasture-based AMS have a higher level of variation in their baseline activity levels, which may influence the accuracy of activity monitoring devices in these systems.

Milk and body temperature measurement

The physiological body temperature of a healthy cow ranges between 37.8 and 39.4 °C. Changes in body temperature during the oestrous cycle have been shown to be

associated with the LH surge and ovulation (Rajamahendran *et al.* 1989). Furthermore, an increase in cows' body temperature at the time of the LH peak during oestrus, a decline around ovulation and a subsequent increase and decrease during mid-oestrus and 2 to 3 d before oestrus, respectively is commonly reported (Wrenn *et al.* 1958; Kyle *et al.* 1998; Fisher *et al.* 2008; Figure 3). Previous studies argued that the cause for the observed changes in body temperature might be ascribed to the increased activity at the time of oestrus (Walton and King 1986) or because of central regulation of body temperature influenced by factors that influence GnRH (Kyle *et al.* 1998). Another study suggested that the thermogenic effect of P_4 secreted during the luteal phase contributed to the rise in body temperature (Wrenn *et al.* 1958). However, the mechanism behind the temperature changes during oestrus is not fully clear and the studies (Walton and King 1986; Kyle *et al.* 1998) were not designed to explore the mechanism of temperature changes.

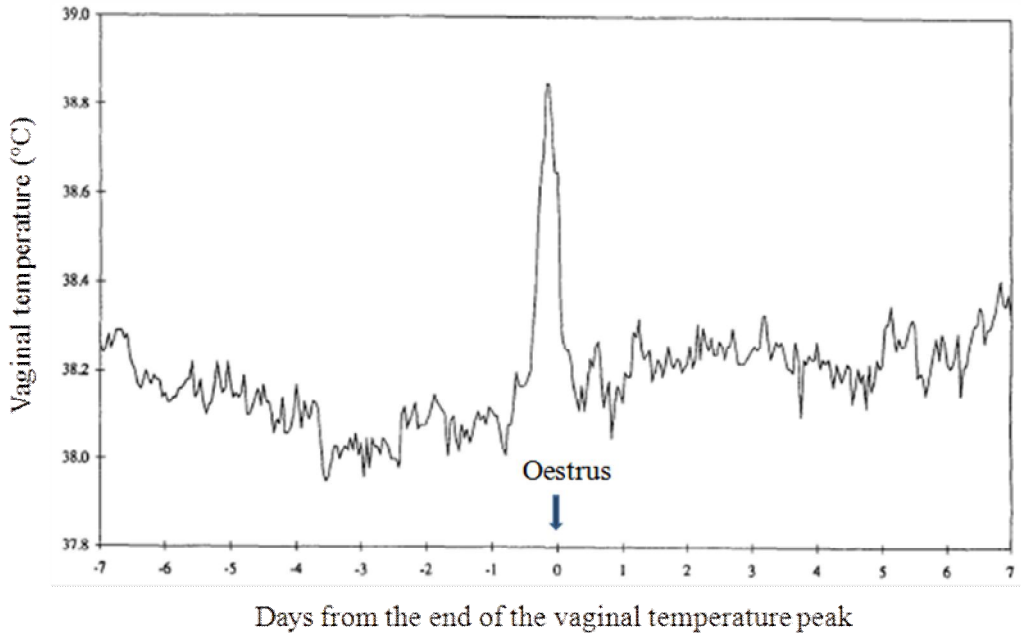

Figure 3. Mean hourly vaginal temperature before and subsequent to oestrus predicted using vaginal temperature (n = 15), adapted from Kyle *et al.* (1998).

The reported magnitude and duration of body temperature rises during the oestrus cycle varies considerably in the literature. Core body temperature has been reported to rise between 0.3°C and 1.1°C (Kyle *et al.* 1998), 0.9°C and 1.3°C (Piccione *et al.* 2003) and

up to 0.5°C (Suthar *et al.* 2011). During oestrus core body temperature can remain elevated for 7 (Rajamahendran *et al.* 1989; Redden *et al.* 1993) to 11 hours (Mosher *et al.* 1990). Such variations in temperature rise and duration among the different studies may be explained by the different cooling strategies adopted in the different housing systems in which the above studies were conducted or even by other factors including nutrition, milk production or cow condition.

In a study of Holstein-Friesian cows in tie stall barns, Schlünsen *et al.* (1987) monitored milk temperatures for a period of six years to detect oestrus and health conditions. These researchers observed that the following caused increases in temperature: oestrus 26%, medicinal treatment 16%, metritis 15%, metabolic disorder 11%, mastitis 5%, and inflammation of claws and limbs 5%. The remaining 22% of elevated temperatures were unexplained. Intensive fresh cow management programs have been established based upon using electronic thermometers to detect fever (Aalseth 2005). Udder surface temperature obtained by infrared thermography (IRT) has been evaluated for early detection of mastitis (Berry *et al.* 2003).

Measurements of deep body temperature can be performed directly by vaginal thermometry, rectal probe (Piccione *et al.* 2003), placing microprocessor controlled data loggers into the vaginal cavity, temperature boluses placed in the reticulum and rumen (Bewley *et al.* 2008; AlZahal *et al.* 2011), implanting transmitters in the abdominal cavity (Brown-Brandl *et al.* 2005) and implanting transmitters in the udder (Lefcourt *et al.* 1999). Milk temperature can be used as an indirect measurement of deep body temperature with significant positive correlations reported between the two measurements (Fordham *et al.* 1988; McArthur *et al.* 1992).

Whilst there has been no published literature of studies measuring deep body temperature of pastured cows for oestrus detection it is expected that temperature variations caused by a variation in environmental temperature, disease-related hyperthermia and⁄or some systemic or local inflammation would alter the accuracy of oestrus detection based on body or milk temperature measurement regardless of whether cows are pastured or housed (Firk *et al.* 2002; Fisher *et al.* 2008; Saint-Dizier and Chastant-Maillard 2011).

Whilst electronic temperature loggers lend themselves to automated data capture, the cost, accuracy and battery life are all factors that will impact on the viability of integration into commercial herds. More manual temperature measurements require the restraint of animals to measure the rectal/vaginal temperatures. The insertion of implants within the vaginal cavity/rumen/reticulum to record body temperature may cause discomfort and stress to animals that may alter the actual body temperature although it is expected that the duration of any discomfort/stress would be short term with well designed implants.

With the development of non-invasive diagnostic tools, such as IRT, it is now possible to measure temperature of body surfaces non-invasively and with minimal discomfort to the cow. Because a robust physiologically based oestrus detection system may offer a more direct measurement of the changes that precede oestrus than monitoring animal behaviour, there is value in re-examining the predictive relationship between cow body temperature and oestrus (Fisher *et al.* 2008). If IRT shows value in detection of body temperature rises for oestrus detection it should be possible to automate the data capture at milking to allow individual cow baseline data to be developed. This may help to ensure that robust and accurate algorithms are developed to result in high levels of sensitivity and specificity of oestrus alerts.

Factors affecting oestrus detection

Regardless of the method, the accuracy and ease of oestrus detection is impacted by factors such as genetics, milk yield, parity, housing system, herd size, physiological status of herd mates and ambient conditions (Diskin and Sreenan 2000).

The heritability of oestrus expression is usually low (0.21) and subjected to individual variation. However, genetic lines both within and between breeds do differ in oestrus expression (Orihuela 2000).

The influence of milk yield on the magnitude and duration of oestrus expression varies from antagonistic (Lopez *et al.* 2004; Wiltbank *et al.* 2006) to non-existent (van Eerdenburg *et al.* 1996; Santos *et al.* 2009). The challenge with investigating the influence of milk yield on oestrus expression is accounting for all other confounding factors (particularly body reserves, nutritional status, hormone clearance rates and

energy balance; Meadows 2005*a*) and it is likely that these influence the findings and create the discrepancies. Milk production has been shown to be negatively correlated with E_2 concentration and follicle diameter, with high producing cows (≥39.5 kg/day) having shorter oestrous duration, less standing events and reduced standing time compared to lower producing herd mates (<39.5 kg/day; Lopez *et al*, 2004). Decreased E_2 could cause increased follicular size by delaying the time to E_2-induction of oestrus, GnRH/LH surge, and ovulation in high-producing cows (Wiltbank *et al.* 2006). A descriptive study (Shrestha *et al.* 2004) following return to ovarian cyclicity in a population of high-yielding Holsteins found that 63% had delayed resumption (resumption did not occur until >45 days after calving). It is possible that an increased metabolic clearance rate of hormones occurs in high producing cows (Sangsritavong *et al.* 2002) which could explain the increased interval from calving to resumption of ovarian cyclicity. Negative energy balance has been shown to be associated with decreased pulsatile LH secretion and IGF-1 concentration (Diskin *et al.* 2003).

Conflicting findings of the influence of parity on oestrus expression may be attributed to a host of factors including milk production, nutritional status, size of young stock (in relation to mature cows) and social hierarchy. Whilst Roelofs *et al*. (2005a) observed that primiparous cows had higher oestrus intensity and 3 h longer duration of oestrus than multiparous, other researchers have shown primiparous cows to have shorter and less intense oestrus events (van Vliet and van Eerdenburg 1996; Walker *et al.* 1996). López-Gatius *et al*. (2005) reported a dramatic impact of parity with each additional lactation number (1-9 lactations) being associated with a 21.4% decrease in walking activity at oestrus. In a review, Orihuela (2000) reported similar findings with the number of cows standing to be mounted (the best indication that a cow is in oestrus) increased with parity.

Expression of oestrus activity also depends on the housing system and flooring. A comparison of oestrus behaviour of dairy cows in cubicle housing with that of pastured cows reported that the former did not exhibit the same increase in mounting behaviour during standing oestrus period compared to cows at pasture and received fewer mounts during standing oestrus in observation sessions (Palmer *et al.* 2012). Whilst the flooring might explain the difference in mounting behaviour the reduced frequency of chin resting and ano-genital sniffing in the housed cows would be more likely explained by

the housing design itself which may have limited the cows' freedom of movement and ability to interact with each other. In a separate study (Palmer *et al.* 2010) comparing the expression of oestrus between housed and pastured cows, it was reported that the mean number of mounts recorded by HeatWatch® for the standing oestrus events was 5.4 vs 8.2 mounts and the mean duration of the standing oestrus period was 264 vs 326 min in the housed vs pastured animals, respectively. Some cows may evade mounting attempts because of unsafe conditions in the cubicles, which may explain the reduction in the mounting success rate (Phillips and Schofield 1990). In a study aimed at determining differences in intensity of oestrus, de Silva found no difference in the rate of mounting between animals housed in a free stall barn and those kept at pasture (de Silva *et al.* 1981). Animals in de Silva's study (both housed and pastured) were herded to dirt gravel areas for detection of oestrus thus removing the environmental differences while in the Palmer's study cows were observed in their respective treatment locations (housed vs pasture) which may explain the variability between these two studies.

The substrate on which cattle are housed can affect the frequencies of oestrus behaviours (Orihuela 2000). Cows are more likely to express mounting behaviour on underfoot surfaces such as grass, dirt or straw bedded yards compared to concrete floor (Diskin and Sreenan 2000). The preference for a dirt surface is further exacerbated when foot ailments are prevalent in a herd (Britt 1982; Orihuela 2000).

Cows in oestrus are inclined to congregate in sexually active groups. This is only possible when more than one animal is in oestrus (or immediately pre or post oestrus) at any one time. Numerous studies have shown that the intensity and accuracy of oestrus detection is influenced by the number of cows being in oestrus at the same time with number of oestrus cows being positively related to walking activity (Roelofs *et al.* 2005a) and number of mounts (van Vliet and van Eerdenburg 1996). Roelofs *et al.* (2005a) reported that the detection accuracy of pedometers was significantly higher when more than two animals were in behavioural oestrus at the same time (95%) compared to just two animals (85%) or one animal (67%) was in behavioural oestrus at the same time. Hurnik *et al.* (1975) observed that the number of mounts per cow increased from the average 11.2 with only one oestrus cow to 52.6 with three cows in oestrus in the same day.

Increased ambient temperature increases the incidence of anoestrus and silent ovulation (de Rensis and Scaramuzzi 2003). A higher percentage of abnormal luteal activity, delayed luteal cyclicity and reduced oestrus detection rate has been reported during hot seasons compared to cold seasons (Kornmatitsuk *et al.* 2008). Hot climatic conditions also resulted in a shorter duration of standing mount activity compared to cooler weather [2.97 h vs 6.76 h; (At-Taras and Spahr 2001)]. However, with less dramatic maximum environmental temperatures (range from 5.6 to 24.4°C) no significant effect of ambient temperature was observed (Walker *et al.* 1996) suggesting that the impact of environmental temperature is likely to be limited below a certain threshold. It is likely that this is also influenced by relative humidity as well.

The influence of ambient temperature variation on cow body temperature may obscure oestrus associated body temperature peaks in such a way as to make detection by this method somewhat challenging (Fisher *et al.* 2008). In addition, disease-related hyperthermia and ⁄or some systemic or local inflammation may alter oestrus detection based on body or milk temperature measurement thus increasing the number of false positives (Saint-Dizier and Chastant-Maillard 2011). As a result, we expect that ambient temperature and even humidity should be taken into account if body and/or milk temperature are to be measure with the intention of detecting cows in oestrus.

INFRARED THERMOGRAPHY

Infrared thermography (IRT) is a non-contact, non-invasive technique, which enables us to visualize the thermal outline of a definite object or being. It is the process of acquisition and analysis of thermal information from non-contact thermal imaging devices (Scolari *et al.* 2011).

Infrared spectrum

The optical region of the electromagnetic spectrum consists of three parts: the visible, the ultraviolet (UV) and the infrared (IR) (Kastberger and Stachl 2003). Humans can detect the visible waves by the naked eye but neither the UV nor IR light (Balaras and Argiriou 2002) can be detected with the naked eye. The wavelength of IR radiation is between 0.7 to 1 μm which is between the visible and microwave areas of the electromagnetic spectrum (Balaras and Argiriou 2002; Figure. 4). The IR spectrum can

be further categorized into three regions: near IR (0.75-1.4 μm), middle IR (1.5-<15 μm) and far IR (15-1,000 μm). Near IR is close to visible wavelength while far IR is close to microwave wavelength. The middle and far IR spectrums can be measured by thermal detectors and are useful for IRT (Kastberger and Stachl 2003).

Measurement principle of infrared thermography

All objects emit infrared radiation due to internal mechanical movement of molecules in the form of rays (Kastberger and Stachl 2003). Infrared thermography transforms the radiant energy of objects in the IR band of the electromagnetic spectrum into an electronic video signal and finally into a visible data image with black/blue tunes at low temperatures and red/white tones at higher temperature (Lamprecht *et al.* 2007). The obtained IRT data can be processed by dedicated software and is visualized as temperature maps with details of the temperature fields (Knizkova and Kunc 2007). Infrared thermography is sensitive enough to detect 0.1°C difference and with spatial distances to a few millimetres to scan effectively and report temperature measurements with high accuracy (Lamprecht *et al.* 2007).

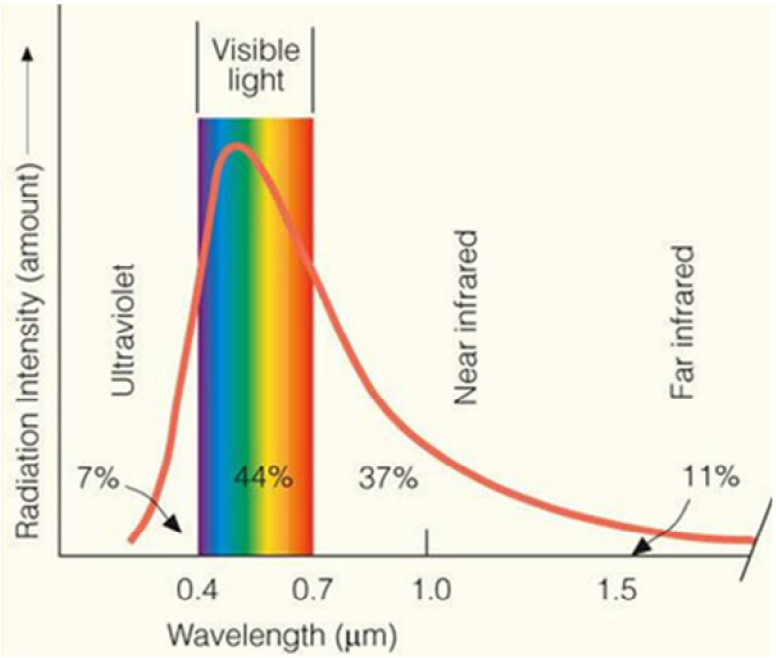

Figure 4. The optical region of the electromagnetic spectrum. Percentages describe the radiation intensity at the various wavelengths (Source: http://www.veterinary-thermal-imaging.com).

Veterinary applications of IRT

Thermal imaging was initially developed for industrial, medical, and military applications (Burnay *et al.* 1988) but the technology has been used vastly to examine radioactive temperatures of animals (McCafferty *et al.* 1998). One of the most significant advantages of IRT when applied in animal research is that it is a non-invasive technique and measurements can be made without touching or disturbing the animal. Furthermore, measurements can be made at close range (<1 m) right through to far distances (>1000 m) (McCafferty 2007) depending on the instrument type and application. Some of the examples of veterinary use of IRT are presented in Table 4 and discussed below.

Reproduction

In the field of female reproduction, IRT has been shown to have some value in pregnancy diagnosis and oestrus detection in livestock (Table 4). For pregnancy detection/confirmation in late gestation mares, IRT has been used to confirm higher average flank temperatures compared to those in nonpregnant mares (Bowers *et al.* 2009).

The use of IRT for oestrus detection is predominantly reported in sows, gilts and cows. Scolari *et al.* (2011) and Sykes *et al.* (2012) have reported the use of IRT measuring the vulval temperature for oestrus detection in sows and gilts respectively and both concluded that this technology shows promise as an adjunct or aid for oestrus detection in pigs. Hurnik *et al.* (1985), Hellebrand *et al.* (2003) and Jones *et al.* (2005) have all investigated the potential for use of IRT as a tool for oestrus detection in cattle with varying results. The reality is that IRT as an oestrus detection tool in cattle will have its greatest application if the data/image capture can be automated. Bearing this is mind it is meaningful to understand the link between the IRT measurement and the reproductive physiology and inflammation (ovulation and oxidative stress). This requires studies that focus on defining the physiological state of the cows whilst also capturing IRT data throughout the reproductive cycle. It is possible that the hormonal state, the behavioural expression or the physical 'inflammation' of the cow causes an increase in temperature. Whether just one or a combination of these explanations is the case may impact on the accuracy of the technology as a detection aid and the physical site of data capture (e.g.

vulva, muzzle or other body parts). Furthermore, understanding the sensitivity of the measurements to climatic conditions, temperature rises at other times during the oestrus cycle, skin colour and moisture/dirt contamination will be important. Another factor that may be important could be whether the oestrus event occurs naturally or is induced. It is this factor, which may explain why Jones *et al.* (2005) were able to accurately discriminate the first oestrus and diestrus after oestrus synchrony but not in subsequent unsynchronised cycles.

Thermal physiology and nutritional studies

IRT has been used to evaluate many different aspects of thermoregulation in veterinary science. Such research in bulls has included scrotal surface temperature (Kastelic *et al.* 1996; Lunstra and Coulter 1997) and determination of the contribution of the scrotum, testes, and the testicular artery to scrotal/testicular thermoregulation at two ambient temperatures (Kastelic *et al.* 1997). Under pasture based systems several studies used IRT as a non-invasive technology for assessing feed efficiency in dairy cattle (Montanholi *et al.* 2008) and beef steers (Montanholi *et al.* 2010).

In horse research, IRT also been used to detect intrasynovial injections of bupivacaine hydrochloride in fetlock and middle carpal joints (Figueiredo *et al.* 2013), performance enhancing techniques (van Hoogmoed *et al.* 2000) and the scintigraphic, radiographic and thermographic appearance of the metacarpal and metatarsal regions of healthy adult horses treated with non-focused extracorporeal shock wave therapy (Verna *et al.* 2004; Kunc and Knizkova 2012). Simon *et al.* (2006) has evaluated the use of IRT on thermoregulation in exercised horses.

In an experimental study in sheep, Capraro *et al.* (2008) used IRT to detect a significant level of gonadal cooling of hemiscrotum of sheep after experimental testicular torsion. Interestingly (particularly in relation to potential for image/data capture automation) George *et al.* (2014) showed high correlations between IRT eye temperature and vaginal and rectal temperature as measures of core body temperature in hair sheep. The eye may lend itself to automated data capture due to the fact that it is unlikely to be obscured (as the vulva may be with the tail) and if moisture and faecal/dirt contamination influence the accuracy of IRT data this would not be an issue with eye measurements.

Disease and injury

Infrared thermography has been investigated as a non-invasive aid for detection of hoof disorders (Nikkhah *et al.* 2005), screening subclinical mastitis (Polat *et al.* 2010) and pain in welfare research (Stewart *et al.* 2010) in cattle. Schaefer *et al.* (2004) showed the value of IRT as an early detection tool for the identification of calves with bovine viral diarrhoea virus observing significant changes in eye temperature several days to one week before other clinical signs of infection manifested. The positive welfare implications of such non-invasive, early disease detection would have particular value if the data capture could be automated on a regular basis allowing for timely management decisions, which might prevent the onset of clinical disease. Infrared thermography has shown valuable application as a screening technology giving immediate and valuable confirmatory diagnostic testing during foot and mouth disease outbreaks (Rainwater-Lovett *et al.* 2009). The potential use of IRT to diagnose foot lesions in sheep on endemic farms (Talukder and Celi, 2013) was another example of early diagnosis of disease. These authors recommended that longitudinal studies investigating the progression or regression of foot/hoof lesions in a larger population of sheep were required to identify temperature thresholds for early diagnosis and quantify the effectiveness of intervention measures (Talukder and Celi 2013).

Table 4. Veterinary application of infrared thermography, adapted from Mccafferty (2007).

Species	Measurement	Distance (meter)	Camera type	IRT deemed useful (or not) by authors	Author
Cattle	Effects of transportation	–	AGEMA 782	Yes	Schaefer *et al.* (1988)
Cattle	Scrotal temperature	1	AGA Thermovision 782	Yes	Kastelic *et al.* (1996)
Cattle	Inflammation and branding	–	Thermovision 470	Yes	Schwartzkopf-Genswein and Stookey (1997)
Cattle	Barn management	3	AGA 570 DEMO	Yes	Kní žková*et al.* (2002)
Cattle	Variation in udder temperature	2 to 2.5	Inframetrics 760	Yes	Berry *et al.* (2003)
Cattle	Infection detection	1.3	FLIR Inframetrics 760	Yes	Schaefer *et al.* (2004)
Cattle	Climate and housing conditions	–	Thermotracer 6T62 NEC	Not defined	Zähner (2004)
Cattle	Health and condition of hooves	1.5 to 2.0	FLIR Inframetrerics 760	Yes	Nikkhah *et al.* (2005)
Cattle	Tail docking and pain	–	Merge, Sebastian FL	Yes	Eicher *et al.* (2006)
Cattle	Foot lesions, digital dermatitis	-	ThermaCAM E2, FLIR Systems	Yes	Stokes *et al.* (2012)
Cattle	Subclinical mastitis	0.5	IR FlexCam S	Yes	Polat *et al.* (2010)

Table 4 continued…

Species	Measurement	Distance (meter)	Camera type	IRT deemed useful (or not) by authors	Author
Cattle	Hoof and especially coronary band temperatures	1-2	TIR1 imager manufactured by Fluke	Yes	Gloster *et al.* (2011)
Cattle	Subclinical mastitis	0.5	IR FlexCam S	Yes	Polat *et al.* (2010)
Cattle	Health and fertility diagnosis	-	-	Yes	Hellebrand *et al.* (2003)
Cattle	Oestrus detection	2.5	AGA Thermovision 7504 unit	No	Hurnik *et al.* (1985)
Cattle	Discriminate between oestrus and dioestrus phases	-	-	Yes	Jones *et al.* (2005)
Cattle	Scrotal temperature	1	Thermovision 782 System	Yes	Lunstra and Coulter (1997)
Cattle	Scrotal/testicular thermoregulation	1	Thermovision 782	Not defined	Kastelic *et al.* (1997)
Cattle	Bovine respiratory disease complex	-	FLIR S60 broadband camera	Yes	Schaefer *et al.* (2012)
Cattle	Eye temperature	0.5	ThermaCam S60	Yes	Stewart *et al.* (2010)
Cattle	Early prediction index for infection in calves	1 to 3	Inframetrics broadband 760 infrared scanner	Yes	Schaefer *et al.* (2004)
Horse	Clinical disorders	–	AGA Thermovision 80	Yes	Purohit (1980)

Table 4 continued…

Species	Measurement	Distance (meter)	Camera type	IRT deemed useful (or not) by authors	Author
Horse	Posture and anaesthesia	0.01	Mikron 25	Yes	Palmar (1981)
Horse	Rug and whip damage	–	AGEMA 450	Not defined	Holah (1995)
Horse	Podotrochlosis	–	Dynarad 209a	Yes	Turner (1983)
Horse	Back pain	–	Insight Visions	Yes	Colles (1995)
Horse	Lameness diagnosis		FLIR P45	Yes	Cetinkaya and Demirutku (2012)
Horse	Thermoregulation in exercised horses	4	-	Yes	Simon *et al.* (2006)
Horse	Intrasynovial Injections of anaesthetic agents	1	ThermaCAM i40	Yes	Figueiredo *et al.* (2013)
Horse	Performance enhancement technique	1.2	DTIS-500 EmergeVisionl	Yes	van Hoogmoed *et al.* (2000)
Horse	Non-focused extracorporeal shock wave therapy	-	DTIS 500 thermal camera	Not defined	Verna *et al.* (2004)

Table 4 continued…

Species	Measurement	Distance (meter)	Camera type	IRT deemed useful (or not) by authors	Author
Horse	Whether surface temperature and pregnancy	1.5 to 1.6	Meditherm vet2000	Yes	Bowers *et al.* (2009)
Pig	Nutritional study	2	Radiometric IR thermal maging camera	Yes	Loughmiller *et al.* (2005)
Pig	Febrile responses	2	PM-280 Thermacam	Yes	Loughmiller *et al.* (2001)
Pig	Vulval temperature and oestrus	0.61	Fluke IR FlexCam Termal Imager, model Ti55	Yes	Scolari *et al.* (2011)
Pig	Vulval temperature and oestrus	1.2 to 1.5	FLIR ThermoCAM S60	Yes	Sykes *et al.* (2012)
Sheep	Diagnosis of interdigital dermatitis	0.5	FLIR, 620 series	Yes	Talukder and Celi (2013)
Sheep	Detection of gonadal cooling	24 inches	-	Yes	Capraro *et al.* (2008)
Sheep	Measuring core body temperature	-	-	Yes	George *et al.* (2014)

Limitations of IRT

There are some limitations and factors, which may influence the accuracy of IRT measurements and which need to be considered during the use of IRT. Environmental conditions such as ambient temperature and relative humidity can affect the accuracy of IRT. In a recent porcine study, Sykes *et al.* (2012) reported the greatest differences in vulval temperature were captured at ambient temperatures <10°C. On the other hand, Love and Linsted (1976) and Turner *et al.* (1986) reported that ambient temperatures of approximately 20°C and <20°C (respectively) were ideal for capturing IRT images. The difference in reported 'ideal' ambient temperatures are likely to be caused by other factors that were not reported in those studies including (but not limited to) humidity, nutritional status of the animals, moisture or contamination of skin at site of measurement, skin colour, angle and distance of measurement.

Relative humidity changes the components of the atmosphere, which needs to be taken into account during thermography. At particular wavelengths, certain components of the atmosphere such as vapour and carbon dioxide absorb IR radiation (Kastberger and Stachl 2003; Stelletta *et al.* 2012). If environmental humidity is not taken into account, the displayed temperature may be overestimated (Kastberger and Stachl 2003). The known impacts of ambient temperature and humidity may create the need for comparison of control versus typical management environments to improve and develop new strategies for accurate IRT measurements.

Sunlight and distance from object of measurement are also factors that have been reported to influence IRT eye temperature suggesting the need to ensure these two factors are managed with regard to consistency (i.e. capture images from a set distance and conduct the monitoring in a shaded environment; Johnson *et al.* 2011). Additional factors for example angle of measurement, wind velocity, drafts, foreign material on the hair coat and eye injury/disease may also have a significant influence (Hoffmann *et al.* 2013).

Despite the influence of above mentioned factors on the accuracy of IRT data capture, the technology shows much promise and is considered worthy of further investigation. The use of IRT in female reproduction is relatively limited and needs further intensive

studies on mapping the body temperature differences through the duration of the oestrus cycle. These temperatures should be correlated with plasma progesterone level to ensure that the biological status of the animals is captured throughout the temperature mapping. By doing this, it is more likely that a sound understanding of the cause of core temperature rises are developed i.e. whether it is the hormonal status, the increased behavioural activity or the physical 'inflammation' of the animal which are most responsible for causing the increase in temperature associated with the oestrus event. If it is the 'inflammation' caused by the process of ovulation and expressed as oxidative stress then it is also possible that a more direct measure of this is more appropriate and less prone to data capture challenges.

FREE RADICALS AND ANTIOXIDANTS

Measuring surface temperature with IRT might be possible to infer underlying circulation related to physiology and behaviour (McCafferty 2007). Like IRT, measuring other physiological parameters for example free radicals and antioxidants may also create opportunities to monitor normal and abnormal physiological events. The mechanism of ovulation (an important physiological event for reproduction) has been compared to inflammation, which causes an increase of temperature of inflamed tissue/area. Any technologies or sensors that are able to identify the increased temperature related to inflammation might be a reliable indicator of ovulation. Free radicals (produced as by-products of cellular metabolism) have been reported to be important mediators of inflammatory reactions involved in ovulation (Sugino 2005). Therefore, there is value in investigating the measurement of the free radicals in biological fluids as an indication of ovulation.

Normally free radicals (oxidants) and antioxidants remain in balance, but when this balance is disrupted as a consequence of overproduction of free radicals or over-depletion of antioxidants, oxidative stress occurs (Agarwal *et al.* 2005; Celi 2011*b*). Numerous studies have reported the physiological as well as pathological involvement of oxidants and antioxidants in mammals. Since reproductive and developmental processes accompany dynamic changes in metabolism and energy consumption, by products are generated on an extraordinary scale (Fujii *et al.* 2005). Among such by

products, free radicals are inevitably generated during the physiological process of oxygen consumption (Fujii *et al.* 2005). However, cells under aerobic conditions have a defence system (antioxidants) against these free radicals (Sugino 2005). The types of free radicals and antioxidants, their role in female reproduction and potential in the measurement of oxidative stress in relation to reproduction are discussed here and presented in Table 5 and 6.

Free radicals

Free radical species are unstable and highly reactive molecules (Agarwal *et al.* 2005). There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (Atsumi *et al.* 2008). The biologically most important ROS are superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$). Reactive oxygen species (ROS) have been implicated in more than 100 diseases and also involved in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, corpus luteal function and luteolysis (Agarwal *et al.* 2005). The two common examples of reactive nitrogen species are nitric oxide (NO) and nitrogen dioxide (Szczepańska *et al.* 2003). Reactive nitrogen species has been associated with asthma, ischemic/reperfusion injury, septic shock and atherosclerosis (Schrier and Wang 2004; Agarwal *et al.* 2005; Reynaert *et al.* 2005; Schulman *et al.* 2005).

Antioxidants

Under normal conditions, scavenging molecules known as antioxidants convert ROS to H_20 to prevent the potential toxic effects of ROS (Agarwal *et al.* 2005). There are two types of antioxidants: enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (Nordéus *et al.* 2012). Non-enzymatic antioxidants are known as synthetic antioxidants or dietary supplements such as vitamin C, vitamin E, β-carotene and carotene, selenium, zinc, taurine, hypotaurine, glutathione (Pierce *et al.* 2004).

Oxidative stress

Under normal circumstances, an appropriate balance is maintained between oxidants and antioxidants (Agarwal *et al.* 2005). When ROS are produced rapidly and are not neutralized by antioxidant mechanisms, these substances accumulate in the tissues resulting in OS (Agarwal *et al.* 2005). Reactive oxygen species have been reported to cause DNA damage, lipid peroxidation (which principally affects membrane structure and function), and protein damage (Sugino 2005).

Role of free radicals and antioxidants in female reproduction

The ovary is a metabolically active organ and, hence, it generates ROS (Fujii *et al.* 2005). Many animal and human studies have demonstrated the presence of ROS in the female reproductive tract. Reactive oxygen species play both physiological and pathological roles in the female reproductive tract (Table 5 and 6; Agarwal *et al.* 2005). They are involved in several reproductive functions such as the regulation of follicular fluid environment, folliculogenesis, steroidogenesis, CL function, and luteolysis. Growing follicles, granulose cells of Graffian follicles and ovulated follicles all produce both enzymatic and non-enzymatic antioxidants to preserve themselves from the oxidative damage of ROS (Rizzo *et al.* 2012). This section addresses the vital role of ROS and antioxidants in physiological processes of the female reproductive tract including folliculogenesis, ovulation, CL function, steroidogenesis and maintenance of pregnancy.

Whilst there is a considerable volume of literature relating to the role of OS in human reproduction, there is a great deal to be discovered about its role in ruminant reproduction, particularly in dairy cattle. A deeper understanding of the physiological role of OS on dairy cow reproduction may allow for the development of farm monitoring tools for reproductive events such as ovulation, embryo mortality, and cystic ovarian disease.

Table 5. Physiological roles of reactive oxygen species (ROS) and antioxidant enzymes in female reproductive processes in various mammalian species [Source: (Al-Gubory *et al.* 2010)].

Remarks	Suggested role	Species	Reference
H_2O_2 stimulates uterine contractions	Peri-partum regulation of prostaglandin production	Rat	Cherouny *et al.* (1988)
High GSH levels in the oocyte	Reduction of disulfide bonds and sperm nucleus decondensation during fertilization	Hamster	Perreault *et al.* (1988)
High levels of O_2^- in Day 5 uterine pregnancy	Regulation of vascular permeability at the initiation of implantation	Mouse	Laloraya *et al.* (1989*a*); Laloraya *et al.* (1989*b*)
Growing and ovulated follicles exhibit high SOD Activity	Regulation of follicular development, ovulation and luteal functions	Rat	Laloraya *et al.* (1989*a*); Laloraya *et al.* (1989*b*)
Increased SOD activity during uterine deciduoma Development	Control of decidual cell differentiation	Rat	Devasagayam *et al.* (1990)
Levels of O_2^- and SOD exhibit marked changes in the uterus during the oestrous cycle	Regulation of uterine oedema and cell proliferation	Rat	Laloraya *et al.* (1991)
Blastocoel fluid contains amounts of H_2O_2 toxic to malignant pretrophectodermal cells	Regulation of blastocyst tissue mass by apoptosis	Mouse	Pierce *et al.* (1991)
Inhibition of ovulation by SOD in hCG-treated animals	Role of O_2^- in the mechanism of gonadotropin-induced ovulation	Rat	Sato *et al.* (1992)
Recovery of GSH after depletion in two-cell and Blastocyst stage embryos	Protective role for GSR in the GSH redox cycle	Mouse	Gardiner and Reed (1995)
Decreased SOD activity and increased lipid peroxide in the endometrium of the late secretory phase	Endometrium shedding	Human	Sugino *et al.* (1996)

Table 5 continued…

Remarks	Suggested role	Species	Reference
Early expression of GST isoenzymes in embryonic tissues	Detoxification of toxic compounds	Human	van Lieshout *et al.* (1998)
Enhanced CAT, SOD and GSH-PX activities in placental and fetal tissues	Protection against ROS toxicity in the feto-placental system	Human	Qanungo and Mukherjea (2000)
Enhanced CAT and GSH-PX activities, and GSH levels in placental tissue	Control of H_2O_2 and stimulation of placental differentiation	Human	Jauniaux *et al.* (2000)
High SOD expression and activity in CL during early pregnancy	Regulation of luteal function	Human	Sugino *et al.* (2000)
Enhanced CAT and GSH-PX activities and GSH levels in oviduct during the oestrous cycle	Control of H_2O_2 during fertilization	Cow	Lapointe and Bilodeau (2003)
Enhanced SOD and GSH-PX activities in CL during early pregnancy	Rescue of CL from apoptosis	Sheep	Al-Gubory *et al.* (2004)
H_2O_2 or O_2^- reduce oxytocin-induced myometrial Contractility	Control of uterine contraction	Human	Warren *et al.* (2005)
Enhanced GSH-PX and GSR activities and concomitant drop in B-cell lymphoma 2 associated X protein expression in early developing placentomes	Control of H_2O_2 and cell death during placental development	Sheep	Garrel *et al.* (2010)
SOD1 knock-out females exhibit marked increase in post-implantation embryo death	Protection against O_2^- during implantation	Mouse	Ho *et al.* (1998)

Table 6. Pathological roles of reactive oxygen species (ROS) and antioxidant enzymes in female reproductive processes and pregnancy outcomes in various mammalian species.

Remarks	Suggested role	Species	Reference
A burst of placental oxidative stress during establishment of maternal circulation	Early pregnancy loss	Human	Jauniaux *et al.* (2000)
Oxidative stress trigger the release of cytokines and prostaglandins from placenta	Development of preeclampsia	Human	Malek *et al.* (2001)
Exposure of free radicals to ovarian surface epithelium due to incessant ovulation	Etiopathogenesis of ovarian cancer	Human	Ness and Cottreau (1999)
Imbalance in prooxidant antioxidant systems in follicular fluid	Abnormal development of oocytes and impaired fertility as well as damage to the oocyte's DNA, cytoskeleton or membrane	Human	Agarwal and Allamaneni (2004)
High oxidatively modified substances in peritoneal fluid and ectopic endometrial tissue	Endometriosis	Human	van Langendonckt *et al.* (2001)
High concentration of ROS in hydrosalpingeal fluid	Embryo blastocyst development	Mouse	Bedaiwy *et al.* (2002)
High concentration of ROS in embryo	Aggregation of cytoskeleton components, condensation of the endoplasmic reticulum and loss of membrane fluidity	Human	Agarwal and Allamaneni (2004)

Oxidative stress, folliculogenesis and oocyte maturation

Superoxide radicals are generated in the cytosol and mitochondria by normal metabolism and steroidogenesis during follicular development (Sugino 2005). The transition of the developing follicles to the antral stage is associated with a marked increase in metabolic function of granulose cells, most notably in the production of steroids via enhanced activity of cytochrome P450 enzymes (Figure 5). Reactive oxygen species and related compounds may function as intracellular regulators of steroidogenesis and P_4 release in the CL. On the other hand, P_4 is also able to enhance ROS generation as a physiological by-product of steroid synthesis during the follicular and luteal phase (González-Fernández *et al.* 2005).

There are specific metaloenzymes those are known to scavenge superoxide radicals: copper-zinc superoxide dismutase (Cu, Zn-SOD), located in the cytosol; and manganese SOD (Mn-SOD), located in the mitochondria of ovarian follicular cells (Sugino 2005). It is reported that Cu, Zn-SOD may play a role in P_4 production by the theca interna cells via the synthesis of hydrogen peroxide (Sugino 2005). Selenium dependent glutathione peroxidase activity has been demonstrated in human and mouse oviducts and oocytes (El Mouatassim *et al.* 1999). A delicate balance between ROS (superoxide radicals) and antioxidant enzymes (manganese and copper-zinc SOD) has been examined for SOD expression in the cumulus cells that surround the oocytes in all follicular stages including primordial, primary, pre-antral, non-dominant antral follicles in follicular phase, dominant follicles, ovulated follicles and atretic follicles (Suzuki *et al.* 1999). It is suggested that manganese and copper-zinc SOD both play important roles in the prolonged process of follicular atresia or luteal regression, possibly due to the inhibition of apoptosis in follicular or luteal cells (Suzuki *et al.* 1999). If the balance between antioxidants and ROS is disrupted during folliculogenesis, it may cause atrophy of the ovarian follicular atresia and premature resumption of meiosis (Levine and Morita 1984).

Oxidative stress and ovulation

Reactive oxygen species play vital roles in the physiological processes leading to an ovulation event (Figure 5). The mechanism of ovulation has been compared to an inflammatory reaction (Espey 1980) - a localized physical condition in which part of the

body becomes reddened, swollen, hot, and often painful. Inflammation is accompanied by an increases in prostaglandin synthesis and cytokine production, the action of proteolytic enzymes such as matrix metalloproteinases, and increased vascular permeability (Brannstrom 2004). Reactive oxygen species could be important mediators of those inflammatory reactions involved in follicle rupture and have therefore been reported to be involved in ovulation (Sugino 2005).

Possible sources of ROS during the ovulatory process could be the local leukocytes or endothelial cells localized around the preovulatory follicle (Brännström *et al.* 1993; Sugino 2005). Leukocytes accumulate around preovulatory follicles and even infiltrate into the granulose cell layer (Brännström *et al.* 1993; Araki *et al.* 1996). In an investigation to explore the state of female peripheral polymorphonuclear leukocytes (PMN) during menstrual cycles, Shirai *et al.* (2002) found that PMN contained high levels of superoxide, H_2O_2 and NO during at the periovulatory period suggesting the cause of this elevation to be LH receptors. Kodaman and Behrman (2001) assessed the ROS generation during the ovulatory cascade by luminol-amplified chemiluminescence and reported that ROS generated from isolated follicles originate in leukocytes indicating the role for ROS in the periovulatory period.

Several studies have reported a strong interplay amongst ROS, apoptosis and ovulation (Sato *et al.* 1992; Miyamoto *et al.* 2010; Rizzo *et al.* 2012). Rizzo *et al.* (2009) measured reactive oxygen metabolites (ROMs) in follicular fluid of cows with cystic ovarian disease and found that they contained lower ROMs concentrations than the follicular fluid of ovulated cows. This suggests that ROMs in cystic fluid may be insufficient to induce the apoptosis of the cells of the follicular wall, thus inhibiting ovulation in cows with cystic ovarian disease. Future studies are required to evaluate temporal changes in OS markers in plasma as well as in utero-ovarian fluids if a deeper understanding of the role of OS in pathogenesis of cystic ovarian disease in cows is to be developed.

Miyazaki *et al.* (1991) examined the potential role of ROS in human chorionic gonadotrophin-induced ovulation using SOD and/or CAT with in-vitro perfused rabbit ovary preparations. The simultaneous administration of CAT with SOD had no additive effect on ovulation rate compared with SOD alone suggesting that superoxide radicals may be involved in ovulation (Miyazaki *et al.* 1991; Sugino 2005). Whilst the

mechanisms by which superoxide radicals induce follicle rupture are not fully understood yet (Sugino 2005), it seems that they induce prostaglandin production (Cherouny *et al.* 1988; Sugino *et al.* 2001), activation of proteolytic enzymes (Buhimschi *et al.* 2000) and enhancement of vascular permeability (Sugino 2005).

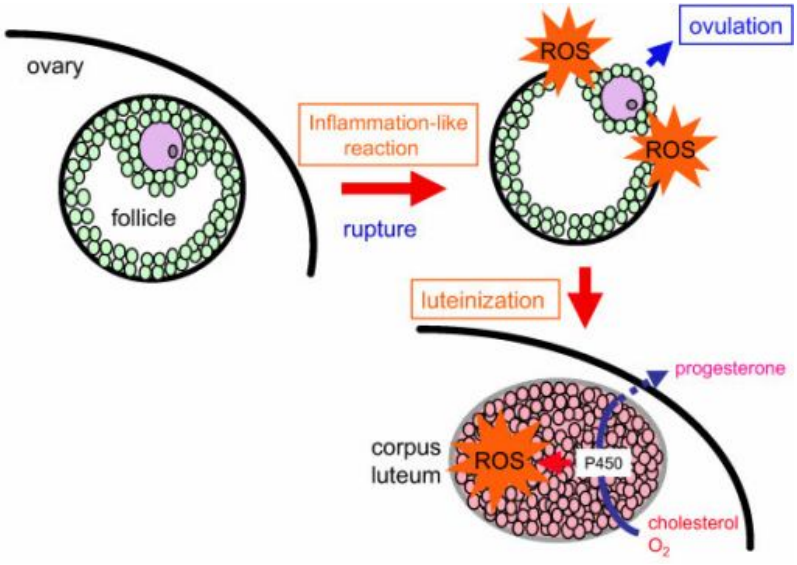

Figure 5. Generation of reactive oxygen species during ovulation and steroidogenesis in corpora lutea. Ovulation appears to be an inflammation like process. Reactive oxygen species are locally produced during follicular rupture and may be involved in the ovulation process. They are also generated by the corpus luteum via the monooxygenase reaction as a by-product during steroid hormone synthesis (Fujii *et al.* 2005).

Regarding the role of SOD in preovulatory follicles during the ovulatory process, Sato *et al.* (1992) and Sasaki *et al.* (1994) reported dynamic aspects of Mn-SOD in follicles during the ovulatory process in rats. Sasaki *et al.* (1994) observed a marked increase of messenger ribonucleic acid levels and a low Mn-SOD activity level (but unchanged Cu/Zn-SOD activity and Cu/Zn-SOD mRNA) and suggested a possible rapid turnover of Mn-SOD (increased rates of synthesis and consumption of Mn-SOD). This indicates high levels of superoxide radical generation due to activated P_4 production while Sugino *et al.* (1998) suggested that such a pattern of increased Mn-SOD expression is due to the response of cytokine rather than the enhanced steroidogenesis mentioned by Sasaki *et al.* (1994). Such a pattern of increased Mn-SOD expression without significant changes in Cu/Zn-SOD expression is often seen in inflammatory reactions (Wong and Goeddel

1988; Sugino *et al.* 1998), suggesting the presence of inflammation like events in preovulatory follicles and the important role of Mn-SOD in protecting luteal cells from inflammation mediated oxidative damage (Sugino *et al.* 1998).

Oxidative stress, CL steroidogenesis and CL function

Steroidogenic cells are potential sources of ROS because these compounds are by-products of steroidogenesis. On the other hand, luteal ROS generated via monooxygenase reaction of the mitochondrial cytochrome P450 also influence P_4 synthesis (Sugino 2005; Rizzo *et al.* 2012). Sawada and Carlson (1996) reported a biphasic effect of ROS, with small levels associated with stimulation and high levels involved in inhibition of P_4 secretion. Although ROS regulates the luteal cell steriodogenesis (positively or negatively depending on concentration, it has been proposed that the uncontrolled generation of ROS such as elicited by $PGF_{2\alpha}$ (Sawada and Carlson 1994) disrupts cellular homeostasis leading to functional and structural luteolysis (Sawada and Carlson 1994). It is therefore possible that the monitoring of oxidative stress in different biological fluids will allow us to monitor CL function. If cow level information of oxidants and antioxidants concentrations could be generated automatically (on a daily basis) it might be possible to monitor CL function through on-farm milk testing although the greatest value might require P_4 data to be generated simulataneously and combine in an algorithm. Unless the combined data significantly enhanced reproductive monitoring and performance then the additional cost and effort required to capture, collate and report the data would not be justified.

Considering that ROS are generated in the CL and that a decline in the expression of SOD, as well as CAT, has been observed during CL regression in the bovine ovary, it is reasonable to speculate that ROS and SOD are involved in the regulation of CL function (Sugino 2006). During recent years, evidence has accumulated to suggest that locally produced antioxidant enzymes within the CL are involved in the maintenance of luteal steroidogenic activity and structure (Al-Gubory *et al.* 2005). Rapoport *et al.* (1998) evaluated the levels of antioxidants during different developmental stages of the bovine CL as well as their correlation with steriodogenic status and reported that SOD and CAT activities showed similar patterns to plasma P_4 concentration. In addition, an increase in the activity of CAT, SOD and β-carotene concentration in the mid luteal phase of the oestrous cycle followed by a decrease during the luteal regression has also been reported

(Rapoport *et al.* 1998). Furthermore, an increase in antioxidants (SOD and GSH-Px) and a decrease of pro-oxidant (nitric oxide synthetase) in the CL (from early to mid luteal phase) has been reported in sheep (Al-Gubory *et al.* 2005). However, no correlation of antioxidants and pro-oxidant enzymatic capacities of luteal cells with cell steroidogenic status and integrity during the late luteal phase were observed. This suggests that the demise and removal of sheep CL at the end of the oestrous cycle may be mediated by apoptosis despite the high local levels of antioxidant enzymes. After the initial functional luteolysis, the rise in secretion of $PGF_{2\alpha}$ and the rapid decline in P_4 production both contribute to the progression of apoptosis in the sheep luteal cells (Al-Gubory *et al.* 2005).

Oxidative stress, embryo development and embryo mortality

Reactive oxygen species may originate from embryo metabolism and/or embryo surroundings but an excess of its production has been associated with retardation of embryo growth often leading to embryo death (Guérin *et al.* 2001; Celi 2010), increasing days open and calving interval in dairy cows. Embryos have both internal (SOD and GSH-Px) and external (transferrin, ascorbic acid present in the oviduct) scavenging mechanisms (Guérin *et al.* 2001; Celi 2010). If fertilization occurs, high levels of luteal SOD and CAT have been observed (Rizzo *et al.* 2012). An increase in advanced oxidative protein products and malondialdehyde has been observed in pastured dairy cows that were diagnosed with embryo mortality after Day 25 of artificial insemination (Celi *et al.* 2011; Celi *et al.* 2012). Miszkiel *et al.* (1999) reported the presence of β-carotene and ascorbic acid in the CL of pregnant cows and sows. However, the continuous and abundant production of ROS during embryo development may result in oxidative stress.

Oxidative stress might also be considered one of the mechanisms that link inflammation with embryo damage including mitochondrial alterations, embryo cell block, ATP depletion and apoptosis (Guérin *et al.* 2001). These embryonic losses appear to follow activation of multiple pathways that disrupt the reproductive axis at points including the hypothalamic-pituitary axis, ovary, oocyte and the embryo (Hansen *et al.* 2004; Celi 2010). Activation of inflammatory and immune responses leads to an increase in the production of cytokines that can in turn increase secretion of other molecules

detrimental for embryo survival and development, such as $PGF_{2\alpha}$ or nitric oxide (Hansen *et al.* 2004).

Oxidative stress and pregnancy

The stromal cells of the endometrium generate ROS as by-products of normal metabolism (Freeman and Crapo 1982). During pregnancy, foetal growth is associated with an increase in placental and maternal metabolism that, in turn, make the dam and the foetus susceptible to oxidative stress (Myatt 2010; Rizzo *et al.* 2012). Uterine, embryonic, feto-placental antioxidant adaptations to OS during pregnancy are now considered as key events for the establishment and outcome of pregnancy (Al-Gubory *et al.* 2010). However, ROS are recognized as physiologically relevant molecules that mediate cell responses to a variety of stimuli, and the activities of several molecules in the embryo (Covarrubias *et al.* 2008) and on placental function including trophoblast proliferation and differentiation and vascular reactivity (Myatt and Cui 2004). It has been reported that hypoxia and OS initiate placental trophoblast apoptosis, which are essential for early embryonic and placental growth via regulation of peri-implantation angiogenesis in humans, sheep and mice (Al-Gubory *et al.* 2010). An excessive production of ROS during early placental development can be associated with various diseases of pregnancy such as embryo resorption, miscarriage, intrauterine growth restriction, and pre-eclampsia in women (Myatt and Cui 2004; Rizzo *et al.* 2012). Oxidative status may also affect key transcription factors (hypoxia-inducible factor Nuclear factor-kB, activator protein-1 and apoptotic factor p53) and hence alter gene expression during development of the embryo (Dennery 2004). Over expression of these transcription factors impacts on vascular development, decreases apoptosis and increases the proinflammatory state, further modifies the cellular redox status and arrests growth of the embryo (Dennery 2004). Garrel *et al.* (2010) reported increased activity of antioxidant enzymes (Mn-SOD, GSH-Px) in sheep placentomes during early placental development, suggesting the changes in the antioxidant enzymatic defences could be a part of placentome adaptation to reactive oxygen species-induced OS at specific early developmental stages of pregnancy. Lista *et al.* (2009) suggested that the antioxidant protective role of the placenta against oxidative damage is in keeping with the large gradient of ROS between mother and foetus and the passage of total antioxidants from the mother to the foetus. Some authors observed only higher GSH concentration in

pregnant women compared to that in non-pregnant women while other antioxidants (GSH-Px and GSH reductase) did not differ between two groups (Zachara *et al.* 1993; Arikan *et al.* 2001). Antioxidant control of ROS is important during embryonic development through cellular signalling pathways involved in proliferation, differentiation and apoptosis, and ROS induced OS can alter embryonic development (Dennery 2007; Al-Gubory *et al.* 2010; Rizzo *et al.* 2012). Induced climatic stress in ruminants can result from an over-abundant production of ROS which has major adverse effects on health and reproduction of grazing ruminant animals, directly through the increased production of ROS in the animal, or indirectly, through the reduced quality of fresh or conserved forage (Aurousseau *et al.* 2006).

Potential of measuring oxidative stress in relation to reproduction

Measuring oxidative stress parameters in human reproduction has brought about substantial insights into the physiological and pathological role of oxidants and antioxidants in a number of reproductive events (folliculogenesis, ovulation, fertilization, embryo development, and pregnancy). The unpaired electron in the outer orbital of free radicals gives them the potential to attack any organic molecule, thus generating a class of compounds which are called reactive oxygen metabolites (ROMs) (Reilly *et al.* 1991). Plasma level concentration of ROMs can be considered as indicators of free radical production (Miller *et al.* 1993). There are numerous ways to measure oxidative stress in plasma (Celi 2011*a*). Reactive oxygen metabolites kits have been developed to assess oxidant levels in plasma and other biological fluids. The ROMs assay is intended for measurement of the concentration of total hydroperoxides in serum or heparin plasma (Ruskovska *et al.* 2014). Earlier studies have reported the use of the ROMs assay in monitoring oxidative stress in goats (Di Trana *et al.* 2006; Celi *et al.* 2008), sheep (Rizzo *et al.* 2008), and dairy cows (Bernabucci *et al.* 2002; Pedernera *et al.* 2010). Celi *et al.* (2012) used the free radical analytical system (FRAS 4) technology for assessing oxidative status in dairy cows. Total antioxidant capacity can be measured by means of several methods, such as Trolox Equivalent Antioxidant Capacity (Miller *et al.* 1993), Total Radical-trapping Antioxidant Parameter (Ghiselli *et al.* 1995), Oxygen Radical Absorbance Capacity (Cao *et al.* 1993) or the Ferric Reducing Ability of Plasma and Biological Antioxidant Potential (BAP; Benzie and Strain 1996). However, each of these techniques for measuring

oxidants/OS/antioxidants has pros and cons. Trolox Equivalent Antioxidant Capacity is probably the least suitable to determine plasma antioxidant capacity due to the reliance on enzymatic activity and hence has not been widely applied (Badarinath *et al.* 2010). Total Radical-trapping Antioxidant Parameter is suitable for measurements of in-vivo antioxidant capacity in both serum or plasma because it measures nonenzymatic antioxidants such as glutathione and ascorbic acid (Huang *et al.* 2005) but it is recognised as a relatively complex, time consuming process which requires a high degree of expertise and experience (Badarinath *et al.* 2010). The advantage of Ferric Reducing Ability of Plasma is simple, relatively quick, inexpensive, and does not require specialized equipment (Benzie and Strain 1999) although it cannot detect species that act by radical quenching, particularly sulfhydryl group containing antioxidants like thiols, such as glutathione and proteins (Prior and Cao 2000; Huang *et al.* 2005). Oxygen Radical Absorbance is suited equally well for the measurement of both antioxidants that exhibit distinct lag phase and those that have no lag phases (Caldwell 2001) but it is limited to measurement of hydrophilic chain and ignores lipophilic antioxidants. The Biological Antioxidant Potential test provides a global measurement of many antioxidants, including uric acid, ascorbic acid, proteins, α-tocopherol and bilirubin (Benzie and Strain 1996). Regardless of the method employed, measuring biomarkers of OS in the laboratory can be time consuming, labour intensive, and costly (Celi 2011*a*). In addition, the measurement of biomarkers in blood or plasma does not lend itself to automation because cows need to be restrained and the blood must be collected by venepuncture. Milk, on the other hand, is less invasive and lends itself to in-line measurements, which has the advantages of convenience and timeliness.

Due to its very complex composition, milk exhibits various biological activities (e.g. antioxidant, antimicrobial and immunomodulatory; Cervato 1999; Bučević-Popović *et al.* 2014). Milk contains many enzymes, which constantly consume metabolites, produce free radicals, and modify its composition (Silanikove *et al.* 2006). The indigenous enzymes in milk are secreted and arise through blood plasma, secretory cell cytoplasm, the apical membrane of the mammary cell and somatic cell (leucocytes; Fox and Kelly 2006). Xanthine oxidase (XO) and lactoperoxidase (LP) activities are also sources of ROS in colostrum (Przybylska *et al.* 2007). Among antioxidative enzymes, SOD, GSH-Px and CAT have been demonstrated in milk (Przybylska *et al.* 2007). In addition to enzymatic substances, several non-enzymatic antioxidants act as radical scavengers in

the lipid phase of milk, such as vitamin E, carotenoids and ubiquinol, whereas vitamin C acts in the water phase (Przybylska *et al.* 2007).

Macrophage and epithelial cells of the mammary gland produce significant amounts of nitric oxide, which mediates inflammation during mastitis (Bouchard *et al.* 1999; Celi 2011*b*). Nitric oxide production is considered as a primer defence system as it has antimicrobial properties due to peroxynitrite, a reactive nitrogen metabolite, derived from oxidation of nitric oxide (Huie and Padmaja 1993; Okamoto *et al.* 1997), however, excess production of peroxynitrite can cause alterations in antioxidant balance (Chaiyotwittayakun *et al.* 2002). Measuring oxidants and antioxidants in milk samples may be a reliable way for monitoring such an imbalance in dairy species.

The "Herd Navigator" in-line milk testing system is based on biosensors for in-line measurement of P_4 in milk (Gillis *et al.* 2002) which uses complex individual cow algorithms to determine appropriate sampling days, analyses samples and creates alerts for the herdsperson through the support software program. The breakthrough of technology like Herd Navigator creates the expectation that one day the automated measurement of oxidative biomarkers in milk may be possible. However, there is considerable work to be done to determine how various biomarkers of OS relate to the different states of the reproductive cycle, which biomarkers in milk best lend themselves as accurate and precise indicators of oestrus, and to establish if and when individual animals are experiencing OS in relation to the various reproductive stages (Celi 2010). As the physiological role of oxidative stress for inducing an ovulation event in humans was previously discussed, there is value in examining whether oxidative stress levels can be used as an indicator that a cow is that about to ovulate. If cow level information of oxidants and antioxidants are available as a routine and particularly if automation is possible and tested along with P_4 concentration, it might be possible to identify pre-imminent ovulation through on-farm milk testing.

CONCLUSIONS AND RESEARCH NEEDS

Conclusions

Accurate and timely oestrus detection in dairy cows is an important indicator of effective reproduction management for the dairy farm. The most commonly used

method for oestrus detection is visual monitoring (with or without aids), but this is particularly difficult for large dairy herds and potentially even more so for herds managed with AMS. Because of technical progress in monitoring cows using computers and sensors, automatic oestrus detection is becoming increasingly possible and popular. Results of oestrus detection vary depending on the threshold values (of the relevant technology) implemented on farm, the number of cows, housing and treatment of cows and the utilised method of time series analysis (Firk *et al.* 2002). A sensitivity of 100% in combination with no false oestrus alerts on the basis of computer aided management devices, is often expected by farmers but is difficult to achieve (de Mol 2000) but with the developments of sensor research and analytical techniques an improvement in oestrus detection is expected (van Asseldonk *et al.* 1998).

Research needs

The reproductive performance of dairy cows has been researched extensively for over 40 years and numerous determinants have been identified (Morton 2004). Fertility data of AMS herds has been evaluated in numerous studies but the core (and still existing) gap within these studies is a lack in literature of reproductive performance of dairy cow under pasture-based system. This review of literature has identified some factors related to reproductive performance of dairy cows in AMS under pasture-based systems that are critical for planning future research and reproductive intervention to increase the reproductive performance in the Australian dairy industry.

Measuring herd reproductive performance

Several parameters have been used to evaluate herd reproductive performance; however, to quantify the reproductive performance in indoor-based AMS a limited number of determinants were used in each study. In addition, measures differ depending on herd management systems. As AMS is a completely new way of farming and different from CMS, it is necessary to assess the reproductive performance in pasture-based AMS to ensure that areas of under performance can be addressed and that farmers considering AMS have appropriate expectations around reproductive performance (a key factor impacting on-farm profitability).

Factors determining the reproductive performance

While numerous determinants of reproductive performance in AMS have been identified, much of this research has been conducted in Europe and research findings may vary from Australian production systems. There is a need to conduct data analysis to evaluate the effect of management and physiological factors such as milk yield and milking frequency on the reproductive performance of pasture-based AMS dairy herds. In addition, it is also necessary to evaluate the effects of several other factors (including management and husbandry techniques) on the reproductive performance of AMS.

Identifying the most important factors

To improve herd reproductive performance, it is necessary to identify the most important factors associated with the measures of reproductive performance in AMS systems. In CMS, herd persons monitor cows for visual sign of oestrus during fetching, yarding, and milking. Since, in AMS, no milkers are present at the time of milking, there is value in reconsidering traditional oestrus detection methods. Accordingly, interest in automatic oestrus detection as a replacement or assistant to manual detection of oestrus continues to be of interest.

Role of technologies in oestrus detection

Irrespective of AMS/CMS, there is a need of replacing visual detection of oestrus considering growing labour cost, increased herd size and shorter duration of oestrus in modern high yielding dairy cows. Progress is ongoing with respect to monitoring cows with electronic devices and biosensors. New technologies need to be tested and validated for oestrus detection in pasture-based Australian dairy herds.

Testing OS as ovulation indicator

Oxidative stress has been reported to be associated with female reproductive events. Developing a more integral understanding of the biological processes and their relationships is essential if the true value/potential of using OS biomarkers as indicators of oestrus to be determined.

Addressing these needs

Many of the above-mentioned gaps in knowledge are addressed in this thesis. Descriptive statistics of reproductive indices and associations between numerous risk factors and reproductive performance of an AMS research herd using the determinants suitable for Australian dairy herds are described in Chapter 2. To improve oestrus detection, the potential of new technologies and their on farm practical use was investigated in Chapter 3 and Chapter 5. Changes in plasma OS biomarkers between ovulatory and anovulatory oestrus cycles were reported in Chapter 4, while differences in milk OS biomarkers in ovulated vs anovulated cows are described in Chapter 6. During the study period detailed in Chapter 3, a cow was diagnosed with a follicular cyst after synchronization of oestrus. Her ovarian follicle diameter, hormonal profiles and OS biomarkers are reported and compared to those in her ovulated cohort, in a case study (Chapter 7). Similarly, the investigation capture in Chapter 5 coincided with the occurrence of a Displaced Abomasum and the data captured allowed the generation of a detailed case report for that cow detailing her milk yield, activity level, and rumination level compared to her herd mates. Chapter 9 discusses the implications of study findings for future research and provides the recommendations for validating the oestrus detection methods and in line milk monitoring systems of OS biomarkers as ovulation indicators.

REFERENCES

Aalseth E (2005) Fresh cow management: What is important, what does it cost, and what does it return. In 'Proceedings of the 7th Western Dairy Management Conference.' pp. 1-12. (Reno, NV)

Agarwal A, Allamaneni SSR (2004) Role of free radicals in female reproductive diseases and assisted reproduction. *Reproductive BioMedicine Online* **9,** 338-347.

Agarwal A, Gupta S, Sharma R (2005) Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology* **3,** 1-21.

Agarwal A, Gupta S, Sikka S (2006) The role of free radicals and antioxidants in reproduction. *Current Opinion in Obstetrics and Gynecology* **18,** 325-332.

Al-Gubory KH, Bolifraud P, Germain G, Nicole A, Ceballos-Picot I (2004) Antioxidant enzymatic defence systems in sheep corpus luteum throughout pregnancy. *Reproduction* **128,** 767-774.

Al-Gubory KH, Ceballos-Picot I, Nicole A, Bolifraud P, Germain G, Michaud M, Mayeur C, Blachier F (2005) Changes in activities of superoxide dismutase, nitric oxide synthase, glutathione-dependent enzymes and the incidence of apoptosis in sheep corpus luteum during the estrous cycle. *Biochimica et Biophysica Acta* **1725,** 348-357.

Al-Gubory KH, Fowler PA, Garrel C (2010) The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *International Journal of Biochemistry and Cell Biology* **42,** 1634-1650.

AlZahal O, AlZahal H, Steele MA, van Schaik M, Kyriazakis I, Duffield TF, McBride BW (2011) The use of a radiotelemetric ruminal bolus to detect body temperature changes in lactating dairy cattle. *Journal of Dairy Science* **94,** 3568-3574.

Araki M, Fukumatsu Y, Katabuchi H, Shultz LD, Takahashi K, Okamura H (1996) Follicular development and ovulation in macrophage colony-stimulating factor-deficient mice homozygous for the osteopetrosis (op) mutation. *Biology of Reproduction* **54,** 478-484.

Arikan S, Dildar K, Arikan Ç, Akçay T, Davas I (2001) Lipid peroxidation and antioxidant status in maternal and cord blood. *Gynecologic and Obstetric Investigation* **51,** 145-149.

Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R, Sinclair KD (2001) Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. *Biology of Reproduction* **64,** 1624-1632.

Arnott WJ (1961) Problems of artificial breeding of cattle. *Australian Veterinary Journal* **36,** 140-145.

Atsumi T, Tonosaki K, Fujisawa S (2008) Salivary free radical-scavenging activity is affected by physical and mental activities. *Oral Diseases* **14,** 490-496.

At-Taras EE, Spahr SL (2001) Detection and characterization of estrus in dairy cattle with an electronic heatmount detector and an electronic activity tag. *Journal of Dairy Science* **84,** 792-798.

Aurousseau B, Gruffat D, Durand D (2006) Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant. *Reproduction, Nutrition, Development* **46,** 601-620.

Badarinath AV, Rao KM, Chetty CMS, Ramkanth S, Rajan TVS, Gnanaprakash K (2010) A review on in-vitro antioxidant methods: comparisions, correlations and consideration. *International Journal of PharmTech Research* **2,** 1276-1285.

Balaras CA, Argiriou AA (2002) Infrared thermography for building diagnostics. *Energy and Buildings* **34,** 171-183.

Ball PJH, Peters AR (2004) Oestrus behavior and its detection. In 'Reproduction in Cattle.' Chapter 8, Third edition. pp 92-109. (Blackwell Publishing, Oxford, UK).

Beam S, Butler W (1998) Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *Journal of Reproduction and Fertility. Supplement* **54,** 411-424.

Bedaiwy MA, Goldberg JM, Falcone T, Singh M, Nelson D, Azab H, Wang X, Sharma R (2002) Relationship between oxidative stress and embryotoxicity of hydrosalpingeal fluid. *Human Reproduction* **17,** 601-604.

Bela B, Nagy G, Vinczeffy I (1995) The influence of grazing on milk production and productive lifetime. In 'Poster presentation at 46th Annual Meeting of the European Association for Animal Production.' (Prague, Czech Republic)

Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry* **239,** 70-76.

Benzie IFF, Strain JJ (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In 'Methods in Enzymology.' (Ed. P Lester) pp. 15-27. (Academic Press)

Bernabucci U, Ronchi B, Lacetera N, Nardone A (2002) Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. *Journal of Dairy Science* **85,** 2173-2179.

Bernard DJ, Fortin J, Wang Y, Lamba P (2010) Mechanisms of FSH synthesis: what we know, what we don't, and why you should care. *Fertility and Sterility* **93,** 2465-2485.

Berry RJ, Kennedy AD, Scott SL, Kyle BL, Schaefer AL (2003) Daily variation in the udder surface temperature of dairy cows measured by infrared thermography: Potential for mastitis detection. *Canadian Journal of Animal Science* **83,** 687-693.

Bewley JM, Einstein ME, Grott MW, Schutz MM (2008) Comparison of reticular and rectal core body temperatures in lactating dairy cows. *Journal of Dairy Science* **91,** 4661-4672.

Bouchard L, Blais S, Desrosiers C, Zhao X, Lacasse P (1999) Nitric oxide production during endotoxin-induced mastitis in the cow. *Journal of Dairy Science* **82,** 2574-2581.

Bowers S, Gandy S, Anderson B, Ryan P, Willard S (2009) Assessment of pregnancy in the late-gestation mare using digital infrared thermography. *Theriogenology* **72,** 372-377.

Brandt M, Haeussermann A, Hartung E (2010) Invited review: Technical solutions for analysis of milk constituents and abnormal milk. *Journal of Dairy Science* **93,** 427-436.

Brannstrom M (2004) Potential role of cytokines in ovarian physiology: The case for interleukin-1. In 'The Ovary.' (Eds PCK Leung, EY Adashi) pp. 261–265. (Elsevier Academic Press: New York)

Brännström M, Mayrhofer G, Robertson SA (1993) Localization of leukocyte subsets in the rat ovary during the periovulatory period. *Biology of Reproduction* **48,** 277-286.

Brightling P, Larcombe MT, Malmo J (1990) Investigating shortfalls in reproductive performance in dairy herds. (Dairy Research Council: Australia)

Britt JH (1982) Foot problems affect heat detection. *Hoard's Dairyman* **127,** p. 824.

Brown-Brandl TM, Eigenberg RA, Nienaber JA, Hahn GL (2005) Dynamic response indicators of heat stress in shaded and non-shaded feedlot cattle, part 1: Analyses of indicators. *Biosystems Engineering* **90,** 451-462.

Bučević-Popović V, Delaš I, Međugorac S, Pavela-Vrančić M, Kulišić-Bilušić T (2014) Oxidative stability and antioxidant activity of bovine, caprine, ovine and asinine milk. *International Journal of Dairy Technology* **67,** 394-401.

Buhimschi IA, Kramer WB, Buhimschi CS, Thompson LP, Weiner CP (2000) Reduction-oxidation (redox) state regulation of matrix metalloproteinase activity in human fetal membranes. *American Journal of Obstetrics and Gynecology* **182,** 458-464.

Bulman DC, Lamming GE (1978) Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. *Journal of Reproduction and Fertility* **54,** 447-458.

Burnay SG, Williams TL, Jones CH (1988) Applications of thermal imaging. (Adam Hilger: Bristol)

Caldwell CR (2001) Oxygen radical absorbance capacity of the phenolic compounds in plant extracts fractionated by high-performance liquid chromatography. *Analytical Biochemistry* **293,** 232-238.

Canfield RW, Sniffen CJ, Butler WR (1990) Effects of excess degradable protein on postpartum reproduction and energy balance in dairy cattle. *Journal of Dairy Science* **73,** 2342-2349.

Cao G, Alessio HM, Cutler RG (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine* **14,** 303-311.

Capraro GA, Coughlin BF, Mader TJ, Smithline HA (2008) Testicular cooling associated with testicular torsion and its detection by infrared thermography: An experimental study in sheep. *The Journal of Urology* **180,** 2688-2693.

Cavalieri J, Eagles VE, Ryan M, Macmillan KL (2003) Comparison of four methods for detection of oestrus in dairy cows with resynchronised oestrous cycles. *Australian Veterinary Journal* **81,** 422-425.

Celi P (2010) The role of oxidative stress in small ruminants' health and production. *Revista Brasileira de Zootecnia* **39,** 348-363.

Celi P (2011*a*) Biomarkers of oxidative stress in ruminant medicine. *Immunopharmacology and Immunotoxicology* **33** 233-240.

Celi P (2011*b*) Oxidative Stress in Ruminants. In 'Studies on Veterinary Medicine. Vol. 5.' (Eds L Mandelker, P Vajdovich.) pp. 191-231. (Humana Press, Totowa, NJ)

Celi P, di Trana A, Quaranta A (2008) Metabolic profile and oxidative status in goats during the peripartum period. *Australian Journal of Experimental Agriculture* **48,** 1004-1008.

Celi P, Merlo M, Barbato O, Gabai G (2012) Relationship between oxidative stress and the success of artificial insemination in dairy cows in a pasture-based system. *The Veterinary Journal* **193,** 498-502.

Celi P, Merlo M, Da Dalt L, Stefani A, Barbato O, Gabai G (2011) Relationship between late embryonic mortality and the increase in plasma advanced oxidised protein products (AOPP) in dairy cows. *Reproduction, Fertility and Development* **23,** 527-533.

Cervato RC, Benvenuto Cestaro, Giovanna (1999) Studies on the antioxidant activity of milk caseins. *International Journal of Food Sciences and Nutrition* **50,** 291-296.

Cetinkaya MA, Demirutku A (2012) Thermography in the assessment of equine lameness. *Turkish Journal of Veterinary and Animal Sciences* **36,** 43-48.

Chagas LM, Bass JJ, Blache D, Burke CR, Kay JK, Lindsay DR, Lucy MC, Martin GB, Meier S, Rhodes FM, Roche JR, Thatcher WW, Webb R (2007) Invited review: New perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. *Journal of Dairy Science* **90,** 4022-4032.

Chagas LM, Rhodes FM, Blache D, Gore PJS, Macdonald KA, Verkerk GA (2006) Precalving effects on metabolic responses and postpartum anestrus in grazing primiparous dairy cows. *Journal of Dairy Science* **89,** 1981-1989.

Chagunda MGG, Friggens NC, Rasmussen MD, Larsen T (2006) A model for detection of individual cow mastitis based on an indicator measured in milk. *Journal of Dairy Science* **89,** 2980-2998.

Chaiyotwittayakun A, Erskine RJ, Bartlett PC, Herdt TH, Sears PM, Harmon RJ (2002) The effect of ascorbic acid and L-histidine therapy on acute mammary inflammation in dairy cattle. *Journal of Dairy Science* **85,** 60-67.

Cherouny PH, Ghodgaonkar RB, Niebyl JR, Dubin NH (1988) Effect of hydrogen peroxide on prostaglandin production and contractions of the pregnant rat uterus. *American Journal of Obstetrics and Gynecology* **159,** 1390-1394.

Coleman J, Pierce KM, Berry DP, Brennan A, Horan B (2009) The influence of genetic selection and feed system on the reproductive performance of spring-calving dairy cows within future pasture-based production systems. *Journal of Dairy Science* **92,** 5258-5269.

Colles C (1995) Thermal imaging as an aid to the diagnosis of back pain in the horse. In 'The Thermal Image in Medicine and Biology.' (Eds K Ammer, EFJ Ring) pp. 164-167. (Uhlen-Verlag: Wien, Austria)

Covarrubias L, Hernández-García D, Schnabel D, Salas-Vidal E, Castro-Obregón S (2008) Function of reactive oxygen species during animal development: Passive or active? *Developmental Biology* **320,** 1-11.

Dairy Australia (2011) Improvement of the reproductive performance of Victorian dairy herds.

de Koning CJM, Rodenburg J (2004) Automatic milking: State of the art in Europe and North America. In 'Automatic milking—A better understanding.' (Eds A Meijering, H Hogeveen, CJAM de Koning) pp. 27–40. (Wageningen Academic Publishers: Wageningen, The Netherlands)

de Koning CJND (2010) Automatic milking – Common practice on dairy farms. In 'Proceedings of the 1st North American Conference, Precision Dairy Management and 2nd North American Conference, Robotic Milking.' pp. 52-67. (Wageningen Academic Publishers: Wageningen, The Netherlands)

de Mol RM (2000) 'Automated detection of oestrus and mastitis in dairy cows.' PhD thesis, University of Wageningen, The Netherlands.

de Rensis F, Scaramuzzi RJ (2003) Heat stress and seasonal effects on reproduction in the dairy cow—a review. *Theriogenology* **60,** 1139-1151.

de Silva AWMV, Anderson GW, Gwazdauskas FC, McGilliard ML, Lineweaver JA (1981) Interrelationships with estrous behavior and conception in dairy cattle. *Journal of Dairy Science* **64,** 2409-2418.

Dearing J, Hilerton JE, Poelarends JJ, Neijenhuis F, Sampimon OC, Fossing C (2004) Effects of automatic milking on body condition score and fertility of dairy cows. In 'A better understanding automatic milking.' (Eds. A Meijering, H Hogeveen, CJAM de Koning) pp. 135–140. (Wageningen Academic Publishers: Wageningen, The Netherlands)

Dennery PA (2004) Role of redox in fetal development and neonatal diseases. *Antioxidants and Redox Signaling* **6,** 147-153.

Dennery PA (2007) Effects of oxidative stress on embryonic development. *Birth Defects Research Part C: Embryo Today: Reviews* **81,** 155-162.

Devasagayam TP, Sivabalan R, Tarachand U (1990) Lipid peroxidation in the rat uterus during deciduoma induced cell differentiation. *Biochemistry International* **21,** 27-32.

Di Trana A, Celi P, Claps S, Fedele V, Rubino R (2006) The effect of hot season and nutrition on the oxidative status and metabolic profile in dairy goats during mid lactation. *Animal Science* **82,** 717-722.

Diskin MG, Mackey DR, Roche JF, Sreenan JM (2003) Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Animal Reproduction Science* **78,** 345-370.

Diskin MG, Sreenan JM (2000) Expression and detection of oestrus in cattle. *Reproduction, Nutrition, Development* **40,** 481-491.

Dobson H, Walker SL, Morris MJ, Routly JE, Smith RF (2008) Why is it getting more difficult to successfully artificially inseminate cows? *Animal* **2,** 1104–1111.

Dransfield MBG, Nebel RL, Pearson RE, Warnick LD (1998) Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. *Journal of Dairy Science* **81,** 1874-1882.

Eddy R (1980) Analysing dairy herd fertility. *In Practice* **2,** 25-30.

Eicher SD, Cheng HW, Sorrells AD, Schutz MM (2006) Short Communication: Behavioral and physiological indicators of sensitivity or chronic pain following tail docking. *Journal of Dairy Science* **89,** 3047-3051.

El Mouatassim S, Guérin P, Ménézo Y (1999) Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. *Molecular Human Reproduction* **5,** 720-725.

Espey Ll (1980) Ovulation as an inflammatory reaction—a hypothesis. *Biology of Reproduction* **22,** 73-106.

Esslemont RJ (1992) Measuring dairy herd fertility. *Veterinary Record* **131,** 209-212.

Ferguson JD (1996) Diet, production and reproduction in dairy cows. *Animal Feed Science and Technology* **59,** 173-184.

Ferguson JD, Galligan DT (2000) Assessment of reproductive efficiency in dairy herds. *Compendium on Continuing Education for the Practicing Veterinarian* **22,** 150-159.

Fetrow J, McClary D, Harman R, Butcher K, Weaver L, Studer E, Ehrlich J, Etherington W, Guterbock W, Klingborg D, Reneau J, Williamson N (1990) Calculating

selected reproductive indices: Recommendations of the American association of bovine practitioners. *Journal of Dairy Science* **73,** 78-90.

Figueiredo T, Dzyekanski B, Pimpão CT, Silveira AB, Capriglione LG, Michelotto Jr, Vicente P (2013) Use of infrared thermography to detect intrasynovial injections in horses. *Journal of Equine Veterinary Science* **33,** 257-260.

Firk R, Stamer E, Junge W, Krieter J (2002) Automation of oestrus detection in dairy cows: A review. *Livestock Production Science* **75,** 219-232.

Fisher AD, Morton R, Dempsey JMA, Henshall JM, Hill JR (2008) Evaluation of a new approach for the estimation of the time of the LH surge in dairy cows using vaginal temperature and electrodeless conductivity measurements. *Theriogenology* **70,** 1065-1074.

Forde N, Beltman ME, Lonergan P, Diskin M, Roche JF, Crowe MA (2011) Oestrous cycles in *Bos taurus* cattle. *Animal Reproduction Science* **124,** 163-169.

Fordham DP, Rowlinson P, McCarthy TT (1988) Oestrus detection in dairy cows by milk temperature measurement. *Research Veterinary Science* **44,** 366-374.

Fox PF, Kelly AL (2006) Indigenous enzymes in milk: Overview and historical aspects—Part 1. *International Dairy Journal* **16,** 500-516.

Freeman BA, Crapo JD (1982) Biology of disease: free radicals and tissue injury. *Laboratory investigation* **47,** 412-426.

Friggens NC, Chagunda MGG (2005) Prediction of the reproductive status of cattle on the basis of milk progesterone measures: Model description. *Theriogenology* **64,** 155-190.

Fujii J, Iuchi Y, Okada F (2005) Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reproductive Biology and Endocrinology* **3,** 1-10.

Gardiner CS, Reed DJ (1995) Glutathione redox cycle-driven recovery of reduced glutathione after oxidation by tertiary-butyl hydroperoxide in preimplantation mouse embryos. *Archives of Biochemistry and Biophysics* **321,** 6-12.

Garrel C, Fowler PA, Al-Gubory KH (2010) Developmental changes in antioxidant enzymatic defences against oxidative stress in sheep placentomes. *Journal of Endocrinology* **205,** 107-116.

George WD, Godfrey RW, Ketring RC, Vinson MC, Willard ST (2014) Relationship among eye and muzzle temperatures measured using digital infrared thermal imaging and vaginal and rectal temperatures in hair sheep and cattle. *Journal of Animal Science* **92,** 4949-4955.

Ghiselli A, Serafini M, Maiani G, Azzini E, Ferro-Luzzi A (1995) A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biology and Medicine* **18,** 29-36.

Gillis EH, Gosling JP, Sreenan JM, Kane M (2002) Development and validation of a biosensor-based immunoassay for progesterone in bovine milk. *Journal of Immunological Methods* **267,** 131-138.

Gloster J, Ebert K, Gubbins S, Bashiruddin J, Paton D (2011) Normal variation in thermal radiated temperature in cattle: implications for foot-and-mouth disease detection. *BMC Veterinary Research* **7,** 1-10.

González-Fernández R, Gaytán F, Martínez-Galisteo E, Porras P, Padilla CA, Sánchez Criado JE, Bárcena JA (2005) Expression of glutaredoxin (thioltransferase) in the rat ovary during the oestrous cycle and postnatal development. *Journal of Molecular Endocrinology* **34,** 625-635.

Guérin P, El Mouatassim S, Ménézo Y (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update* **7,** 175-189.

Hafez ESE, Jainudeen MR, Rosnina Y (2000) Hormones, growth factors and reproduction, In 'Reproduction in farm animals.' (Eds ESE Hafez and B Hafez) pp. 33–54. (Lippincott Williams and Wilkins: Philadelphia)

Hansen PJ, Soto P, Natzke RP (2004) Mastitis and fertility in cattle – possible involvement of inflammation or immune activation in embryonic mortality. *American Journal of Reproductive Immunology* **51,** 294-301.

Harris B (2005) Multiple trail fertility model for the national genetic evaluation. *Livestock Improvement Corporation* 1–24.

Hellebrand HJ, Brehme U, Beuche H, Stollberg U, Jacobs H (2003) Application of thermal imaging for cattle management. In 'Proceddings of the First European conference on Precision Livestock Farming.' pp. 761-763. (Berlin, Germany)

Henry ET (1986) Dairy herd reproductive efficiency. In 'Current veterinary therapy, food animal practice.' (2nd edition, Ed. JL Howard) pp. 803-808. (WB Saunders, Philadelphia)

Hettinga KA, van Valenberg HJF, Lam TJGM, van Hooijdonk ACM (2008) Detection of mastitis pathogens by analysis of volatile bacterial metabolites. *Journal of Dairy Science* **91,** 3834-3839.

Ho YS, Gargano M, Cao J, Bronson RT, Heimler I, Hutz RJ (1998) Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *Journal of Biological Chemistry* **273,** 7765-7769.

Hockey CD, Morton JM, Norman ST, McGowan MR (2010*a*) Evaluation of a neck mounted 2-hourly activity meter system for detecting cows about to ovulate in two paddock-based australian dairy herds. *Reproduction in Domestic Animals* **45,** 107-117.

Hockey CD, Morton JM, Norman ST, McGowan MR (2010*b*) Improved prediction of ovulation time may increase pregnancy rates to artificial insemination in lactating dairy cattle. *Reproduction in Domestic Animals* **45,** 239-248.

Hoffmann G, Schmidt M, Ammon C, Rose-Meierhöfer S, Burfeind O, Heuwieser W, Berg W (2013) Monitoring the body temperature of cows and calves using video recordings from an infrared thermography camera. *Veterinary Research Communications* **37,** 91-99.

Hogeveen H, Kamphuis C, Steeneveld W, Mollenhorst H (2010) Sensors and clinical mastitis—The quest for the perfect alert. *Sensors* **10,** 7991-8009.

Holah GD (1995) Some practical aspects of thermography in competition horses. In 'The Thermal Image in Medicine and Biology.' (Eds K Ammer, EFJ Ring) pp. 156–163. (Uhlen-Verlag: Wien, Austria)

Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry* **53,** 1841-1856.

Huie RE, Padmaja S (1993) The reaction of NO with superoxide. *Free Radical Research* **18,** 195-199.

Hurnik JF, King GJ, Robertson HA (1975) Estrous and related behaviour in postpartum Holstein cows. *Applied Animal Ethology* **2,** 55-68.

Hurnik JF, Webster AB, DeBoer S (1985) An investigation of skin temperature differentials in relation to estrous in dairy cattle using a thermal infrared scanning technique. *Journal of Animal Science* **61,** 1095-1102.

InCalf (2007) InCalf project. Dairy Australia. Available at http://www.incalf.com.au [verified 2 December 2013]

Ireland JJ, Roche JF (1987) Hypotheses regarding development of dominant follicles during a bovine estrous cycle. In 'Follicular Growth and Ovulation Rate in Farm Animals.' (Eds JF Roche, NM Callaghan) pp. 1-18.

Jago JG, Davis KL, Jensen R (2007) Future innovative dairy technologies to address constraints of labour, information collection and decision-making on farms. In 'Australasian Dairy Science Symposium Conference Proceedings (Meeting the Challenges for Pasture-Based Dairying).' (Eds DF Chapman, DA Clark, KL Macmillan, DP Nation) pp. 493-507. (National Dairy Alliance: Australia)

Jauniaux E, Watson AL, Hempstock J, Bao Y-P, Skepper JN, Burton GJ (2000) Onset of maternal arterial blood flow and placental oxidative stress. *The American Journal of Pathology* **157,** 2111-2122.

Johnson SR, Rao S, Hussey SB, Morley PS, Traub-Dargatz JL (2011) Thermographic eye temperature as an index to body temperature in ponies. *Journal of Equine Veterinary Science* **31,** 63-66.

Jones M, Denson A, Bowers S, Moulton K, Williams E, Graves K, Dos Santos A, Kouba A, Willard S (2005) Thermography of the vulva in Holstein dairy cows: A comparison of estrus vs. diestrus. In 'ASAS/ADSA National Meeting.' (Cincinnati: Ohio, USA)

Kamphuis C, DelaRue B, Burke CR, Jago J (2012) Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. *Journal of Dairy Science* **95,** 3045-3056.

Kastberger G, Stachl R (2003) Infrared imaging technology and biological applications. *Behavior Research Methods* **35,** 429-439.

Kastelic JP, Cook RB, Coulter GH (1997) Contribution of the scrotum, testes, and testicular artery to scrotal/testicular thermoregulation in bulls at two ambient temperatures. *Animal Reproduction Science* **45,** 255-261.

Kastelic JP, Cook RB, Coulter GH, Wallins GL, Entz T (1996) Environmental factors affecting measurement of bovine scrotal surface temperature with infrared thermography. *Animal Reproduction Science* **41,** 153-159.

Kerbrat S, Disenhaus C (2004) A proposition for an updated behavioural characterisation of the oestrus period in dairy cows. *Applied Animal Behaviour Science* **87,** 223-238.

Knizkova I, Kunc P (2007) Applications of infrared thermography in animal production. *Journal of the Faculty of Agriculture* **22,** 329-336.

Kní žková Į Kunc P, Koubková M, Flusser J, Dolezal O (2002) Evaluation of naturally ventilated dairy barn management by a thermographic method. *Livestock Production Science* **77,** 349-353.

Knopf L, Kastelic JP, Schallenberger E, Ginther OJ (1989) Ovarian follicular dynamics in heifers: Test of two-wave hypothesis by ultrasonically monitoring individual follicles. *Domestic Animal Endocrinology* **6,** 111-119.

Kodaman PH, Behrman HR (2001) Endocrine-regulated and protein kinase C-dependent generation of superoxide by rat preovulatory follicles. *Endocrinology* **142,** 687-693.

Kolver ES, Macmillan KL (1994) Variation in selected blood plasma constituents during the post-partum and breeding periods in dairy cows. *New Zealand Veterinary Journal* **42,** 161-166.

Kornmatitsuk B, Chantaraprateep P, Kornmatitsuk S, Kindahl H (2008) Different types of postpartum luteal activity affected by the exposure of heat stress and subsequent reproductive performance in Holstein lactating cows. *Reproduction in Domestic Animals* **43,** 515-519.

Kruip TM, Stefanowska J, Ouweltjes W (2000) Robot milking and effect on reproduction in dairy cows: a preliminary study. *Animal Reproduction Science* **60,** 443-447.

Kunc P, Knizkova I (2012) The use of infrared thermography in livestock production and veterianry field. In 'Infrared Thermography: Recent Advances and Future Trends.' (Ed. C Meola) pp. 85-101. (Bentham Science: United Arab Emirates)

Kyle BL, Kennedy AD, Small JA (1998) Measurement of vaginal temperature by radiotelemetry for the prediction of estrus in beef cows. *Theriogenology* **49,** 1437-1449.

Laloraya M, Kumar GP, Laloraya MM (1989*a*) Histochemical study of superoxide dismutase in the ovary of the rat during the oestrous cycle. *Journal of Reproduction and Fertility* **86,** 583-587.

Laloraya M, Kumar GP, Laloraya MM (1991) Changes in the superoxide radical and superoxide dismutase levels in the uterus of *Rattus norvegicus* during the estrous cycle and a possible role for superoxide radical in uterine oedema and cell proliferation at proestrus. *Biochemistry and Cell Biology* **69,** 313-316.

Laloraya M, Pradeep kumrr G, Laloraya MM (1989*b*) A possible role of superoxide anion radical in the process of blastocyst implantation in *Mus musculus*. *Biochemical and Biophysical Research Communications* **161,** 762-770.

Lamming GE, Darwash AO (1998) The use of milk progesterone profiles to characterise components of subfertility in milked dairy cows. *Animal Reproduction Science* **52,** 175-190.

Lamprecht I, Maierhofer C, Röllig M (2007) Infrared thermography and thermometry of phototropic plants. *Journal of Thermal Analysis and Calorimetry* **87,** 49-54.

Lapointe J, Bilodeau J (2003) Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. *Biology of Reproduction* **68,** 1157-1164.

Lean IJ, Celi P, Raadsma H, McNamara J, Rabiee AR (2012) Effects of dietary crude protein on fertility: Meta-analysis and meta-regression. *Animal Feed Science and Technology* **171,** 31-42.

Leblanc S (2010) Assessing the association of the level of milk production with reproductive performance in dairy cattle. *Journal of Reproduction and Development* **56,** 1-7.

Lefcourt AM, Huntington JB, Akers RM, Wood DL, Bitman J (1999) Circadian and ultradian rhythms of body temperature and peripheral concentrations of insulin and nitrogen in lactating dairy cows. *Domestic Animal Endocrinology* **16,** 41-55.

Levine M, Morita K (1984) Ascorbic acid in endocrine systems. *Vitamins and Hormones* **42,** 1-64.

Lista G, Castoldi F, Compagnoni G, Maggioni C, Cornélissen G, Halberg F (2009) Neonatal and maternal concentrations of hydroxil radical and total antioxidant system: Protective role of placenta against fetal oxidative stress. *Neuro Endocrinology Letters* **31,** 319-324.

Löf E, Gustafsson H, Emanuelson U (2007) Associations between herd characteristics and reproductive efficiency in dairy herds. *Journal of Dairy Science* **90,** 4897-4907.

Lof E, Gustafsson H, Emanuelson U (2012) Evaluation of two dairy herd reproductive performance indicators that are adjusted for voluntary waiting period. *Acta Veterinaria Scandinavica* **54,** 1-11.

Lopez H, Satter LD, Wiltbank MC (2004) Relationship between level of milk production and estrous behavior of lactating dairy cows. *Animal Reproduction Science* **81,** 209-223.

López-Gatius F, Santolaria P, Mundet I, Yániz JL (2005) Walking activity at estrus and subsequent fertility in dairy cows. *Theriogenology* **63,** 1419-1429.

Louca A, Legates JE (1968) Production losses in dairy cattle due to days open. *Journal of Dairy Science* **51,** 573-583.

Loughmiller JA, Spire MF, Dritz SS, Fenwick BW, Hosni MH, Hogge SB (2001) Relationship between mean body surface temperature measured by use of infrared thermography and ambient temperature in clinically normal pigs and pigs inoculated with *Actinobacillus pleuropneumoniae*. *American Journal of Veterinary Research* **62,** 676-681.

Loughmiller JA, Spire MF, Tokach MD, Dritz SS, Nelssen JL, Goodband RD, Hogge SB (2005) An evaluation of differences in mean body surface temperature with infrared thermography in growing pigs fed different dietary energy intake and concentration. *Journal of Applied Animal Research* **28,** 73-80.

Love TJ, Linsted RD (1976) Theoretical basis for use of skin temperature as a plethysmographic indicator. *American Society of Mechanical Engineering* **6,** 1-4.

Lovendahl P, Chagunda MG (2010) On the use of physical activity monitoring for estrus detection in dairy cows. *Journal of Dairy Science* **93,** 249-259.

Lucy MC (2001) Reproductive physiology and management of high-yielding dairy cattle. In 'Proceedings of the New Zealand Society of Animal Production.' pp. 120-127. (New Zealand Society of Animal Production)

Lunstra DD, Coulter GH (1997) Relationship between scrotal infrared temperature patterns and natural-mating fertility in beef bulls. *Journal of Animal Science* **75,** 767-774.

Maatje K, Loeffler SH, Engel B (1997) Predicting optimal time of insemination in cows that show visual signs of estrus by estimating onset of estrus with pedometers. *Journal of Dairy Science* **80,** 1098-1105.

Macmillan KL, Watson JD (1975*a*) Factors influencing A.B. conception rates. V. Cow age and stage of oestrus at insemination. *New Zealand Journal of Experimental Agriculture* **3,** 29-35.

Macmillan KL, Watson JD (1975*b*) Fertility differences between groups of sires relative to the stage of oestrus at the time of insemination. *Animal Production Science* **21,** 243-249.

Malek A, Sager R, Schneider H (2001) Effect of hypoxia, oxidative stress and lipopolysaccharides on the release of prostaglandins and cytokines from human term placental explants. *Placenta* **22,** 45-50.

McArthur AJ, Easdon MP, Gregson K (1992) Milk temperature and detection of oestrus in dairy cattle. *Journal of Agricultural Engineering Research* **51,** 29-46.

McCafferty DJ (2007) The value of infrared thermography for research on mammals: Previous applications and future directions. *Mammal Review* **37,** 207-223.

McCafferty DJ, Moncrieff JB, Taylor IR, Boddie GF (1998) The use of IR thermography to measure the radiative temperature and heat loss of a barn owl (*Tyto alba*). *Journal of Thermal Biology* **23,** 311-318.

Meadows CVMD (2005*a*) 'Evaluation of the effects of a contract breeding program on reproductive efficiency in Ohio dairy herds.' PhD thesis, Ohio State University, USA.

Meadows CVMD (2005*b*) Reproductive record analysis. *Veterinary Clinics Food Animal Practice* **21,** 305–323.

Miller JK, Brzezinska-Slebodzinska E, Madsen FC (1993) Oxidative stress, antioxidants, and animal function. *Journal of Dairy Science* **76,** 2812-2823.

Miszkiel G, Skarzynski D, Bogacki M, Kotwica J (1999) Concentrations of catecholamines, ascorbic acid, progesterone and oxytocin in the corpora lutea of cyclic and pregnant cattle. *Reproduction Nutrition Development* **39,** 509-516.

Miyamoto K, Sato EF, Kasahara E, Jikumaru M, Hiramoto K, Tabata H, Katsuragi M, Odo S, Utsumi K, Inoue M (2010) Effect of oxidative stress during repeated

ovulation on the structure and functions of the ovary, oocytes, and their mitochondria. *Free Radical Biology and Medicine* **49,** 674-681.

Miyazaki T, Sueoka K, Dharmarajan AM, Atlas SJ, Bulkley GB, Wallach EE (1991) Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. *Journal of Reproduction and Fertility* **91,** 207-212.

Molfino J (2014) Impact of automatic milking systems (AMS) on labour and life style on commercial farms in Australia. In 'Proceedings of the Dairy Research Foundation Symposium.' pp. 105-110. (The University of Sydney: Camden, Australia)

Montanholi YR, Odongo NE, Swanson KC, Schenkel FS, McBride BW, Miller SP (2008) Application of infrared thermography as an indicator of heat and methane production and its use in the study of skin temperature in response to physiological events in dairy cattle (*Bos taurus*). *Journal of Thermal Biology* **33,** 468-475.

Montanholi YR, Swanson KC, Palme R, Schenkel FS, McBride BW, Lu D, Miller SP (2010) Assessing feed efficiency in beef steers through feeding behavior, infrared thermography and glucocorticoids. *Animal* **4,** 692-701.

Morton J (2004) 'Determinants of reproductive performance of dairy cows in commercial herds in Australia.' PhD thesis, The University of Melbourne, Australia.

Morton J (2011). 'Report on InCalf Fertility Data Project, 2011.' (Dairy Australia: Southbank, Vic.)

Mosher MD, Ottobre JS, Haibel GK, Zartman DL (1990) Estrual rise in body temperature in the bovine II. The temporal relationship with ovulation. *Animal Reproduction Science* **23,** 99-107.

Moss N, Lean IJ, Reid SWJ, Hodgson DR (2002*a*) The epidemiology of subfertility in non-seasonal calving dairy herds in the Camden region of New South Wales: Preliminary investigation of risk factors. *Australian Veterinary Journal* **80,** 432-436.

Moss N, Lean IJ, Reid SWJ, Hodgson DR (2002*b*) Risk factors for repeat-breeder syndrome in New South Wales dairy cows. *Preventive Veterinary Medicine* **54,** 91-103.

Mottram T, Rudnitskaya A, Legin A, Fitzpatrick JL, Eckersall PD (2007) Evaluation of a novel chemical sensor system to detect clinical mastitis in bovine milk. *Biosensors and Bioelectronics* **22,** 2689-2693.

Mottram T, Velasco-Garcia M, Berry P, Richards P, Ghesquiere J, Masson L (2002) Automatic on-line analysis of milk constituents (urea, ketones, enzymes and hormones) using biosensors. *Comparative Clinical Pathology* **11,** 50-58.

Myatt L (2010) Review: Reactive oxygen and nitrogen species and functional adaptation of the placenta. *Placenta* **31,** 66-69.

Myatt L, Cui X (2004) Oxidative stress in the placenta. *Histochemistry and Cell Biology* **122,** 369-382.

Ness RB, Cottreau C (1999) Possible role of ovarian epithelial inflammation in ovarian cancer. *Journal of the National Cancer Institute* **91,** 1459-1467.

Neves RCDF (2011) Investigation of automated activity monitoring systems for reproduction in dairy cattle. *The Faculty of Graduate Studies* 1-107.

Nikkhah A, Plaizier JC, Einarson MS, Berry RJ, Scott SL, Kennedy AD (2005) Short Communication: Infrared thermography and visual examination of hooves of dairy cows in two stages of lactation. *Journal of Dairy Science* **88,** 2749-2753.

Nordéus K, Båge R, Gustafsson H, Humblot P, Söderquist L (2012) The influence of oestrous substances on cyclicity and oestrous behaviour in dairy heifers. *Acta Veterinaria Scandinavica* **54,** 1-10.

Okamoto T, Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H (1997) Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. *Archives of Biochemistry and Biophysics* **342,** 261-274.

Ordolff D (2001) Introduction of electronics into milking technology. *Computers and Electronics in Agriculture* **30,** 125-149.

Orihuela A (2000) Some factors affecting the behavioural manifestation of oestrus in cattle: a review. *Applied Animal Behaviour Science* **70,** 1-16.

Palmar SE (1981) Use of the portable infrared thermometer as a means of measuring limb surface temperature in the horse. *American Journal of Veterinary Research* **42,** 105-108.

Palmer MA, Olmos G, Boyle LA, Mee JF (2010) Estrus detection and estrus characteristics in housed and pastured Holstein–Friesian cows. *Theriogenology* **74,** 255-264.

Palmer MA, Olmos G, Boyle LA, Mee JF (2012) A comparison of the estrous behavior of Holstein-Friesian cows when cubicle-housed and at pasture. *Theriogenology* **77,** 382-388.

Pastell M, Tiusanen J, Hakojärvi M, Hänninen L (2009) A wireless accelerometer system with wavelet analysis for assessing lameness in cattle. *Biosystems Engineering* **104,** 545-551.

Pedernera M, Celi P, García SC, Salvin HE, Barchia I, Fulkerson WJ (2010) Effect of diet, energy balance and milk production on oxidative stress in early-lactating dairy cows grazing pasture. *The Veterinary Journal* **186,** 352-357.

Perreault SD, Barbee RR, Slott VL (1988) Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. *Developmental Biology* **125,** 181-186.

Petersson KJ, Berglund B, Strandberg E, Gustafsson H, Flint APF, Woolliams JA, Royal MD (2007) Genetic analysis of postpartum measures of luteal activity in dairy cows. *Journal of Dairy Science* **90,** 427-434.

Phillips CJC, Schofield SA (1990) The effect of environment and stage of the oestrous cycle on the behaviour of dairy cows. *Applied Animal Behaviour Science* **27,** 21-31.

Piccione G, Caola G, Refinetti R (2003) Daily and estrous rhythmicity of body temperature in domestic cattle. *BioMed Central Physiology* **3,** 1- 8.

Pierce GB, Parchment RE, Lewellyn AL (1991) Hydrogen peroxide as a mediator of programmed cell death in the blastocyst. *Differentiation* **46,** 181-186.

Pierce JD, Cackler AB, Arnett MG (2004) Why should you care about free radicals? *Healthcare Traveler* **12,** 64-69.

Pinedo P, Risco C, Melendez P (2011) A retrospective study on the association between different lengths of the dry period and subclinical mastitis, milk yield, reproductive performance, and culling in Chilean dairy cows. *Journal of Dairy Science* **94,** 106-115.

Polat B, Colak A, Cengiz M, Yanmaz LE, Oral H, Bastan A, Kaya S, Hayirli A (2010) Sensitivity and specificity of infrared thermography in detection of subclinical mastitis in dairy cows. *Journal of Dairy Science* **93,** 3525-3532.

Prior RL, Cao G (2000) Analysis of botanicals and dietary supplements for antioxidant capacity: A review. *Journal of AOAC International* **83,** 950-956.

Przybylska J, Albera E, Kankofer M (2007) Antioxidants in bovine colostrum. *Reproduction in Domestic Animals* **42,** 402-409.

Purohit RC (1980) Thermography in the diagnosis of inflammatory processes in the horse. *American Journal of Veterinary Research* **41,** 1167-1174.

Qanungo S, Mukherjea M (2000) Ontogenic profile of some antioxidants and lipid peroxidation in human placental and fetal tissues. *Molecular and Cellular Biochemistry* **215,** 11-19.

Rae DO (2010) Bovine estrus: Tools for detection and understanding. In 'Factors Affecting Calf Crop: Biotechnology of reproduction.' (Eds FJ Michael , SS Robert, YV Joel) pp. 7-20.

Rainwater-Lovett K, Pacheco JM, Packer C, Rodriguez LL (2009) Detection of foot-and-mouth disease virus infected cattle using infrared thermography. *The Veterinary Journal* **180,** 317-324.

Raizman EA, Santos JEP (2002) The effect of left displacement of abomasum corrected by toggle-pin suture on lactation, reproduction, and health of Holstein dairy cows. *Journal of Dairy Science* **85,** 1157-1164.

Rajamahendran R, Robinson J, Desbottes S, Walton JS (1989) Temporal relationships among estrus, body temperature, milk yield, progesterone and luteinizing hormone levels, and ovulation in dairy cows. *Theriogenology* **31,** 1173-1182.

Rapoport R, Sklan D, Wolfenson D, Shaham-Albalancy A, Hanukoglu I (1998) Antioxidant capacity is correlated with steroidogenic status of the corpus luteum during the bovine estrous cycle. *Biochimica et Biophysica Acta* **1380,** 133-140.

Redden KD, Kennedy AD, Ingalls JR, Gilson TL (1993) Detection of estrus by radiotelemetric monitoring of vaginal and ear skin temperature and pedometer measurements of activity. *Journal of Dairy Science* **76,** 713-721.

Reilly PM, Schiller HJ, Bulkley GB (1991) Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. *The American Journal of Surgery* **161,** 488-503.

Reynaert NL, Ckless K, Wouters EF, van Der Vliet A, Janssen-Heininger YM (2005) Nitric oxide and redox signaling in allergic airway inflammation. *Antioxidants & redox signaling* **7,** 129-143.

Rizzo A, Minoia G, Trisolini C, Manca R, Sciorsci RL (2007) Concentrations of free radicals and beta-endorphins in repeat breeder cows. *Animal Reproduction Science* **100,** 257-263.

Rizzo A, Minoia G, Trisolini C, Mutinati M, Spedicato M, Jirillo F, Sciorsci RL (2009) Reactive Oxygen Species (ROS): Involvement in bovine follicular cysts etiopathogenesis. *Immunopharmacology and Immunotoxicology* **31,** 631-635.

Rizzo A, Mutinati M, Spedicato M, Minoia G, Trisolini C, Jirillo F, Sciorsci RL (2008) First demonstration of an increased serum level of reactive oxygen species during the peripartal period in the ewes. *Immunopharmacology and Immunotoxicology* **30,** 741-746.

Rizzo A, Roscino MT, Binetti F, Sciorsci RL (2012) Roles of reactive oxygen species in female reproduction. *Reproduction in Domestic Animals* **47,** 344-352.

Roche JR, Burke CR, Meier S, Walker CG (2011) Nutrition × reproduction interaction in pasture-based systems: is nutrition a factor in reproductive failure? *Animal Production Science* **51,** 1045–1066.

Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ, Berry DP (2009) Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. *Journal of Dairy Science* **92,** 5769-5801.

Roche JR, Macdonald KA, Burke CR, Lee JM, Berry DP (2007) Associations among body condition score, body weight, and reproductive performance in seasonal-calving dairy cattle. *Journal of Dairy Science* **90,** 376-391.

Rodenburg J (2014) Time for technology herd navigator in-line milk testing. Available at http://www.dairylogix.com [verified 2 December 2014]

Roelofs J, López-Gatius F, Hunter RHF, van Eerdenburg FJCM, Hanzen C (2010) When is a cow in estrus? Clinical and practical aspects. *Theriogenology* **74,** 327-344.

Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp B (2005*a*) Pedometer readings for estrous detection and as predictor for time of ovulation in dairy cattle. *Theriogenology* **64,** 1690-1703.

Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp B (2005*b*) Various behavioral signs of estrous and their relationship with time of ovulation in dairy cattle. *Theriogenology* **63,** 1366-1377.

Rossing W, Hogewerf PH (1997) State of the art of automatic milking systems. *Computers and Electronics in Agriculture* **17,** 1-17.

Royal MD, Pryce JE, Woolliams JA, Flint APF (2002) The genetic relationship between commencement of luteal activity and calving interval, body condition score, production, and linear type traits in Holstein-Friesian dairy cattle. *Journal of Dairy Science* **85,** 3071-3080.

Ruskovska T, Jansen EHJM, Antarorov R (2014) Evaluation of assays for measurement of serum (anti)oxidants in hemodialysis patients. *BioMed Research International* **2014, 1**-8.

Saint-Dizier M, Chastant-Maillard S (2011) Towards an automated detection of oestrus in dairy cattle. *Reproduction in Domestic Animals* **47,** 1056–1061.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC (2002) High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17[beta] in dairy cattle. *Journal of Dairy Science* **85,** 2831-2842.

Santos JEP, Rutigliano HM, Filho MFS (2009) Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. *Animal Reproduction Science* **110,** 207-221.

Sasaki J, Nomura T, Mori H, Watanabe S, Kanda S, Sato E, Inoue M, Watanabe H, Utsumi K (1994) Detection of manganese superoxide dismutase mRNA in the theca interna cells of rat ovary during the ovulatory process by in situ hybridization. *Histochemistry* **102,** 173-176.

Sato EF, Kobuchi H, Edashige K, Takahashi M, Yoshioka T, Utsumi K, Inoue M (1992) Dynamic aspects of ovarian superoxide dismutase isozymes during the ovulatory process in the rat. *FEBS Letters* **303,** 121-125.

Sawada M, Carlson JC (1994) Studies on the mechanism controlling generation of superoxide radical in luteinized rat ovaries during regression. *Endocrinology* **135,** 1645-1650.

Sawada M, Carlson JC (1996) Intracellular regulation of progesterone secretion by the superoxide radical in the rat corpus luteum. *Endocrinology* **137,** 1580-1584.

Schaefer AL, Cook N, Tessaro SV, Deregt D, Desroches G, Dubeski PL, Tong AKW, Godson DL (2004) Early detection and prediction of infection using infrared thermography. *Canadian Journal of Animal Science* **84,** 73-80.

Schaefer AL, Cook NJ, Bench C, Chabot JB, Colyn J, Liu T, Okine EK, Stewart M, Webster JR (2012) The non-invasive and automated detection of bovine respiratory

disease onset in receiver calves using infrared thermography. *Research in Veterinary Science* **93,** 928-935.

Schaefer AL, Jones SDM, Tong AKW, Vincent BC (1988) The effects of fasting and transportation on beef cattle. 1. Acid-base-electrolyte balance and infrared heat loss of beef cattle. *Livestock Production Science* **20,** 15-24.

Schlünsen D, Roth H, Schön H, Paul W, Speckmann H (1987) Automatic health and oestrus control in dairy husbandry through computer aided systems. *Journal of Agricultural Engineering Research* **38,** 263-279.

Schrier RW, Wang W (2004) Acute renal failure and sepsis. *New England Journal of Medicine* **351,** 159-169.

Schulman IH, Zhou M-S, Raij L (2005) Nitric oxide, angiotensin II, and reactive oxygen species in hypertension and atherogenesis. *Current Hypertension Reports* **7,** 61-67.

Schwartzkopf-Genswein KS, Stookey JM (1997) The use of infrared thermography to assess inflammation associated with hot-iron and freeze branding in cattle. *Canadian Journal of Animal Science* **77,** 577-583.

Scolari SC, Clark SG, Knox RV (2011) Vulvar skin temperature changes significantly during estrus in swine as determined by digital infrared thermography. *Journal of Swine Health and Production* **19,** 151-155.

Senger PL (1999) Pathways to pregnancy and parturition. pp. 1-368. (Current Conceptions Inc., Moscow, ID)

Shirai F, Kawaguchi M, Yutsudo M, Dohi Y (2002) Human peripheral blood polymorphonuclear leukocytes at the ovulatory period are in an activated state. *Molecular and Cellular Endocrinology* **196,** 21-28.

Shrestha HK, Nakao T, Higaki T, Suzuki T, Akita M (2004) Resumption of postpartum ovarian cyclicity in high-producing Holstein cows. *Theriogenology* **61,** 637-649.

Silanikove N, Merin U, Leitner G (2006) Physiological role of indigenous milk enzymes: an overview of an evolving picture. *International Dairy Journal* **16,** 533-545.

Simon EL, Gaughan EM, Epp T, Spire M (2006) Influence of exercise on thermographically determined surface temperatures of thoracic and pelvic limbs in horses. *Journal of the American Veterinary Medical Association* **229,** 1940-1944.

Stelletta C, Gianesella M, Vencato J, Fiore E, Morgante M (2012) Thermographic applications in veterinary medicine. In: 'Infrared thermography.' (Ed. RV Prakash) pp. 117-140. (InTech: China) Available at http://www.Intechopen.Com/books/infraredthermography/thermographic-applications-in-veterinary-medicine [verified 30 August 2013]

Stevenson JS, Hill SL, Nebel RL, DeJarnette JM (2014) Ovulation timing and conception risk after automated activity monitoring in lactating dairy cows. *Journal of Dairy Science* **97,** 4296-4308.

Stevenson MA, Lean IJ (1998) Descriptive epidemiological study on culling and deaths in eight dairy herds. *Australian Veterinary Journal* **76,** 482-488.

Stewart M, Webster JR, Stafford KJ, Schaefer AL, Verkerk GA (2010) Technical note: Effects of an epinephrine infusion on eye temperature and heart rate variability in bull calves. *Journal of Dairy Science* **93,** 5252-5257.

Stokes JE, Leach KA, Main DCJ, Whay HR (2012) An investigation into the use of infrared thermography (IRT) as a rapid diagnostic tool for foot lesions in dairy cattle. *The Veterinary Journal* **193,** 674-678.

Sugino N (2005) Reactive oxygen species in ovarian physiology. *Reproductive Medicine and Biology* **4,** 31-44.

Sugino N (2006) Roles of reactive oxygen species in the corpus luteum. *Animal Science Journal* **77,** 556-565.

Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H (2001) Reactive oxygen species stimulate prostaglandin F2α production in human endometrial stromal cells in vitro. *Human Reproduction* **16,** 1797-1801.

Sugino N, Shimamura K, Takiguchi S, Tamura H, Ono M, Nakata M, Nakamura Y, Ogino K, Uda T, Kato H (1996) Changes in activity of superoxide dismutase in the

human endometrium throughout the menstrual cycle and in early pregnancy. *Human Reproduction* **11,** 1073-1078.

Sugino N, Takiguchi S, Kashida S, Karube A, Nakamura Y, Kato H (2000) Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. *Molecular Human Reproduction* **6,** 19-25.

Sugino N, Telleria CM, Gibori G (1998) Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase in the rat corpus luteum: Induction of manganese superoxide dismutase messenger ribonucleic acid by inflammatory cytokines. *Biology of Reproduction* **59,** 208-215.

Suthar VS, Burfeind O, Patel JS, Dhami AJ, Heuwieser W (2011) Body temperature around induced estrus in dairy cows. *Journal of Dairy Science* **94,** 2368-2373.

Suzuki T, Sugino N, Fukaya T, Sugiyama S, Uda T, Takaya R, Yajima A, Sasano H (1999) Superoxide dismutase in normal cycling human ovaries: Immunohistochemical localization and characterization. *Fertility and Sterility* **72,** 720-726.

Sykes DJ, Couvillion JS, Cromiak A, Bowers S, Schenck E, Crenshaw M, Ryan PL (2012) The use of digital infrared thermal imaging to detect estrus in gilts. *Theriogenology* **78,** 147-152.

Szczepańska M, Koźlik J, Skrzypczak J, Mikołajczyk M (2003) Oxidative stress may be a piece in the endometriosis puzzle. *Fertility and Sterility* **79,** 1288-1293.

Talukder S, Celi P (2013) Use of digital infrared thermography and oxidative stress biomarkers as a diagnostic tool to diagnose interdigital dermatitis in sheep. *Journal of Dairy Science* **96,** E-Suppl. 1, p. 382.

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2014*a*) Factors affecting reproductive performance of dairy cows in a pasture-based, automatic milking system research farm: a retrospective, single-cohort study. *Animal Production Science* **55,** 31-41.

Talukder S, Ingenhoff L, Kerrisk KL, Celi P (2014*b*) Plasma oxidative stress biomarkers and progesterone profiles in a dairy cow diagnosed with an ovarian follicular cyst. *Veterinary Quarterly* **34,** 113-117.

Turner T, Purohit R, Fessler J (1986) Thermography: a review in equine medicine. *The Compendium on continuing education for the practicing veterinarian* **8,** 855-861.

Turner TA (1983) Thermographic evaluation of horses with podotrochlosis. *American Journal of Veterinary Research* **44,** 535-539.

Uchida K, Mandebvu P, Ballard CS, Sniffen CJ, Carter MP (2001) Effect of feeding a combination of zinc, manganese and copper amino acid complexes, and cobalt glucoheptonate on performance of early lactation high producing dairy cows. *Animal Feed Science and Technology* **93,** 193-203.

van Asseldonk MAPM, Huirne RBM, Dijkhuizen AA (1998) Quantifying characteristics of information-technology applications based on expert knowledge for detection of oestrus and mastitis in dairy cows. *Preventive Veterinary Medicine* **36,** 273-286.

van Eerdenburg FJ, Loeffler HS, van Vliet JH (1996) Detection of oestrus in dairy cows: a new approach to an old problem. *Veterinary Quarterly* **18,** 52–54.

van Hoogmoed L, Snyder JR, Allen AK, Waldsmith JD (2000) Use of infrared thermography to detect performance enhancing techniques in horses. *Equine Veterinary Education* **12,** 102-107.

van Langendonckt A, Casanas-Roux F, Donnez J (2001) Oxidative stress and peritoneal endometriosis. *Fertility and Sterility* **77,** 861-870.

van Lieshout EM, Knapen MF, Lange WP, Steegers EA, Peters WH (1998) Localization of glutathione S-transferases alpha and pi in human embryonic tissues at 8 weeks gestational age. *Human Reproduction* **13,** 1380-1386.

van Vliet JH, van Eerdenburg FJCM (1996) Sexual activities and oestrus detection in lactating Holstein cows. *Applied Animal Behaviour Science* **50,** 57-69.

Verkerk G (2003) Pasture-based dairying: Challenges and rewards for New Zealand producers. *Theriogenology* **59,** 553-561.

Verna M, Turner T, Anderson K (2004) Scintigraphic, radiographic, and thermographic appearance of the metacarpal and metatarsal regions of adult healthy horses treated with nonfocused extracorporeal shock wave therapy--a pilot study. *Veterinary therapeutics: Research in applied veterinary medicine* **6,** 268-276.

Walker WL, Nebel RL, McGilliard ML (1996) Time of ovulation relative to mounting activity in dairy cattle. *Journal of Dairy Science* **79,** 1555-1561.

Walsh S, Buckley F, Pierce K, Byrne N, Patton J, Dillon P (2008) Effects of breed and feeding system on milk production, body weight, body condition score, reproductive performance, and postpartum ovarian function. *Journal of Dairy Science* **91,** 4401-4413.

Walsh SW, Williams EJ, Evans ACO (2011) A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science* **123,** 127-138.

Walton JS, King GJ (1986) Indicators of estrus in Holstein cows housed in tie stalls. *Journal of Dairy Science* **69,** 2966-2973.

Warren AY, Matharoo-Ball B, Shaw RW, Khan RN (2005) Hydrogen peroxide and superoxide anion modulate pregnant human myometrial contractility. *Reproduction* **130,** 539-544.

Washburn SP, Silvia WJ, Brown CH, McDaniel BT, McAllister AJ (2002*a*) Trends in reproductive performance in Southeastern Holstein and Jersey DHI herds. *Journal of Dairy Science* **85,** 244-251.

Washburn SP, White SL, Green Jr JT, Benson GA (2002*b*) Reproduction, mastitis, and body condition of seasonally calved Holstein and Jersey cows in confinement or pasture systems. *Journal of Dairy Science* **85,** 105-111.

Weiss D, Reist M, Bruckmaier RM (2004) The acyclic period postpartum in automatic and conventional milking. *Journal of Veterinary Medicine Series A* **51,** 268-272.

Westwood CT, Lean IJ, Garvin JK, Wynn PC (2000) Effects of genetic merit and varying dietary protein degradability on lactating dairy cows. *Journal of Dairy Science* **83,** 2926-2940.

White S (2000) 'Investigation of pasture and confinement dairy feeding systems using Jersey and Holstein cattle.' MS thesis, North Carolina State University, Raleigh, NC.

Williamson NB, Morris RS, Anderson GA (1978) Pregnancy rates and non-return rates following artificial and natural breeding in dairy herds. *Australian Veterinary Journal* **54,** 111-114.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A (2006) Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology* **65,** 17-29.

Wong G, Goeddel DV (1988) Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science* **242,** 941-944.

Wrenn TR, Bitman J, Sykes JF (1958) Body temperature variations in dairy cattle during the estrous cycle and pregnancy. *Journal of Dairy Science* **41,** 1071-1076.

Zachara B, Wardak C, Didkowski W, Maciag A, Marchaluk E (1993) Changes in blood selenium and glutathione concentrations and glutathione peroxidase activity in human pregnancy. *Gynecologic and Obstetric Investigation* **35,** 12-17.

Zähner M (2004) The influence of climatic conditions on physiological and behavioural parameters in dairy cows kept in open stables. *Acta agriculturae Scandinavica. Section A, Animal science* **78,** 139-147.

CHAPTER 2

Factors Affecting Reproductive Performance of Dairy Cows in a Pasture-Based, Automatic Milking System Research Farm: A Retrospective Single Cohort Study

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OVERVIEW OF CHAPTER 2

Chapter 2 is a retrospective analysis to investigate the relationships between milk yield and milking frequency during early lactation on reproductive performance parameters based on historic data captured from Australia's first AMS research herd. In addition, the effect of a number of other factors (including management and husbandry techniques, cow factors, disease events, season of calving and insemination details) on the reproductive performance of the pasture based AMS research herd has been evaluated in this chapter.

ABSTRACT

A retrospective, single-cohort study was conducted to identify production and health factors associated with reproductive performance in a pasture-based, automatic milking system research farm. The calving system of this herd shifted from split calving to year-round calving gradually during the study period. Data from 365 cows with 798 lactations were analysed. Reproductive outcome variables of interest were intervals from calving to first oestrus, to first insemination, and to conception, as well as number of inseminations per conception, probability of submission for insemination by 80 days in milk, probability of conception by 100 days in milk and probability of conception at first insemination. Production factors (milk yield and its composition, milking frequency), record of periparturient disease, parity and season of calving were considered as predictor variables. The associations between predictor and outcome variables were assessed by multivariable linear regression, logistic regression, and survival analyses, for quantitative, binary and time-to-event outcomes, respectively. Average milk yield and milking frequency during 100 days in milk were not significantly associated with any of the reproductive measures. The likelihood of conception by 100 days in milk decreased gradually with year of automatic milking system commissioning. Cows calved in autumn were 43% (hazard ratio 1.43, $P < 0.05$) more likely to conceive than cows calved in summer. Multiparous cows were more likely ($P < 0.05$) to be recorded for oestrus than primiparous cows. Twinning was negatively associated with the reproductive outcomes measured in the automatic milking system research herd. Milk yield and milking frequency during 100 days in milk had no effect on reproductive measures in the pasture-based, automatic milking system research herd.

Keywords: reproduction, automatic milking system, pasture-based system.

INTRODUCTION

The introduction of automatic milking systems is one of the most significant technological changes in the dairy industry since the late 19th Century. In Europe, automatic milking systems have been commercially available since 1992. Since then, the number of automatic milking system units installed throughout the world has increased steadily. In late 1997, >100 farms were using automatic milking systems, and by early 2004, the number had increased to >2200 farms (de Koning and Rodenburg 2004). Currently, >10,000 farms globally are using automatic milking systems (de Koning 2011). Automatic milking systems are becoming increasingly popular due to the growing cost of labour, reduced labour availability and increased desire for improved lifestyle in many dairy countries (Rossing *et al.* 1985). More recently, the ability to manage the herd at an individual level through the improvement of real-time and individualised data has also likely influenced the uptake of this technology in some markets. Automatic milking systems are often described as a completely new way of farming because the voluntary cow traffic and resultant distribution of milkings throughout the day and night affects most aspects of herd and farm management. For example, cows managed with conventional milking systems are generally inspected for oestrus behaviour at the time of milking. Milking occurs largely without human intervention with automatic milking systems; therefore, traditional oestrus detection methods and/or routines require reconsideration.

In intensive-housing systems, several studies have been conducted to identify the factors influencing reproductive performance in automatic milking systems, but findings are inconclusive. Under research conditions with an individual feeding and management system in an automatic milking system (regardless of the milking frequency), significant association has been reported with longer interval from calving to first insemination but no effect on non-return rate at 56 days after first insemination (Kruip *et al.* 2002). Weiss *et al.* (2004) reported a neutral effect of milking system (automatic *v.* conventional) on the interval from calving to conception and resumption of ovarian cyclicity for cows kept in a naturally ventilated, loose housing barn. On the other hand, Dearing *et al.* (2004) monitored six farms in The Netherlands, from up to 6 months before automatic milking system installation through to 12 months post-installation, and showed an increase in interval from calving to conception as well as reduced conception rate. By

contrast, Löf *et al*. (2007) reported that automatic milking system herds in Sweden had shorter calving intervals and shorter intervals from calving to first insemination or to conception compared with conventional milking system herds. It is possible that the combined effect of individual cow performance, management policy (e.g. voluntary waiting period, the method of oestrus detection) and herd size are all factors contributing to the different findings.

We are not aware of any studies that have evaluated the reproductive performance of dairy cows in pasture-based, automatic milking system herds. Therefore, we conducted a retrospective, single-cohort study to investigate the effects of milk yield and milking frequency during early lactation on reproductive performance. In addition, we evaluated effects of several other factors (including management and husbandry techniques, cow factors, periparturient disease events, season of calving and insemination details) on the reproductive performance of the pasture-based, automatic milking system research herd.

MATERIALS AND METHODS

Farm and animal management

This retrospective, single-cohort study involved 365 cows with calving dates between 1 May 2006 and 30 April 2011, as mentioned in Talukder *et al*. (2012). Only cows that had at least one recorded oestrus or insemination event were included in the analyses (n = 798). The pasture-based, automatic milking system research herd (Elizabeth Macarthur Agricultural Institute, Camden, NSW, Australia) consisted of 82% (n = 660) Holstein-Friesian cows, 16% (n = 125) Illawarra, and 2% (n = 13) crossbreds. From May 2006 to March 2009, all milkings were conducted through two single-box milking units (VMS; DeLaval, Tumba, Sweden). In March 2009, a prototype robotic rotary (Automatic Milking Rotary™; DeLaval; Kolbach *et al*. 2013) was commissioned and milkings were conducted in a combination of single-box milking units and RR as the prototype equipment was co-developed and tested in a pasture-based system. From early February 2011, all milkings were conducted in the RR, and in May 2011, the single-box milking units were removed completely.

During the study period, cows were always subjected to a voluntary waiting period of 50 days in milk, during which time observed oestrus events were recorded but cows were

not inseminated. The primary oestrus detection method was visual behavioural observation, aided by the use of a secondary method, Kamar Heatmount Detectors (Kamar Products Inc., Zionsville, IN, USA). The Kamar Heatmount Detector was applied to the tail head of each cow at Day 45 of lactation and was monitored for colour changes (assumed to be caused by mounting behaviour). Cows that were not recorded for oestrus by 60 days in milk were drafted and checked by the veterinarian and a Kamar Heatmount Detector was reapplied if the first had been lost. Non-cyclers (cows that did not show oestrus signs during the voluntary waiting period) were checked by the veterinarian at 60–65 days in milk and synchronised according to the following protocol only if they were diagnosed with ovarian abnormalities (e.g. persistent corpus luteum or follicular cyst). Oestrus was synchronised with 1 mL (100 µg) intramuscular injection of gonadorelin acetate (Gonabreed®; Parnell Laboratories Pty Ltd, Alexandria, NSW) followed by 2 mL (500 µg) synthetic prostaglandin analogue, cloprostenol sodium (Estrumate®; Schering-Plough Animal Health Ltd, Baulkham Hills, NSW) 7 days later. An additional 1 mL Gonabreed injection was administered 48 h after the Estrumate injection, and fixed-time insemination was conducted 8–24 h later. Farm staff programmed the system to have cows in oestrus automatically drafted at the dairy; inseminations were conducted twice daily before cows were released back with the main herd. Pregnancy status was diagnosed by rectal palpation by veterinarians ~6 weeks after insemination. When cows were diagnosed not pregnant, they were injected with Estrumate and then monitored for oestrus signs as described above. If any cow was diagnosed pregnant to different services, rechecking was performed by a veterinarian to determine the actual date of insemination that resulted in pregnancy. During the study period, a transitional and gradual change in calving pattern was implemented by shifting from split to year-round calving. Animals were routinely vaccinated against clostridial diseases (Ultravac®; Pfizer Animal Health, West Ryde, NSW) and were regularly treated for parasites.

Data management

Individual cow records were maintained electronically on the automatic milking system or RR support software (VMSClient and DelPro; DeLaval). In addition to milk production and milking session records, the herd database included cow identification, date of birth, parity, breed, calving history, date and details of recorded oestrus events,

inseminations, dry-off events, periparturient problems (milk fever, retained foetal membrane, assisted calving), and all treatments. Data related to pregnancy diagnosis and any hormonal interventions were also recorded in the support software. Three lactations recorded with abortions and mummified foetuses were excluded from the final dataset.

Cumulative milk yield and average milking frequency from calving to 100 days in milk were calculated using the electronic data captured in the support software VMSClient. Average milking frequency from calving to 100 days in milk was calculated using the average daily number of milkings through the first 100 days in milk. Fortnightly herd tests (analysed milk samples) were conducted until January 2011. Herd test samples and production parameter data were submitted to Dairy Express (University of New England, Armidale, NSW). Milk fat and protein percentage data at 100 days in milk were obtained from the closest herd test event. If the date for 100 days in milk was <15 days from a record for milk solids content, the nearest date was considered. If the 100-days date was >15 and ≤20 days from a record, an average was obtained using the preceding and succeeding recorded data according to the procedure followed by Moss *et al*. (2002), and when the nearest sample result was >20 days before or after the 100 days date, the data point was considered as a missing value. All data were collated in an electronic spreadsheet (Microsoft Excel v12, 2007) to allow the outcome variables to be calculated.

Outcome variables

The key reproductive performance measurements of interest were intervals from calving to first oestrus, to first insemination, and to conception, as well as number of inseminations per conception, submission for insemination by 80 days in milk (yes/no), conception to first insemination (yes/no), and conception by 100 days in milk (yes/no). Probability of submission for insemination by 80 days in milk describes the proportion of cows that were inseminated by 80 days after calving, and probability of conception by 100 days in milk describes the proportion of cows that conceived by 100 days after calving (InCalf 2007).

Periods from calving to first oestrus, to first insemination, and to conception were measured in days on a continuous scale and number of inseminations per conception was measured as counts. Where a positive pregnancy diagnosis was recorded, the last

insemination date was considered as the day of conception; all other insemination dates were assumed to have resulted in no conception. Number of inseminations per conception and the interval from calving to conception were analysed only for the cows that were recorded with a positive pregnancy diagnosis.

Predictor variables

Predictor variables investigated in this study were milk yield, milking frequency, milk-fat percentage, and milk protein percentage during early lactation (0–100 days in milk). Additional variables assessed included season of calving and insemination, parity, breed, and periparturient diseases (e.g. assisted calving, retained foetal membrane, milk fever, stillborn and twin birth). The predictor variables are described in Table 1. Interval from calving to first oestrus was also tested as a predictor variable in the model to identify its effect on interval from calving to first insemination and to conception.

Statistical analyses

Among the 798 lactations investigated, cows had a positive pregnancy diagnosis for 86% (n = 686) of lactations and the dataset of those cows was used for analyses of interval from calving to conception, conception to first insemination, and number of inseminations per conception. Frequency tables were created for binary outcome variables. All of the continuous predictor and outcome variables were initially assessed using descriptive statistics. Continuous predictors were then tested for linear association with continuous outcome variables; if there was no linear effect, continuous predictors were then categorised into quartiles for easier interpretation. Continuous predictor variables (except the interval from calving to first interval) such as 100-days milk yield, 100-days milking frequency, 100-days milk-fat percentage and 100-days milk protein percentage were categorised into quartiles that ensured that each group had an approximate similar number of observations using statistical software,SAS which generated the category values as described in Table 1.

Linear and logistic regression analyses were performed for continuous and binary outcome variables, respectively. Initially, univariable analyses were conducted, and variables significant at a P-value 0.25 were selected for multivariable analyses following the procedure reported by Dhand (2009, 2010). Multivariable analyses were performed

to identify groups of predictor variables associated with outcome variables. Biologically relevant interactions were subsequently added to the model following forward stepwise selection procedure, and variables significant at $P < 0.05$ (based on the Wald's test for logistic regression and partial *P*-value for linear regression) were included in the model. Multivariable models were built using a manual stepwise approach, assisted by the MultiLogistic macro (Dhand 2009). At each step of the multivariable model, all of the variables were tested in the model by excluding them one at a time, whilst all other variables were tested for exclusion in the model by including them in the model one at a time. A *P*-value of 0.05 was used as a cut-off for both entry and exclusion. So, if any of the tested variables in the model was not significant, it was removed, and if one of the tested variables not in the model was significant, it was included.

Table 1. Descriptions and values of variables evaluated.

Name	Descriptions	Values
Year	Year of automatic milking system operation in which lactations started	Year 1, Year 2, Year 3, Year 4, Year 5[A]
Season of calving	Season in which cow calved	Summer, Autumn, Winter, Spring[B]
Season of first insemination	Season in which cow was first inseminated	Summer, Autumn, Winter, Spring[B]
Season of conception	Season in which cow conceived	Summer, Autumn, Winter, Spring[B]
100-day milk yield	Cumulative milk yield (kg) during first 100 days in milk as recorded by inline milk meters	≤ 1950, 1950 to 2521, 2522 to 3044, >3044
100-day milking frequency	Average daily number of milkings through first 100 days in milk	≤1.5, 1.6 to 1.9, 2.0 to 2.2, ≥2.3
100-day milk fat percent	Milk fat percent at first 100 days in milk	≤3.28, 3.29 to 3.64, 3.65 to 4.01, >4.01
100-day milk protein percent	Milk protein percent at first 100 days in milk	≤2.88, 2.89 to 3.04, 3.05 to 3.19, >3.19
Interval from calving to first oestrus	Interval (d) from date of calving to date of first detected oestrus	Continuous
Parity	Lactation number	1, 2, 3, 4, 5, ≥6
Twin	Record of twin calving	Yes, No
Assisted calving	Record of requiring any assistance during calving	Yes, No
Retained foetal membrane	Retention of placenta for at least 24 hours post- parturition	Yes, No
Still birth	Parturition of dead calf	Yes, No
Milk fever	Record of any clinical sign of milk fever within four weeks of parturition	Yes, No

[A]Year 1, Calving 1 May 2006–30 April 2007; Year 2, 1 May 2007–30 April 2008; Year 3, 1 May 2008–30 April 2009; Year 4, 1 May 2009–30 April 2010; Year 5, 1 May 2010–30 April 2011.

[B]Summer, December–February; autumn, March–May; winter, June–August; spring, September–November.

Only one measure of milk solids (either fat percentage or protein percentage separately) and milk yield were included in each multivariable model to avoid the problem of collinearity. Parity, calving season, milk yield, and milking frequency during the first 100 days of lactation were included in each final model to avoid any confounding effects of these factors according to Gröhn and Rajala-Schultz (2000) and to obtain effect estimates for those variables. Each final model was then tested by linear mixed model (PROC MIXED; SAS Institute Inc., Cary, NC, USA) for continuous outcome variables, and generalised linear mixed model (PROC GLIMMIX; SAS Institute Inc.) for binary outcome variables after adjusting for the clustering effect of cow. Assumptions were evaluated using residual diagnostics. Models have been refitted twice by including all current variables as in the final models but first excluding milk yield and then excluding milking frequency for all of the reproductive outcome variables evaluated in this study. Intervals from calving to first insemination had skewed distributions, so logarithmic transformation was performed for this variable before conducting the univariable analyses (available as Supplementary Material in the online version of this paper), and the means were back-transformed for presentation of results. The back-transformed means represent the geometric means.

The associations between the predictor variables and number of inseminations per conception were tested by Poisson regression using GENMOD followed by PROC GLIMMIX (SAS Institute Inc.) procedure after adjusting for clustering due to cow. Regression coefficient of each predictor was exponentiated for the interval from calving to first insemination and number of inseminations per conception to calculate ratios of geometric mean and counts, respectively. Times to reproductive events (i.e. interval from calving to first oestrus and to conception) were tested by survival analyses with Cox's proportional hazard model (PHREG procedure; SAS Institute Inc.) and adjusted for the clustering effect of cow. Hazard ratios (HR) and their confidence intervals (CI) are presented in the *Results*. Cows that were not recorded for at least one oestrus by 49 days in milk were considered as right-censored for the first oestrus event while cows that were not pregnant by 6 weeks before date of death or date of culling or 6 weeks before the last data entry date in the dataset were censored for the conception event.

RESULTS

Only cows that had at least one recorded oestrus or insemination event were included in the analyses (n = 798). We excluded the cows that had no records after calving as they either had died or were culled from the herd during the very early stages of lactation. We speculated that inclusion of cows with at least one oestrus or insemination event caused minimal selection bias.

Descriptive analyses

Descriptive statistics of the continuous outcome variables are presented in Table 2. For binary outcome variables, in 49% (n = 391) and 36% (n = 247) of lactations, cows were inseminated by 80 days in milk and were pregnant by 100 days in milk, respectively, while in 50% (n = 399) of lactations, cows conceived to the first insemination. Among the 798 lactations investigated, cows had a positive pregnancy diagnosis for 86% (n = 686) of lactations, and these lactations were used to analyse conception at first insemination, interval from calving to conception and number of inseminations per conception.

Interval from calving to first oestrus

Among the 798 lactations investigated, for 38% (n = 305) of lactations, the cow had at least one oestrus event by 49 days in milk. Of the 15 variables listed in Table 1, six (year, season of calving, parity, 100-days milk yield, 100-days milking frequency and retained foetal membrane) were shortlisted after univariable survival analyses ($P < 0.25$) and were tested by multivariable survival analyses. Year and parity were significant ($P < 0.05$) in the final model.

In the final model, 100-days milking frequency was not associated with the interval from calving to first oestrus event; however, cows with milking frequencies ≥2.3 times/day had a greater likelihood of being observed in oestrus by 49 days in milk than cows with lower milking frequencies (Table 3). The relative likelihood of recording the first oestrus event by 49 days in milk decreased gradually with the year of automatic milking system operation, with cows in year 5 being less (HR 0.32; $P < 0.001$) likely to be observed in oestrus by 49 days in milk than cows in year 1. In addition, primiparous cows had a lower rate of coming into oestrus than did their multiparous herd mates.

When the final model was fitted after excluding 100-days milking frequency, no significant association was observed between 100-days milk yield and the interval from calving to first oestrus. The final model was also fitted after excluding 100-days milk yield but no significant association was observed between 100-days milking frequency and the interval from calving to first oestrus.

Table 2. Descriptive statistics of continuous production and outcome variables for 798 lactations from 365 cows in a pasture-based, automatic milking system research herd from May 2006 to April 2011.

Variables	Mean	SD	Median	Q1	Q3
100-days milk yield, kg	2491	706.6	2523	1959	3049
100-days milking frequency	1.9	0.5	1.9	1.6	2.2
100-days milk fat percent	3.7	0.6	3.7	3.3	4.0
100-days milk protein percent	3.0	0.3	3.0	2.9	3.2
Estimated 305-days milk yield, kg	6093	1706	6200	4922	7281
Interval from calving to first oestrus, d	-	-	57	40	175
Interval from calving to first insemination, d	87	33	79	61	200
Interval from calving to conception, d	-	-	112	76	298
Number of inseminations per conception	1.9	1.2	1.0	1	6

Q1, lower quartile; Q3, upper quartile. In 38% (n = 305) of lactations, cows were recorded for at least one oestrus event by 49 days in milk and their data were used for the analyses of the interval calving–first oestrus. In 86% (n = 686) of lactations, cows had a positive pregnancy diagnosis and were used for the analyses of the interval calving–conception and number of inseminations per conception. For other variables, 798 lactations were used for descriptive statistics

Table 3. Factors affecting the interval from calving to first oestrus in dairy cows in a pasture-based, automatic milking system research herd based on Cox's proportional regression model analyses.

Predictor variables	Class	HR	95% CI for HR	*P*-value
Year	Year 1	1.00	–	<0.001
	Year 2	1.28	0.9–1.8	
	Year 3	0.87	0.6–1.3	
	Year 4	0.55	0.4–0.8	
	Year 5	0.32	0.2–0.5	
Parity	1	1.00	–	0.045
	2	1.24	0.8–2.0	
	3	1.62	1.0–2.6	
	4	1.40	0.8–2.4	
	5	2.21	1.3–3.7	
	≥6	1.22	0.7–2.0	
100-days milking frequency	≤1.5	1.00	–	0.120
	1.6–1.9	1.56	1.1–2.3	
	2.0–2.2	1.43	0.9–2.2	
	≥2.3	1.62	1.0–2.5	
100-days milk yield (kg)	≤1950	1.00	–	0.800
	1950–2521	0.86	0.6–1.3	
	2522–3044	0.92	0.6–1.5	
	>3044	0.81	0.5–1.3	

Covariates in the final model included season of calving. Results are presented as hazard ratio (HR), confidence interval (CI) and probability values. The HR is the ratio of the hazard rates corresponding to the event (e.g. oestrus) described by different levels of an explanatory variable, e.g. if a cow has a hazard ratio of 1.28 (Year 2), then she has a 28% increased likelihood of recorded oestrus compared with a cow in Year 1; the class where HR = 1 is the reference category

Interval from calving to first insemination

First insemination event was recorded for all of the lactations examined in this study. Of the 15 predictor variables (listed in Table 1) tested, 10 (year, season of calving, parity, season of first insemination, 100-days milking frequency, 100-days protein percentage, breed, retained foetal membrane, 100-days milk yield and assisted calving) were shortlisted after univariable linear regression analyses ($P < 0.25$) and were further tested by multivariable linear regression analyses. Season of calving and season of first insemination presented a significant ($P < 0.05$) effect on interval from calving to first insemination; however, no significant effect ($P = 0.09$) of their interaction was noted.

No significant association was observed between time to first insemination and milking frequency or milk yield during the first 100 days of lactation (Table 4). Interval from calving to first oestrus was significantly ($P < 0.001$) associated with the interval from calving to first insemination (Table 4). Cows that calved during autumn had the shortest (69 days) average interval from calving to first insemination (Table 4). The final model was fitted after excluding 100-day milking frequency, but no significant association ($P = 0.84$) was observed between 100-days milk yield and the interval from calving to first insemination. The final model was also fitted after excluding 100-days milk yield; however, no significant association was observed in cows between 100-days milking frequency and the interval from calving to first insemination.

Table 4. Factors affecting the interval (days) from calving to first insemination in dairy cows in a pasture-based, automatic milking system research herd based on linear mixed model analyses.

Step	Predictor variables	Class	*n*	Ratios of GM (95% CI)	LSM	s.e.m.	95% CI for LSM	*P*-value
1	Interval calving–first oestrus (days)	Continuous	–	–	0.0063[A]	0.0004[B]	0.006–0.007	<0.001
2	Season of calving	Summer	150	–	88.1[a]	3.0	82.3–94.2	<0.001
		Autumn	169	0.8 (0.7–0.8)	68.7[b]	2.1	64.7–73.1	
		Spring	101	1.0 (0.9–1.1)	83.1[a]	3.0	77.4–89.3	
		Winter	132	1.0 (0.9–1.1)	83.4[a]	3.0	77.7–89.5	
3	Season of insemination	Summer	90	–	76.3[b]	3.0	70.6–82.4	0.001
		Autumn	207	1.1 (1.0–1.2)	80.7[b]	2.3	76.3–85.3	
		Spring	129	1.0 (0.9–1.1)	74.9[b]	2.8	69.6–80.6	
		Winter	126	1.2 (1.1–1.4)	91.1[a]	3.1	85.2–97.4	
4	100-days milking frequency	≤1.5	136	–	80.6	2.3	76.3–85.1	0.600
		1.6–1.9	159	1.0 (0.9–1.1)	80.6	1.9	75.9–85.4	
		2.0–2.2	139	1.0 (1.0–1.1)	82.5	2.2	78.3–86.9	
		≥2.3	118	1.0 (0.9–1.1)	79.4	2.5	74.6–84.4	
5	100-days milk yield (kg)	≤1950	147	–	79.2	2.5	74.4–84.4	0.800
		1950–2521	139	1.0 (0.9–1.1)	79.8	2.2	75.7–84.2	
		2522–3044	133	1.0 (1.0–1.1)	80.7	2.1	76.6–84.9	
		>3044	138	1.1 (1.0–1.2)	82.3	2.3	77.9–86.9	

Covariates in the final model included parity. Results are presented as ratio of geometric means, least-squares means (LSM), standard error of mean (s.e.m.), confidence interval (CI) and probability values. Ratios of geometric means (GM) were obtained by exponentiation of the regression coefficients. Step refers to the step at which each variable was entered in the statistical model. Within a column and step, means followed by different letters are significantly different ($P \leq 0.05$). [A]Estimate. [B]Standard error.

Interval from calving to conception

Among the 798 lactations investigated, cows had a positive pregnancy diagnosis for 86% (n = 686) of lactations. Of the 15 predictor variables (Table 1) tested by univariable analysis, seven (twin, assisted calving, interval from calving to first oestrus, calving season, retained foetal membrane, milk fever and 100-days milk protein percentage) were shortlisted after univariable analyses ($P < 0.25$) and were further tested by multivariable survival analyses. Interval from calving to first oestrus and season of calving were significant ($P < 0.05$) in the final model.

No significant association was observed between time to conception and milking frequency or milk yield during the first 100 days of lactation (Table 5). Season of calving played a significant role in influencing the likelihood of conception. Conceptions occurred at a 43% greater rate in autumn than in summer, at any point in time ($P < 0.05$). For each additional day between calving and first oestrus, the rate of conception reduced by 1%. The final model was refitted after including all of the significant predictors and excluding 100-days milking frequency. No significant association ($P = 0.71$) was noted between 100-days milk yield and the interval from calving to conception. The final model was also fitted after excluding 100-days milk yield; however, no significant association was observed.

Table 5. Factors affecting the interval from calving to conception in dairy cows in a pasture-based, automatic milking system research herd based on Cox's proportional regression model analyses.

Predictor variables	Class	HR	95% CI for HR	*P*-value
Interval calving–first oestrus (days)	Continuous	0.99	0.991–0.996	<0.001
Season of calving	Summer	1.00	–	0.003
	Autumn	1.43	1.2–1.8	
	Spring	1.11	0.9–1.4	
	Winter	1.00	0.8–1.3	
100-days milking frequency	≤1.5	1.00	–	0.640
	1.6–1.9	0.95	0.8–1.2	
	2.0–2.2	0.85	0.6–1.1	
	≥2.3	0.91	0.7–1.2	
100-days milk yield (kg)	≤1950	1.00	–	0.650
	1950–2521	1.19	0.9–1.6	
	2522–3044	1.13	0.8–1.6	
	>3044	1.16	0.8–1.7	

Covariates in the final model included parity. Results are presented as hazard ratio (HR), confidence interval (CI) and probability values. The HR is the ratio of the hazard rates corresponding to the event (e.g. conception) described by different levels of an explanatory variable, e.g. if a cow has a hazard ratio of 1.43 (calving season: autumn), then she had a 43% increased likelihood of conception compared with a cow calved in summer; the class where HR = 1 is the reference category

Number of inseminations per conception

Among the 798 lactations investigated, 686 (86%) of 'cow lactations' had a positive pregnancy diagnosis, and the dataset of those cows was used for analysis of number of inseminations per conception.

Of the 15 predictor variables (Table 1) initially tested by Poisson regression, seven (season of conception, twin, year, 100-days milk yield, retained foetal membrane, parity and milk fever) were shortlisted after univariable analyses ($P < 0.25$) and were further tested by multivariable analysis. Season of conception and twin birth ($P < 0.05$) but not their interaction had a significant effect ($P = 0.60$) on the number of inseminations per conception.

Milking frequency or milk yield during 100 days in milk was not significantly associated with number of inseminations per conception (Table 6). Cows that were inseminated during autumn required significantly less ($P < 0.05$) inseminations per conception than cows that were inseminated during other seasons. Cows that gave birth to twins required more ($P < 0.05$) inseminations per conception than cows that gave birth to a singleton (Table 6). No significant association was noted between 100-days milk yield and number of inseminations per conception after excluding 100-days milking frequency before fitting the final model. The final model was also fitted after excluding 100-days milk yield; however, no significant association was observed in cows between 100-days milking frequency and the number of inseminations per conception.

Probability of submission for insemination by 80 days in milk

Initially predictor variables (listed in Table 1, except the interval from calving to first oestrus) were tested in univariable analyses, and of these, eight variables (season of calving, year, parity, season of insemination, 100-days milk yield, assisted calving, 100-days milking frequency and breed) significant at $P < 0.25$ were tested by multivariable survival analyses. Two variables (season of calving and season of first insemination) were significant ($P < 0.05$) but the interaction of these two variables was not significant ($P = 0.99$).

Neither 100-days milk yield nor milking frequency was significantly associated with the probability of submission for insemination by 80 days in milk (Table 7). Cows that calved and were inseminated during autumn were more likely (odds ratio 5.59 and 1.81, respectively) to be inseminated by 80 days in milk than those that calved or were inseminated in summer (Table 7). No significant association ($P = 0.49$) was observed between 100-days milk yield and probability of submission for insemination by 80 days in milk. The final model was also fitted after excluding 100-days milk yield; however, no significant association ($P = 0.33$) was observed.

Table 6. Factors affecting the number of inseminations per conception in dairy cows in a pasture-based automatic milking system research herd based on Poisson regression analyses.

Step	Predictor variables	Class	*n*	Ratios of counts (95% CI)	LSM	s.e.m.	95% CI for LSM	*P*-value
1	Season of conception	Summer	86	–	2.5[b]	0.3	2.0–3.0	0.006
		Autumn	187	0.8 (0.6–0.9)	1.9[a]	0.2	1.6–2.2	
		Spring	149	0.9 (0.8–1.1)	2.3[b]	0.2	1.9–2.7	
		Winter	211	1.0 (0.8–1.2)	2.4[b]	0.2	2.0–2.8	
2	Twin	Yes	22	1.4 (1.0–1.8)	2.6	0.3	2.0–3.4	0.028
		No	611	–	1.9	0.1	1.8–2.0	
3	100-days milk yield (kg)	≤1950	154	–	2.2	0.2	1.8–2.6	0.059
		1950–2521	160	1.0 (0.8–1.1)	2.1	0.2	1.7–2.5	
		2522–3044	163	1.2 (1.0–1.4)	2.5	0.2	2.1–3.0	
		>3044	156	1.0 (0.8–1.3)	2.2	0.2	1.8–2.6	
4	100-days milking frequency	≤1.5	144	–	2.2	0.2	1.8–2.6	0.700
		1.6–1.9	185	1.1 (0.9–1.3)	2.3	0.2	2.0–2.7	
		2.0–2.2	162	1.0 (0.9–1.3)	2.3	0.2	1.9–2.7	
		≥2.3	142	1.0 (0.8–1.2)	2.1	0.2	1.7–2.6	

Covariates in the final model included parity, season of calving. Results are presented as the ratio of counts, least-squares means (LSM), standard error of mean (s.e.m.), confidence interval (CI) and probability values. Ratios were obtained by exponentiation of the regression coefficients. Step refers to the step at which each variable was entered in the statistical model. Within a column and step, means followed by different letters are significantly different ($P \leq 0.05$)

Table 7. Factors affecting submission by 80 days in milk in dairy cows in a pasture-based, automatic milking system research herd based on logistic mixed model analyses.

Step	Predictor variables	Class	*n*	OR	95% CI for OR	*P*-value
1	Season of calving	Summer	171	1.00	–	<0.001
		Autumn	198	5.59	3.1–10.1	
		Spring	114	1.75	0.9–3.6	
		Winter	151	2.48	1.1–5.4	
2	Season of first insemination	Summer	97	1.00	–	0.001
		Autumn	240	1.81	0.9–3.8	
		Spring	160	0.93	0.5–1.9	
		Winter	137	0.45	0.2–1.0	
		No	609	2.98	1.1–8.0	
3	100-days milk yield (kg)	≤1950	155	1.00	–	0.440
		1950–2521	160	0.84	0.5–1.5	
		2522–3044	163	1.16	0.6–2.1	
		>3044	156	0.79	0.4–1.6	
4	100-days milking frequency	≤1.5	144	1.00	–	0.330
		1.6–1.9	185	1.46	0.9–2.5	
		2.0–2.2	162	0.99	0.6–1.8	
		≥2.3	143	1.25	0.7–2.4	

Covariates in the final model included parity. Results are presented as the odds ratio (OR), confidence interval (CI) and probability values. Step refers to the step at which each variable was entered in the statistical model. The class where OR = 1 is the reference category

Probability of conception by 100 days in milk

Of the 15 predictor variables (Table 1, except the interval from calving to first oestrus) tested by univariable analyses, eight (year, season of calving, season of conception, season of insemination, parity, twin, retained foetal membrane and milk fever) were shortlisted after univariable logistic regression analyses ($P < 0.25$) and were tested by multivariable analyses. Year and season of calving had a significant ($P < 0.05$) effect on the probability of conception by 100 days in milk. Year and season of calving were tested for the interactions but no significant ($P < 0.22$) interaction was observed.

No significant association was observed between the probability of conception by 100 days in milk and milking frequency or milk yield during 100 days in milk (Table 8). Probability of conception by 100 days in milk decreased gradually with the time of automatic milking system operation ($P < 0.05$). Compared with the summer calving season, calving in autumn increased (odds ratio 2.15) the likelihood of a cow being in calf by 100 days in milk (Table 8). The final model was fitted after excluding 100-days milking frequency; however, no significant association was noted between 100-days milk yield and conception by 100 days in milk. The final model was also fitted after excluding 100-days milk yield; however, no significant association was observed for cows in different quartiles of 100-days milking frequency and conception by 100 days in milk.

Neither milk yield nor milking frequency from calving to 100 days in milk was significantly associated with the probability of conception at first insemination. The outcome of the univariable analysis indicated that year of automatic milking system commissioning, parity and retained foetal membrane were significant at $P < 0.25$; however, no significant association of these variables was noted with the probability of conception at first insemination.

Table 8. Factors affecting conception by 100 days in milk in dairy cows in a pasture-based automatic milking system research herd based on logistic mixed model analyses.

Step	Predictor variables	Class	n	OR	95% CI for OR	*P*-value
1	Year	Year 1	117	1.00	–	0.001
		Year 2	122	0.72	0.4–1.3	
		Year 3	109	0.63	0.4–1.1	
		Year 4	131	0.33	0.2–0.6	
		Year 5	155	0.41	0.2–0.7	
2	Season of calving	Summer	171	1.00	–	0.005
		Autumn	198	2.15	1.4–3.4	
		Spring	114	1.05	1.6–1.8	
		Winter	151	1.40	0.8–2.3	
3	100-days milking frequency	≤1.5	144	1.00	–	0.180
		1.6–1.9	185	1.15	0.7–1.9	
		2.0–2.2	162	0.92	0.5–1.6	
		≥2.3	143	1.61	0.8–3.0	
4	100-days milk yield (kg)	≤1950	155	1.00	–	0.670
		1950–2521	160	0.84	0.5–1.4	
		2522–3044	163	0.73	0.4–1.3	
		>3044	156	0.67	0.3–1.3	

Covariates in the final model included parity. Results are presented as the odds ratio (OR), confidence interval and probability values. Step refers to the step at which each variable was entered in the statistical model. The class where OR = 1 is the reference category

DISCUSSION

Descriptive statistics

The average intervals from calving to first insemination and calving to conception in our study (87 and 128 days, respectively) are longer than the findings of other Australian researchers, who reported corresponding intervals of 76 and 101 days (Stevenson and Lean 1998) and 83 and 118 days (Moss *et al.* 2002), respectively. In the current study, submission for insemination by 80 days in milk and conception rate by 100 days in milk

(36% and 49%, respectively) were lower than the industry benchmarks (58% and 73%, respectively; InCalf 2007). Morton (2004) analysed the data of Australian dairy herds during 1996 and 1997 and reported a 66% submission rate for insemination by 80 days in milk and a 53% pregnancy rate by 100 days in milk. However, the declining trend of reproductive performance during the last 10 years (Morton 2011) supports and could explain, at least in part, the relative reduction of the reproductive outcomes in the current study.

Milking frequency

No significant influence of 100-day milking frequency on reproductive measures was observed in this study; however, the probability of first oestrus being recorded by 49 days in milk ($P = 0.13$) and conception by 100 days in milk ($P = 0.18$) tended to be highest for cows milked at least 2.3 times/day, on average, compared with cows milked <1.5 times/day. The association between milking frequency during early lactation and reproductive outcome is a matter of debate. In a conventional milking system, McNamara *et al*. (2008) observed no association between milking frequency and reproductive performance. Increased milking frequency in automatic milking systems did not cause a delay in resumption in ovarian cyclicity, nor was it related to an increase in interval from calving to conception (Weiss *et al*. 2004). However, Patton *et al*. (2006) observed a shorter interval from calving to first oestrus for cows milked once a day compared with cows milked three times per day. Similarly Blevins *et al*. (2006) reported an increase in days from calving to first oestrus in cows milked four times per day compared with cows milked twice per day. It has been reported that cows milking themselves in automatic milking systems (regardless of the milking frequency) had an increase in the interval from calving to first insemination compared with the cows milked in conventional milking systems (Kruip *et al.* 2002). The low incidence of very high milking frequencies in our study may explain the lack of significant effect of milking frequency on reproductive performance; indeed, only 1% of cows averaged three or more milkings per day during the first 100 days in milk. The fact that higher milking frequencies tended to be associated with increased likelihood of first oestrus could be explained by the concept that these cows moved around the system voluntarily more frequently and may have gained access to more fresh allocations of feed, thereby reducing the duration of early-lactation, negative energy balance. It is also possible that

cows with higher milking frequencies were inadvertently observed by farm staff more frequently, which could have increased the chance of an oestrus event being detected.

Milk volume and milk solids

One of the primary objectives of the study was to determine the association between reproductive performance and milk yield. Milk yield was not significantly associated with any of the reproductive measures evaluated here. While managed in a conventional milking system, using the same herd for cows lactating from February 2005 until January 2006, Pedernera *et al*. (2008) also reported a non-significant relationship between milk yield and reproductive performance. This finding is consistent with other studies indicating a neutral effect of milk production on the interval from calving to conception in Holstein cows in the United States (Gröhn and Rajala-Schultz 2000) and on sub-fertility in Australian dairy herds (Moss *et al.* 2002).

Discrepancies exist in literature explaining the effect of milk yield on fertility. Previous studies have reported negative (Mann *et al.* 2005; Ranasinghe *et al.* 2010) and positive (López-Gatius *et al.* 2006) effects of milk production on fertility. However, the relationship between milk yield and reproductive performance varies at the herd level as well as the individual animal level (Löf *et al.* 2007). For example, Windig *et al*. (2005) reported that herds with high average milk yield had shorter calving–first artificial insemination (AI) intervals, but within herds, cows with high production had longer calving–first AI intervals. Moreover, several factors such as negative energy balance, hormonal concentrations in blood, fertility of sire, accuracy of oestrus detection, and insemination technique are interrelated for fertility (Gröhn and Rajala-Schultz 2000; Lucy 2001) and obviously healthy cows producing more milk are inseminated sooner than are less healthy cows (Gröhn and Rajala-Schultz 2000). Thus, conception and first insemination are potentially influenced more by managerial decisions and individual animal disease history rather than milk production level *per se* (Eicker *et al.* 1996). Moreover, the association between milk yield and conception is difficult to interpret due to the confounding effect of culling (Gröhn and Rajala-Schultz 2000). Cows that do not conceive with repeated inseminations and have low milk yield are more prone to be culled (Gröhn and Rajala-Schultz 2000).

Whilst there was no consistent trend in the number of inseminations per conception for the different milk yield groups, cows with a 100-days milk yield in the range 1950–2521 kg had the lowest number of inseminations per conception ($P = 0.06$), whereas cows in the next production group (2522–3044 kg milk) had the highest number of inseminations per conception. One possible explanation for these differences could be the slightly higher (31 vs 27%) incidence of diagnosed mastitis before conception in the cows producing milk in the range 2522–3044 kg compared with cows producing milk in the range 1950–2521 kg during 100 days in milk.

Previous studies (Fahey *et al.* 2003; Haile-Mariam *et al*. 2003) have shown positive effects of milk protein on reproductive performance. Although 100-days milk protein percentage was associated with the interval from calving to first oestrus by the univariable analyses (data not shown), the final model indicated that neither milk protein nor fat percentage was significantly associated with any of the tested reproductive measures.

Time relative to automatic milking system commissioning

The probability of conception by 100 days in milk decreased gradually with the time of automatic milking system commissioning. Although the likelihood of first oestrus decreased steadily in relation to year of automatic milking system commissioning, the general lack of reported long-term reproductive performance data with automatic milking systems makes it challenging to interpret this result. It is possible that this is a farm-specific finding that could be explained by reduced culling on reproductive performance as the herd size was gradually increased and/or an increased acceptance of low-fertility cows as the herd progressively moved away from split calving to year-round calving. Although the herd was managed as a predominantly split-calving herd early in the study period, there were no months recorded when at least one cow was not inseminated. Certainly, without the inclusion of data from additional farms, there is no ability to conclude that automatic milking systems directly caused the trend. It is also possible that the disruption to the herd and farm-staff routines associated with the installation and testing of the RR during year 3 to year 5 influenced the effectiveness of oestrus detection during these years.

Year of automatic milking system commissioning was not associated with any changes in either interval from calving to first insemination or the likelihood of conception in the current study. However, an earlier study (Dearing *et al.* 2004) reported increased intervals from calving to conception and decreased conception rate during year 1 compared with the year before an automatic milking system was commissioned under research conditions. On the other hand, Löf *et al*. (2007) found that herds with automatic milking systems had shorter duration of calving to first insemination and conception compared with herds milked conventionally.

Season of calving

Number of days to first insemination was significantly shorter in autumn-calved cows than cows calved in other seasons, which is in agreement with data published by Hammoud *et al*. (2010). Those authors explained the positive influence of autumn calving on reproductive performance as via optimal environments and availability of fresh green pastures through the early lactation period (Hammoud *et al.* 2010). On the other hand, in a large scale retrospective study in Spain (López-Gatius 2003) over a 10-year period (1991–2000), average pregnancy rates to first AI were 44 and 27% for the winter and summer, respectively. This finding was explained by the preservation of fertility and reduced risk of reproductive disorders in winter compared to summer. Although cows are not typically classified as seasonal breeders, changes in photoperiodic stimulation (Dahl *et al.* 2000) associated with specific times of the year provide a potential explanation for such an effect of season on the reproductive measures (Santos *et al.* 2009). Alternatively, increased levels of pasture allocation and improved health condition explain (at least in part) such findings. Data relating to pasture allocation and daily herd feeding levels were not incorporated into this analysis.

Twin birth

Association of twin births with an increase in the number of inseminations required per conception in the current study is consistent with other reports (Nielen *et al*. 1989; Bicalho *et al.* 2007). Those authors reported that cows giving birth to twins were less (odds ratio 0.78) likely to conceive and 1.42 times more likely to be culled than dams with singleton births.

Parity

In our study, a significant relationship between calving to first oestrus and parity was observed. Numerous studies have reported negative (Darwash *et al.* 1997; Gröhn and Rajala-Schultz 2000; Moss *et al.* 2002), positive (Alawneh *et al.* 2011) or neutral (Horan *et al.* 2005) effect of parity on reproductive outcomes in conventional milking systems. Poorer reproductive performance in primiparous cows than multiparous cows can be explained by low pre-calving live weight in heifers (Morton 2004), which may cause increased postpartum an-ovulatory interval amongst Friesian heifers (Burke *et al.* 1995). In the present study, parity was included in each final model to avoid any confounding effects. It is possible that the effect reported in the present study was influenced by the nutritional status of the cows and it is possible that the regularity of access to fresh feed was highest for the fourth-parity cows and gradually reduced for both younger and older cows.

Interval from calving to first oestrus

The time to first insemination was influenced by time to first oestrus, in agreement with findings of Galvão *et al*. (2010) and Dubuc *et al*. (2012) who reported that cows cycling by 49 days in milk had an increased median time to first insemination compared with cows that started cycling after 49 days in milk. Abnormal resumption of postpartum ovarian cycles, reduced oestrus detection efficiency and decreased oestrus expression are all common causes for extended interval from calving to insemination (Muhammad *et al.* 2011). Washburn *et al*. (2002) reported an increase in interval from calving to first insemination (84–100 days), which was attributed to a decline in oestrus detection rate (51–42%).

We noted a significant relationship between increasing interval from calving to first oestrus and a reduced number of inseminations per conception on univariable analysis (data not presented). However, on multivariable analysis, the association was not significant. Previous studies have also reported the positive effect of longer interval to first oestrus on probability of pregnancy (Friggens and Labouriau 2010) and a reduction of the incidence of repeated inseminations in a conventional milking system (Moss *et al.* 2002), in agreement with our findings. Such a relationship could be the result of positive effects of an increased number of ovulatory cycles and more cyclic pattern of

reproductive hormones preceding insemination resulting inimproved uterine involution and other physiological changes associated with improved implantation (Butler and Smith 1989; Diskin *et al.* 2003). In addition, increased requirements for milk production during early lactation can lead to a state of negative energy balance which can have adverse effects on reproductive performance in dairy cows (Morton, 2004).

We did not find any significant association ($P < 0.05$) of the periparturient disorders retained foetal membrane, milk fever or stillborn with respect to any outcome variable in the final models, despite these being reportedly potential risk factors for reduced reproductive performance in other studies (Gröhn and Rajala-Schultz 2000; López-Gatius *et al.* 2006). The low incidence of these diseases in the dataset presented here is the most likely reason of the lack of significance.

Among the factors investigated, interval from calving to first oestrus, season of calving, year of automatic milking system commissioning and twin birth were the key factors influencing reproductive performance in the automatic milking system research herd. However, one of the challenges of working with historical data is that often the decision-making that sits behind the management is not captured, which creates some difficulties with data interpretation. It is likely that the dataset presented here was also affected by factors which included (but would not be limited to) shifting from split calving to year-round calving gradually during the study period and the targeted increase in herd size and associated reduced voluntary culling.

CONCLUSIONS

Overall, this study has provided an insight into the factors that have contributed to reproductive performance of dairy cows in a pasture-based, automatic milking system research farm. At the reported production levels and milking frequencies for Australian pastured cows, milk yield and milking frequency during 100 days in milk had no effect on reproductive measures. Interval from calving to first oestrus increased gradually within the study period and consequently influenced other reproductive outcomes. Besides this, season of calving, season of insemination, conception and twin birth all affected the reproductive performance in the automatic milking system research herd. With these results in mind, reviewing management strategies and initiating operating procedures to minimise the identified risk factors would be warranted. Effective oestrus

detection was identified as one of the most significant 'risk' factors impacting on reproductive performance in the dataset investigated here. The evaluation of new technologies is deemed appropriate to enable improved detection of oestrus in pasture-based systems which incorporate automatic milking and voluntary cow traffic.

REFERENCES

Alawneh JI, Laven RA, Stevenson MA (2011) The effect of lameness on the fertility of dairy cattle in a seasonally breeding pasture-based system. *Journal of Dairy Science* **94**, 5487–5493.

Bicalho RC, Cheong SH, Galvão KN, Warnick LD, Guard CL (2007) Effect of twin birth calvings on milk production, reproductive performance, and survival of lactating cows. *Journal of the American Veterinary Medical Association* **231**, 1390–1397.

Blevins CA, Shirley JE, Stevenson JS (2006) Milking frequency, estradiol cypionate, and somatotropin influence lactation and reproduction in dairy cows. *Journal of Dairy Science* **89**, 4176–4187.

Burke CR, Mcdougall S, Macmillan KL (1995) Effects of breed and calving liveweight on postpartum ovarian activity in pasture-fed dairy heifers. In 'Proceedings of the New Zealand Society of Animal Production.' pp. 76–78. (New Zealand Society of Animal Production)

Butler WR, Smith RD (1989) Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *Journal of Dairy Science* **72**, 767–783.

Dahl GE, Buchanan BA, Tucker HA (2000) Photoperiodic effects on dairy cattle: A review. *Journal of Dairy Science* **83**, 885–893.

Darwash AO, Lamming GE, Wooliams JA (1997) The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. *Animal Science* **65**, 9–16.

de Koning K (2011) Automatic milking: common practice on over 10,000 dairy farms worldwide. In 'Proceedings of the Dairy Research Foundation Symposium.' pp. 14–31. (The University of Sydney: Camden, NSW)

de Koning CJAM, Rodenburg J (2004) Automatic milking: State of the art in Europe and North America. In 'Automatic milking—A better understanding.' (Ed. A Meijering, H Hogeveen, C J A M de Koning) pp. 27–40. (Wageningen Academic Publishers: Wageningen, the Netherlands)

Dearing J, Hilerton JE, Poelarends JJ, Neijenhuis F, Sampimon OC, Fossing C (2004) Effects of automatic milking on body condition score and fertility of dairy cows. In 'A better understanding automatic milking.' (Eds A Meijering, H Hogeveen, C J A M de Koning) pp. 135–140. (Wageningen Academic Publishers: Wageningen, The Netherlands)

Dhand NK (2009) SAS macros for statistical modelling. Faculty of Veterinary Science, The University of Sydney. Available at http://sydney.edu.au/vetscience/biostat/macros/multi_about.shtml [verified 2 December 2013]

Dhand NK (2010) UniLogistic: A SAS macro for descriptive and univariable logistic regression analyses. *Journal of Statistical Software* **35**(1), 1–15.

Diskin MG, Mackey DR, Roche JF, Sreenan JM (2003) Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Animal Production Science* **78**, 345–370.

Dubuc J, Duffield TF, Leslie KE, Walton JS, Leblanc SJ (2012) Risk factors and effects of postpartum anovulation in dairy cows. *Journal of Dairy Science* 95, 1845–1854.

Eicker SW, Gröhn YT, Hertl JA (1996) The association between cumulative milk yield, days open, and days to first breeding in New York Holstein cows. *Journal of Dairy Science* **79**, 235–241.

Fahey J, Morton J, Macmillan KL (2003) Relationships between milk protein percentage and reproductive performance in Australian dairy cows. In 'Proceedings

of the New Zealand Society of Animal Production.' pp. 82–86. (New Zealand Society of Animal Production)

Friggens NC, Labouriau R (2010) Probability of pregnancy as affected by oestrus number and days to first oestrus in dairy cows of three breeds and parities. *Animal Reproduction Science* **118**, 155–162.

Galvão KN, Frajblat M, Butler WR, Brittin SB, Guard CL, Gilbert RO (2010) Effect of early postpartum ovulation on fertility in dairy cows. *Reproduction in Domestic Animals* **45**, 207–211.

Gröhn YT, Rajala-Schultz PJ (2000) Epidemiology of reproductive performance in dairy cows. *Animal Reproduction Science* **60–61**, 605–614.

Haile-Mariam M, Morton JM, Goddard ME (2003) Estimates of genetic parameters for fertility traits of Australian Holstein-Friesian cattle. *Animal Science* **76**, 35–42.

Hammoud MH, El-Zarkouny SZ, Oudah EZM (2010) Effect of sire, age at first calving, season and year of calving and parity on reproductive performance of Friesian cows under semiarid conditions in Egypt. *Archiva Zootechnica* **13**, 60–82.

Horan B, Mee JF, O'connor P, Rath M, Dillon P (2005) The effect of strain of Holstein-Friesian cow and feeding system on postpartum ovarian function, animal production and conception rate to first service. *Theriogenology* **63**, 950–971.

InCalf (2007) InCalf project. Dairy Australia. Available at http://www.incalf.com.au [verified 2 December 2013]

Kolbach R, Kerrisk KL, Garcia SC (2013) Short communication: The effect of pre milking with a teat cup-like device, in a novel robotic rotary, on attachment accuracy and milk removal. *Journal of Dairy Science* **96**, 360–365.

Kruip TM, Morice H, Robert M, Ouweltjes W (2002) Robotic milking and its effect on fertility and cell counts. *Journal of Dairy Science* **85**, 2576–2581.

Löf E, Gustafsson H, Emanuelson U (2007) Associations between herd characteristics and reproductive efficiency in dairy herds. *Journal of Dairy Science* **90**, 4897–4907.

López-Gatius F (2003) Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology* **60,** 89-99.

López-Gatius F, García-Ispierto I, Santolaria P, Yániz J, Nogareda C, López-Béjar M (2006) Screening for high fertility in high-producing dairy cows. *Theriogenology* **65**, 1678–1689.

Lucy MC (2001) Reproductive loss in high-producing dairy cattle: where will it end? *Journal of Dairy Science* **84**, 1277–1293.

Mann GE, Mann SJ, Blache D, Webb R (2005) Metabolic variables and plasma leptin concentrations in dairy cows exhibiting reproductive cycle abnormalities identified through milk progesterone monitoring during the post partum period. *Animal Reproduction Science* **88**, 191–202.

McNamara S, Murphy JJ, O'mara FP, Rath M, Mee JF (2008) Effect of milking frequency in early lactation on energy metabolism, milk production and reproductive performance of dairy cows. *Livestock Science* **117**, 70–78.

Morton J (2004) 'Determinants of reproductive performance of dairy cows in commercial herds in Australia.' PhD thesis, The University of Melbourne, Vic., Australia.

Morton J (2011) 'Report on InCalf Fertility Data Project, 2011.' (Dairy Australia: Southbank, Vic.)

Moss N, Lean IJ, Reid SWJ, Hodgson DR (2002) Risk factors for repeat-breeder syndrome in New South Wales dairy cows. *Preventive Veterinary Medicine* **54**, 91–103.

Muhammad Y, Toshihiko N, Chikako Y, Su TL, Gokarna G, Bimalka KR, Kana K, Aki H (2011) Days in milk at first AI in dairy cows; its effect on subsequent reproductive performance and some factors influencing it. *The Journal of Reproduction and Development* **57**, 643–649.

Nielen M, Schukken YH, Scholl DT, Wilbrink HJ, Brand A (1989) Twinning in dairy cattle: A study of risk factors and effects. *Theriogenology* **32**, 845–862.

Patton J, Kenny DA, Mee JF, O'mara FP, Wathes DC, Cook M, Murphy JJ (2006) Effect of milking frequency and diet on milk production, energy balance, and reproduction in dairy cows. *Journal of Dairy Science* **89**, 1478–1487.

Pedernera M, Garcia SC, Horagadoga A, Barchia I, Fulkerson WJ (2008) Energy balance and reproduction on dairy cows fed to achieve low or high milk production on a pasture-based system. *Journal of Dairy Science* **91**, 3896–3907.

Ranasinghe RMSBK, Nakao T, Yamada K, Koike K (2010) Silent ovulation, based on walking activity and milk progesterone concentrations, in Holstein cows housed in a free-stall barn. *Theriogenology* **73**, 942–949.

Rossing W, Ipema AH, Veltman PH (1985) 'The feasibility of milking in a feeding box.' IMAG Research Report 85-2. (Institute of Agricultural Engineers: Wageningen, The Netherlands)

Santos JEP, Rutigliano HM, Filho MFS (2009) Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. *Animal Reproduction Science* **110**, 207–221.

Stevenson MA, Lean IJ (1998) Descriptive epidemiological study on culling and deaths in eight dairy herds. *Australian Veterinary Journal* **76**, 482–488.

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2012) Reproductive performance of dairy cows in a pasture based Automatic Milking System research farm: a retrospective analysis. In 'Proceedings of the Fifth Australasian Dairy Science Symposium'. Melbourne, Vic. pp. 302–305. (Australasian Dairy Science Symposium)

Washburn SP, Silvia WJ, Brown CH, Mcdaniel BT, Mcallister AJ (2002) Trends in reproductive performance in Southeastern Holstein and Jersey DHI herds. *Journal of Dairy Science* **85**, 244–251.

Weiss D, Reist M, Bruckmaier RM (2004) The acyclic period postpartum in automatic and conventional milking. *Journal of Veterinary Medicine Series A* **51**, 268–272.

Windig JJ, Calus MPL, Veerkamp RF (2005) Influence of herd environment on health and fertility and their relationship with milk production. *Journal of Dairy Science* **88**, 335–347.

CHAPTER 3

Infrared Technology for Oestrus Detection and as a Predictor of Time of Ovulation in Dairy Cows in a Pasture-Based System

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OVERVIEW OF CHAPTER 3

In Chapter 2, it was observed that the interval from calving to first oestrus increased over time after the commissioning of the AMS operation. Milking occurs largely without human intervention with automatic milking systems; therefore, traditional oestrus detection methods and/or routines require reconsideration for AMS. The aim of this study was to evaluate the potential of a new technology, Infrared Thermography for oestrus detection and prediction of time of ovulation in oestrus synchronised dairy cows.

ABSTRACT

The development and application of an algorithm to assess the ability of an infrared thermography (IRT) device to predict cows in oestrus and about to ovulate was investigated. Twenty cows were synchronized using a controlled internal drug release (CIDR) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Vulval and muzzle temperatures were measured every 12 h from CIDR insertion to 32 hours after $PGF_{2\alpha}$ treatment and then every 4 hours until ovulation occurred or until 128 hours after $PGF_{2\alpha}$ treatment (whichever occurred first). Thermal images obtained with a FLIR T620 series infrared camera were analysed using ThermaCAM Researcher Professional 2.9 software. Cows were also monitored for behavioural signs of oestrus and colour changes of an Estrotect® applied to the tail head of each cow 36 hours after $PGF_{2\alpha}$ treatment. Algorithms were developed by adjusting body surface temperature of individual animals for ambient temperature and humidity during each observation period and were expressed as a deviation from the baseline temperature. Of the 20 cows enrolled in this study, 12 (60%) ovulated. An IRT oestrus alert was defined using different thresholds (Th; Th = 1°C, 1.25°C, and 1.5°C). Sensitivity and specificity to predict oestrus depended upon the chosen threshold level. At a threshold Th = 1°C, the highest sensitivity (92%, n = 11) and the lowest specificity (29%) and positive predictive value (64%) were observed. Conversely, Th = 1.5°C resulted in sensitivity of 75%, specificity of 57%, and positive predictive value of 69%. The mean ± standard deviation intervals between onset and end of IRT oestrus alert to ovulation were 30.7 ± 8.2 and 13.3 ± 7.7 hours, respectively. Ovulation occurred 24 to 47 hours after the onset of the IRT oestrus alert for eight out of the 11 ovulated cows (73%). Although the sensitivity of the IRT alert was greater than visual observation (67%) and Estrotect activation (67%), the specificity and positive predictive value were lower than these two aids (i.e. the IRT over-predicted the incidence of ovulation). Results presented indicate that, IRT shows some potential as an oestrus detection aid; however, further studies investigating the potential to improve the specificity and capturing data throughout entire 21-day reproductive cycles would be worthwhile.

Keywords: infrared thermography, ovulation, oestrus detection, dairy cow.

INTRODUCTION

A variety of methods can be used to aid the detection of cows in oestrus, e.g., visual observation, changes in body temperature, changes in vaginal mucus resistance, recording of mounting activity (Roelofs *et al.* 2005*b*). Different techniques have been used to measure changes in body temperature during oestrus (Clapper *et al.* 1990; Mosher *et al.* 1990; Redden *et al.* 1993; Kyle *et al.* 1998; Piccione *et al.* 2003; Fisher *et al.* 2008), and these have shown to be associated with the surge in luteinizing hormone (LH) and ovulation. However, restraining animals to measure the rectal and/or vaginal temperatures and using implants within the vaginal cavity to record body temperature may cause discomfort and stress to animals that may alter the actual body temperature. With the development of non-invasive diagnostic tools, such as infrared technology (IRT), it is now possible to measure body surface temperature precisely and with minimal discomfort to the cow.

Infrared thermography is a non-contact technique of thermal visualization through which temperatures are monitored and recorded. It has been used in veterinary science for lameness (Alsaaod and Büscher 2012) and mastitis (Colak *et al.* 2008) in dairy cows. It has also been reported that IRT can be used to detect changes in vulval temperatures between oestrus and di-oestrus sows (Scolari *et al.* 2011; Sykes *et al.* 2012). Hellebrand *et al.* (2003) reported that the vulval temperature changes combined with the body temperature and thermal imaging technology could be used for oestrus detection. A small number of cows were recruited in that study and vulval temperature changes reported during oestrus were not evaluated in relation to the hormonal profile (Hellebrand *et al.* 2003). Jones *et al.* (2005) evaluated thermal imaging technology in dairy cows and were able to discriminate first oestrus from di-oestrus after oestrus synchrony but not in subsequent cycles. However, no information regarding the housing system and methods for differentiating oestrus from di-oestrus groups were reported in that study. To the best of our knowledge, there are no studies that have focused on improving efficiency of oestrus detection using IRT in dairy cows.

Fertilization rates in dairy cows are influenced by the interval between insemination and ovulation (Roelofs *et al.* 2005*a*). Identifying the optimal time to inseminate cows relative to the stage of oestrus requires practical methods (Hockey *et al.* 2010). To

achieve optimal herd conception rates and submission rates, oestrus detection should have high levels of both sensitivity and specificity to ensure that a high proportion of cows are inseminated before an imminent ovulation (Hockey *et al.* 2010).

The aim of this study was to evaluate the potential of IRT temperature monitoring for oestrus detection and prediction of time of ovulation in dairy cows.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee (The University of Sydney, NSW, Australia, approval number: N00/9-2012/1/5829).

Animals, experimental design and oestrus synchronization

Twenty (14 primiparous and 6 multiparous) healthy, lactating, cycling Holstein Friesian dairy cows averaging 65 ± 5 days in milk (DIM), and producing 27 ± 6 kg (mean ± SD [standard deviation]) of milk per day (during the week before study commencement) were enrolled in this study. As oestrous behaviour can be affected by both body condition score (BCS) and lameness (Herlihy *et al.* 2013), all cows were also assessed on the day before commencing the study for BCS (Edmonson *et al.* 1989) and locomotion score (Walker *et al.* 2010) to ensure that all recruited cows had a BCS within the range of 2.5 to 4, and a locomotion score between 1 to 2.

The study was performed during October and November 2012 (Spring) at the University of Sydney's Corstorphine dairy farm, Camden, NSW, Australia. The mean air temperature, maximum air temperature and maximum relative humidity during the experimental period were 19°C, 33°C and 99%, respectively. To conduct intensive measurement during the experimental period, cows were kept in a paddock separate to the main milking herd and fed ad libitum lucerne silage plus concentrate (8 kg/cow.day) at milking.

Oestrus was synchronized in all study cows (on the same date) by inserting a controlled internal drug release (CIDR; Eazi-Breed®, Pfizer Animal Health Limited, West Ryde, NSW, Australia) on Day 0 into the vagina for 7 days. On Day 7, the CIDR was removed, and 2 mL (500 μg) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a synthetic prostaglandin

analogue, cloprostenol sodium (Estrumate®, Schering-Plough Animal Health Limited, Baulkham Hills, NSW, Australia) was administered to each cow (Figure 1).

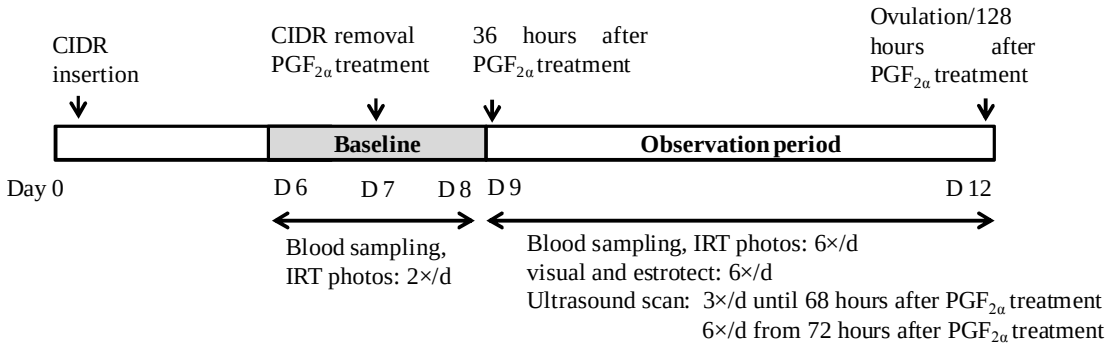

Figure 1. Schematic diagram of experimental procedure and oestrus synchronization protocol. Transrectal ultrasonography was used to assess ovarian structures, and blood samples were collected to assess plasma progesterone concentrations. Infrared thermography (IRT) was performed to measure the surface temperatures of vulva and muzzle. CIDR, controlled internal drug release.

Ultrasound scanning

The day before commencing the study, cows were subjected to ultrasound scanning (Ibex Pro portable ultrasound, E.I. Medical Imaging, Loveland, Co, USA) to confirm the presence of follicle(s) and absence of abnormal structures (cysts). Ovarian activity was also monitored via transrectal ultrasound scanning three times daily between 48 and 68 hours after $PGF_{2\alpha}$ treatment and six times daily thereafter until either ovulation or 128 hours after $PGF_{2\alpha}$ treatment (whichever occurred first; Figure 1). At the first ultrasound examination, both ovaries were examined and the ovary containing the largest follicle and the size of the follicle was recorded. In each subsequent ultrasound examination, only the ovary containing the largest follicle was examined and the size of the follicle was recorded. The presence (or absence) and diameter of corpus luteum (CL) was also recorded. Time of ovulation was defined as the first scanning session at which the dominant follicle had disappeared minus 2 hours.

Infrared thermography of vulva and muzzle

Thermal scanning of cows was performed using an infrared camera (FLIR, 620 series; FLIR Systems Co. Ltd., St Leonards, NSW, Australia) twice daily at 0600 hours and 1400 hours between 24 hours before and 32 hours after $PGF_{2\alpha}$ treatment to establish a baseline temperature for each cow. From 36 hours after $PGF_{2\alpha}$ treatment, IRT was performed six times daily until ovulation or 128 hours after $PGF_{2\alpha}$ treatment (Figure 1). Vulval and muzzle surface temperatures were measured at each IRT scanning session. During thermal scanning of the vulva, the tail was held aside and the vulva was gently wiped with dry paper towels to remove faecal matter. The camera was mounted on a tripod, and thermal imaging was performed from a fixed distance of approximately 1 meter from the animal. Before each thermal scanning session, the emissivity value was set to 0.98 and thermograph resolution was calibrated to ambient temperature and humidity as per manufacturer's recommendation using a solar weather station (Oregon Scientific International Ltd., Los Angeles, CA, USA).

Images were stored in a memory card, and then transferred to a laptop for analysis using ThermaCAM Researcher Professional 2.9. The software allowed the user to obtain temperature at a specific area on the image and calculated the minimum, maximum, and average temperatures and standard deviation for each measuring field. The software calculated the standard deviation of each image within a defined shape by considering each pixel within the given shape. Maximum IRT temperatures of vulva and muzzle were used in the present study. A free hand drawn geometrical polygonal shape covering the entire vulva area was used for calculation of temperature of vulval images, whereas the muzzle temperature was determined in a square area between the two nostrils (Figure 2). Images with standard deviations greater than 1°C (n = 9) and 0.5°C (n = 15) were deemed erroneous for vulva and muzzle respectively and were eliminated from the dataset for this study. A total of 488 and 482 images of vulva and muzzle, respectively, were used in this study.

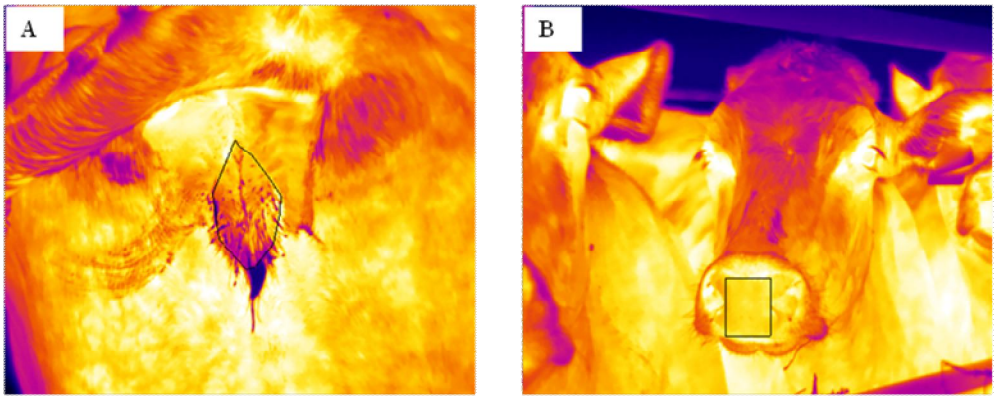

Figure 2. Hand drawn area of vulva (A) and muzzle (B).

Visual observation, Estrotect scoring, and recording of vaginal temperature

Cows were monitored for behavioural signs of oestrus from 36 hours after $PGF_{2\alpha}$ treatment six times daily until either ovulation or 128 hours after $PGF_{2\alpha}$ treatment (whichever occurred first; Figure 1). Oestrus was defined according to the method published by van Eerdenburg *et al.* (1996) with slight modifications. During each observation period, the assigned score was recorded for each displayed behavioural oestrus sign [Table 1 (van Eerdenburg *et al.* 1996)]. If the sum of the scores exceeded or equalled 50 at any observation period, the animal was considered to be in behavioural oestrus. Cows observed sniffing or mounting other cows were considered to be displaying behavioural oestrus if the visual observation score recorded for them (at that observation session) was greater than or equal to 50.

An Estrotect® (oestrus detection devices, Genetics Australia Co-operative Ltd, Bacchus Marsh, VIC, Australia) was applied to the tail head of each cow on Day 7 and monitored from 36 hours after $PGF_{2\alpha}$ treatment at each time of IRT. Estrotect was monitored for colour change and scored as not activated (no colour change), activated (≥ 75% colour change) and inconclusive (< 75% colour change), based on a modification of a published protocol (Holman *et al.* 2011).

Vaginal temperature was recorded with a digital thermometer (Microlife AG, 9443 Widnau, SG, Switzerland) at each IRT data capture session.

Table 1. Scoring system for visual observation of oestrus behaviour/indicators, adapted from van Eerdenburg *et al.* (1996).

Behaviour	Points
Mucous vaginal discharge	3
Flehmen	3
Restlessness	5
Being mounted but not standing	10
Sniffing vagina of other cow	10
Resting with chin on other cow	15
Mounting (or attempting) other cows	35
Mounting head side of other cow	45
Standing heat	100

Blood sampling and hormonal analysis

Blood samples were collected into lithium heparinised (10 mL) vacutainer tubes (BD, North Ryde, NSW, Australia) for analysis of plasma progesterone (P_4) concentration. Blood sampling was conducted according to the following schedule: twice daily from Day 6 to 8, six times daily (at 0200, 0600, 1000, 1400, 1800, and 2200 hours) from 36 hours after $PGF_{2\alpha}$ treatment until confirmation of ovulation or 128 hours after $PGF_{2\alpha}$ treatment (Figure 1). Blood samples were stored on ice for up to 1 h before centrifugation at 1075*g* for 15 minutes. Plasma was separated and stored at -20°C until being analysed via a hormonal assay.

Plasma P_4 was determined by enzyme-linked immunosorbent assay (ELISA) using Progesterone EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The test kits were validated in our laboratory (MC Franklin Laboratory, University of Sydney, Camden, NSW, Australia). A range of internal standards (from 0.007 to 10 ng/mL) of P_4 was prepared by adding standard solutions of known concentration to P_4-free plasma. Progesterone-free plasma was obtained by mixing plasma with activated charcoal (10 mg/mL) overnight. The following day, the charcoal and plasma were separated by centrifugation at 1000 g for 15 min. The mean intra-assay and inter-assay coefficients of variation were 3.64% and 4.55% respectively. The detection limit of the assay was 0.01 ng/mL. Cows were considered to have undergone luteal regression when the P_4

concentration in the sample collected at the time of $PGF_2\alpha$ treatment was 1.5 ng/mL or greater and less than 1.5 ng/mL in the sample collected 48 hours after $PGF_{2\alpha}$ treatment.

Algorithm and steps for detection and description of IRT oestrus temperature

Because of the diurnal variation in ambient temperature and humidity during the different observation periods, vulval and muzzle maximum temperatures were adjusted as described below.

Adjustment 1

Step 1: Fitting linear regression model

A linear mixed model was fitted to the vulval maximum temperature data using a restricted maximum likelihood (REML) procedure with the following form:

$$\text{VulvaT} = \text{constant} + \beta_A\,(\text{AmbT} - \overline{\text{AmbT}}) + \beta_H\,(\text{Hum} - \overline{\text{Hum}}) + \text{Cow} + \varepsilon$$

where VulvaT, vulval temperature; constant, overall mean vulval temperature; AmbT, ambient temperature (covariate); Hum, humidity (covariate); Cow, random effect of cow and ε, random error. The terms $\overline{\text{AmbT}}$ and $\overline{\text{Hum}}$ represent the averages of these values, i.e., the covariates are expressed as deviations from their means in the model. As there was relatively greater variation in vulval temperature during the baseline period than in the posttreatment period, separate residual variances were fitted, $\sigma^2_{\varepsilon B}$ for the baseline, and $\sigma^2_{\varepsilon P}$ for the post-treatment period.

Step 2: Adjustment of vulval temperature using regression coefficient

Vulval temperatures (of individual cow observations) were adjusted to the mean ambient temperature and the mean humidity over the study period as follows:

$$\text{VulvaT}_{adj1} = \text{VulvaT} - b_A\,(\text{AmbT} - \overline{\text{AmbT}}) - b_H\,(\text{Hum} - \overline{\text{Hum}})$$

where, b_A and b_H are the estimated regression coefficients from the REML procedure (step 1).

Step 3: Deviations from the reference value

For each observation period, the deviation ($VulvaT_{inc}$) between the adjusted vulval temperature ($VulvaT_{adj1}$) and constant temperature (from step 1) was calculated as a simple difference.

$VulvaT_{inc} = VulvaT_{adj1} - constant$

Adjustment 2

Adjustment 1 did not take into account differences in individual cow's baseline vulval temperatures. Some cows may have naturally increased body and/or skin temperatures, and hence are at risk of being flagged as entering oestrus when in fact they are not, alternatively, those with naturally lower temperatures risk having oestrus events that go undetected. The empirical baseline temperature data was used as an adjustment to reduce the risk of such occurrences.

$VulvaT_{adj2} = VulvaT_{adj1} - VulvaT_{B}$

where $VulvaT_{B}$ is the average of the baseline vulval temperatures for a cow. Muzzle temperature was adjusted similarly as described above for vulval temperature.

Algorithm optimization

Different thresholds (increase in vulval or muzzle temperature) were set in adjustment 1 and 2 to determine the oestrus-related increase of IRT temperature. A period when the adjusted vulval temperature exceeds the threshold was considered as an indication of an oestrus-related increase of temperature and defined as an IRT oestrus alert. An example of vulval temperature from an individual cow is shown in Figure 3.

Definition of onset, end, and duration of increased IRT vulval temperature

Duration of increased IRT temperature was defined as the time between onset and the end of the period of increased temperature. Times of onset and end of increased IRT temperature were defined respectively as the start times of the first and the last of each 4-hour period, where temperature was increased. Different thresholds were used to define oestrus-related increases in temperature using adjustment 1 and 2. In adjustment 1, three thresholds (≥1.0, 1.2, and 1.4°C) were imposed, and an oestrus event was

considered when temperature increased from the baseline temperature (by the relevant threshold) for three consecutive 4-hour periods. In adjustment 2 the thresholds were set as 1, 1.25, and 1.50 SD above the baseline temperature. The SD was taken as the estimated residual standard deviation during the post treatment period ($\hat{\sigma}_P$).

Definition of true positive, true negative, false positive and false negative increased IRT temperature

Ovulated cows with or without increased vulval temperature (at different thresholds) for three consecutive 4-hour periods from 36 hours after $PGF_{2\alpha}$ treatment were considered as true positive and false negative (FN) respectively. An-ovulated cows with or without increased temperature for three consecutive 4-hour periods on 36 hours after $PGF_{2\alpha}$ treatment were considered as false positive (FP) and true negative (TN), respectively.

Calculation of sensitivity, specificity and positive predictive value

Sensitivity was defined as the proportion of ovulatory periods in which at least one IRT oestrus alert occurred (as defined by the binary classification earlier), and was calculated by the formula, TP/(TP+FN). Specificity was defined as the proportion of an-ovulated cows in which no oestrus alert occurred and was calculated by the formula, TN/(TN+FP). Positive predictive value was calculated by dividing the number of ovulated cows that had at least one IRT oestrus alert by the sum of the number of ovulated and an ovulated cows with at least one IRT oestrus alert (TP+FP).

Sensitivity, specificity and positive predictive value of other oestrus detection aids (binary classification based on visual observation, Estrotect, and vaginal temperature, as defined earlier) were calculated using the same method described for calculating those for IRT oestrus alert.

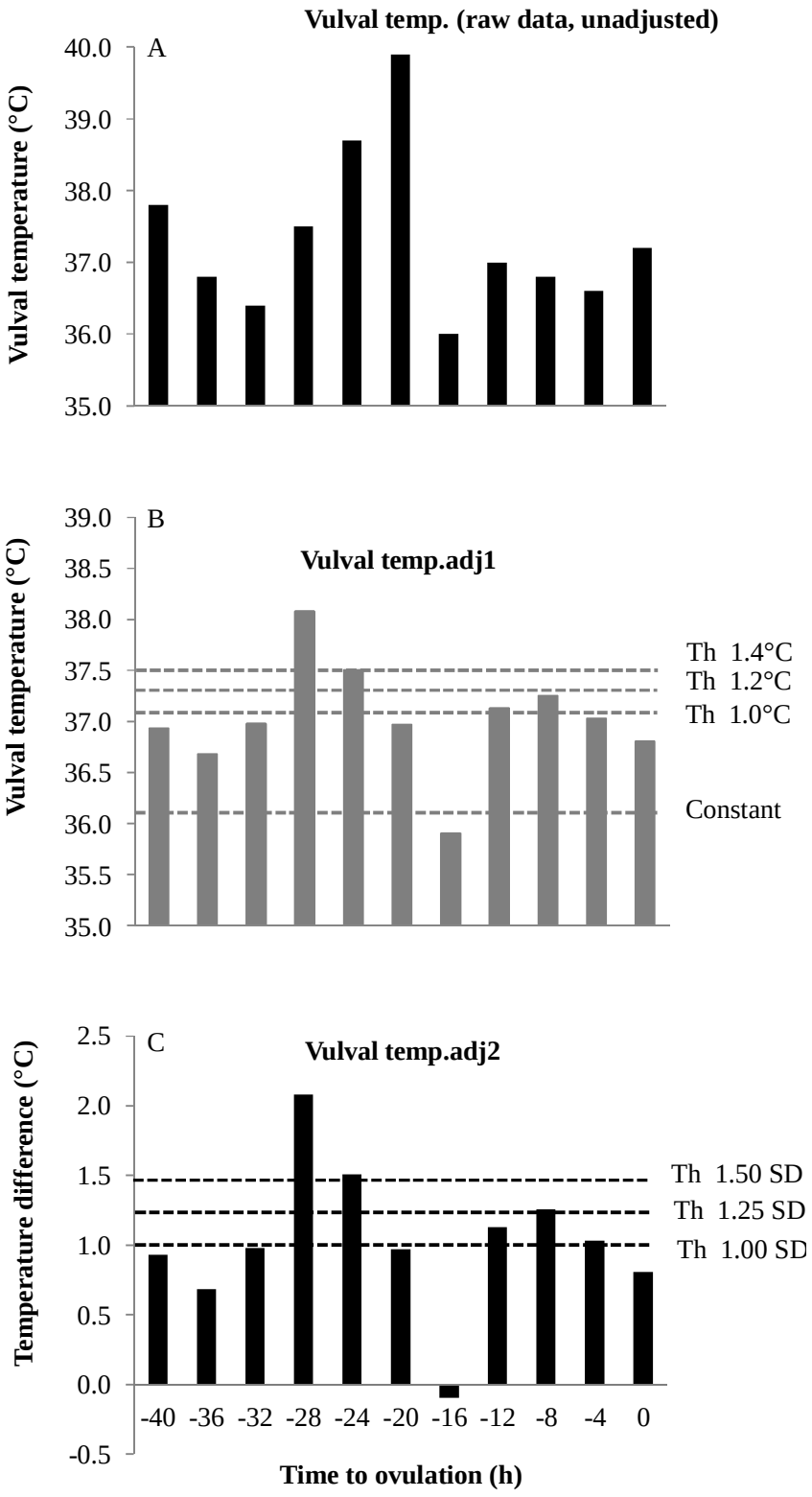

Figure 3. Raw maximum IRT vulval temperature of an individual cow in relation to time to ovulaton. A) Unadjusted data; B) adjusted for ambient temperature and humidity (Vulval temp.adj1, grey bars); C) adjusted as in (B) plus baseline temperature (vulval temp.adj2, black bars). Th denotes threshold. IRT, infrared thermography.

Statistical analysis of adjusted temperature data and duration data

Statistical analyses were performed using GENSTAT 14th Edition (VSN International, Hertfordshire, UK). The variations of adjusted vulval ($VulvaT_{adj2}$) and muzzle ($MuzzleT_{adj2}$) temperatures in relation to time of ovulation were analysed by fitting the following linear mixed model to the data:

$$T_{adj2} = \text{constant} + \text{Time} + \beta_1(\text{Parity} - \overline{\text{Parity}}) + \beta_2(\text{ADMY} - \overline{\text{ADMY}}) + \text{Cow} + \varepsilon$$

where T_{adj2} is either the vulval or muzzle adjusted temperature; Time is the time to ovulation (-84, -72, …, 0 hours) as a fixed effect factor; Parity and the average daily milk yield (ADMY) were fixed effect covariates in the model; and cow is the cow identification number as a random factor in the model. All means are presented as model-based mean ± SD. Note that in the subsequent text, any reference to temperature (muzzle or vulval) will be assumed to be the adjusted temperatures. Data on duration of IRT oestrus alert and the intervals between IRT oestrus and time to ovulation were calculated in Excel (Office 2007, Microsoft Corp., Redmond, WA, USA). A series of separate linear mixed models was fitted to each of these oestrus intensity duration and time interval variables, specifying fixed effects of parity (1 or > 1, factor), BCS (1-5, factor), milk yield (kg, covariate), and locomotion score (1-3, covariate), and with cow being a random effect in each of these models. Each explanatory variable (factor or covariate) was evaluated by univariable analyses using REML.

RESULTS

Of the 20 cows enrolled in this study, 12 ovulated, seven did not ovulate and one cow developed cystic ovarian disease. Data from the cystic cow were excluded from the analyses.

Maximum muzzle, vulval temperature, and P_4 concentration in relation to time to ovulation

Maximum IRT temperature of muzzle and vulva differed significantly ($P < 0.05$) in relation to time to ovulation (Figure 4). The maximum vulval and muzzle temperatures were observed 24 and 72 hours before ovulation, respectively, while the lowest of those temperatures were observed 48 hours before ovulation. Muzzle and vulval temperatures

measured 72 hours before ovulation decreased significantly ($P < 0.05$) at 48 hours before ovulation. An increase in maximum vulval temperature was observed 24 hours before ovulation followed by a decrease at the time of ovulation ($P < 0.05$). However, muzzle temperatures were relatively steady during the last 24 hours before ovulation (Figure 4).

A significant ($P < 0.05$) association was observed between the plasma P_4 concentrations and time to ovulation (Figure 4). The highest plasma P_4 concentration was observed 72 hours before ovulation, with a significant decrease ($P < 0.05$) measured 48 hours before ovulation. No significant differences were observed in P_4 concentrations during the last 48 hours before ovulation (Figure 4).

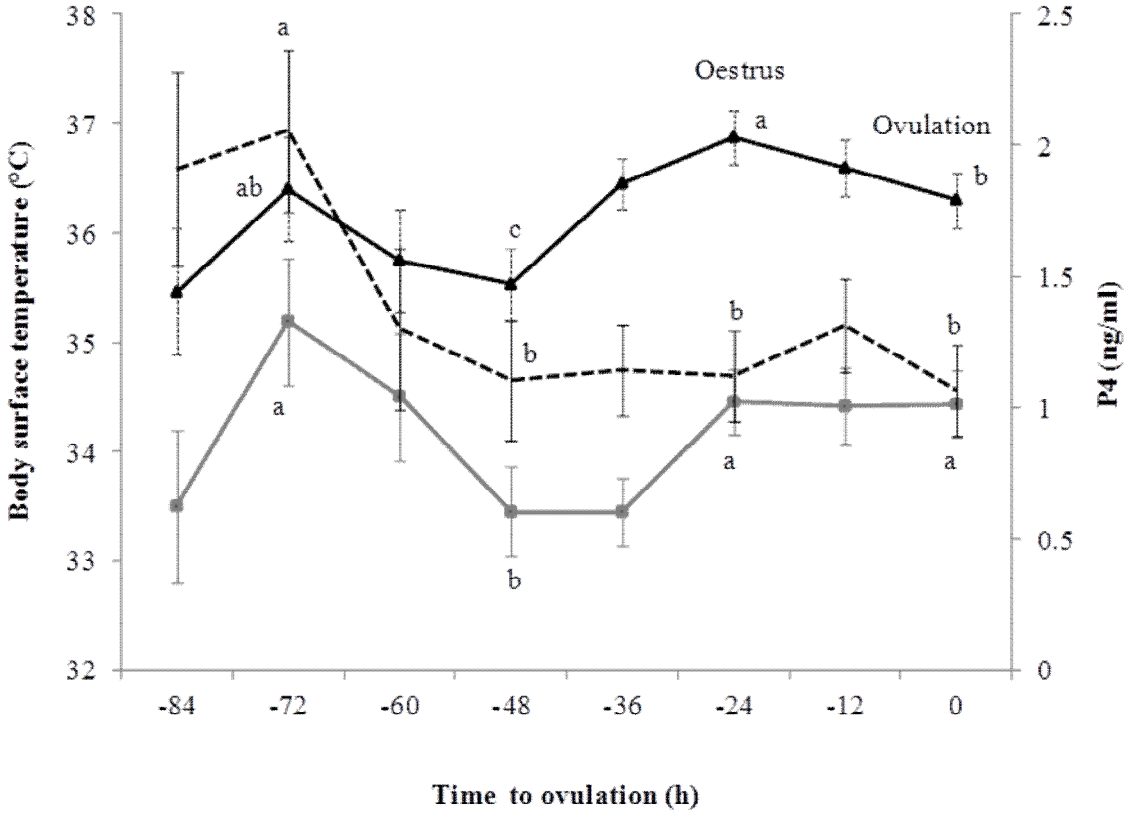

Figure 4. Maximum IRT muzzle (■) temperatures, vulval (▲) temperatures and P_4 (--) concentration in relation to time to ovulation. The values at 4-hour intervals were pooled for every 12 hours. For each parameter, different letters indicate significant differences between time points ($P < 0.05$). IRT, Infrared thermography; P_4, progesterone.

Sensitivity, specificity and positive predictive value of different methods of defining IRT oestrus alert

The algorithms developed for vulval temperatures resulted in greater sensitivity and specificity compared to those for muzzle temperatures. Among the 12 ovulated cows, 83% (10/12) and 92% (11/12) cows were detected by IRT vulval temperature using adjustment 1 and 2, respectively. However, sensitivity and specificity depended on the different thresholds of temperature rise used to define the occurrence of an oestrus alert. In adjustment 1, the threshold 1°C rise resulted in the highest sensitivity, percentage of true IRT oestrus alert (83%), but the lowest specificity (43%; Table 2). Conversely, threshold 1.4°C rise resulted in the lowest sensitivity (58%) and highest specificity (57%). When adjustment 2 was implemented, the threshold of 1 SD resulted in the highest sensitivity (92%) and lowest specificity (29%) for oestrus alerts. However, both 1.25 and 1.5 SD thresholds resulted in similar sensitivity (75%) and positive predictive value (69%) although the 1.5 SD threshold detected more TN cows compared to the 1.25 SD threshold (Table 2).

Relationship of IRT vulval temperature with Estrotect scoring, visual oestrus, and vaginal temperature

Of the 12 ovulated cows, 67% (8/12) were detected by visual behavioural observation and Estrotect scoring. Visual observation resulted in 67% sensitivity, 86% specificity and 89% positive predictive value (Table 2). In contrast, Estrotect resulted in 67% sensitivity and 100% specificity. Of the eight cows recorded for visual signs of oestrus followed by ovulation, 25% (2/8) and 13% (1/8) cows were not detected by IRT vulval temperature adjustment 1 and 2, respectively with any of the thresholds. Vulval temperature of those cows either did not increase for three consecutive periods or the increased temperature was lower than the minimum threshold levels. That is, while there was an increase in vulval temperature, the magnitude and/or duration was too small to generate an IRT oestrus alert. Vaginal temperature was significantly ($P < 0.001$) correlated with IRT vulval and muzzle temperature ($r = 0.68$ and 0.67 respectively). Different thresholds and adjustments of vaginal temperature resulted in different sensitivities and specificities (Table 2).

Table 2. Summary of sensitivity and specificity of maximum IRT vulval temperature, vaginal temperature, Estrotect and behavioural oestrus for detecting cows that are about to ovulate using two adjustments and different thresholds.*

Measure to detect cows about to ovulate	Sensitivity (%)	Specificity (%)	Positive predictive value (%)
IRT vulval maximum temperature, Adjustment 1			
1.0°C	83 ± 11	43 ± 14	71 ±13
1.2°C	67 ± 14	57 ± 14	73 ±13
1.4°C	58 ± 14	57 ± 14	63 ±14
IRT vulval maximum temperature, Adjustment 2			
1.00 SD	92 ± 8	29 ± 13	64 ± 14
1.25 SD	75 ± 13	29 ± 13	69 ± 13
1.5 SD	75 ± 13	57 ± 14	69 ± 13
IRT muzzle maximum temperature, Adjustment 1			
1.0°C	75 ± 13	0	56 ± 14
1.2°C	58 ± 14	14 ± 14	54 ± 14
1.4°C	50 ± 14	14 ± 14	50 ± 14
IRT muzzle maximum temperature, Adjustment 2			
1.00 SD	75 ± 13	0	56 ± 14
1.25 SD	58 ± 14	0	50 ± 14
1.5 SD	58 ± 14	14 ± 10	54 ± 13
Vaginal temperature, Adjustment 1			
0.3°C	83 ± 11	14 ± 10	63 ± 14
0.5°C	58 ± 14	29 ± 13	58 ± 14
1.0°C	33 ± 14	86 ± 10	80 ± 12
Vaginal temperature, Adjustment 2			
0.3°C	92 ± 8	14 ± 10	65 ± 14
0.5°C	58 ± 14	29 ± 13	58 ± 14
1.0°C	33 ± 14	86 ± 10	80 ± 12
Estrotect	67 ± 14	100 ± 0	100 ± 0
Behavioural oestrus	67 ± 14	86 ± 10	89 ± 9
Estrotect + behavioural oestrus	83 ± 11	86 ± 10	91 ± 8
IRT + Estrotect + behavioural oestrus	100 ± 0	29 ± 13	71 ± 13

*Number of ovulated cows = 12. Vaginal temperatures were adjusted according to the protocol followed during adjusting IRT temperature. IRT, infrared thermography; SD, standard deviation. Measures are expressed as percentages.

Interval between onset and end of increased vulval temperature and time of ovulation

Of the 12 cows that ovulated, 73% and 27% of cows were observed in IRT oestrus alert 48 and 72 hours, respectively, after the $PGF_{2\alpha}$ treatment. Ovulation occurred in 33%,

42%, and 25% of cows 72, 96 and 120 hours after $PGF_{2\alpha}$ treatment, respectively. Among the 12 ovulated cows, one cow was not detected by IRT and her data was not used to calculate the onset and end of ovulation. The intervals between $PGF_{2\alpha}$ treatment, the onset, end of IRT oestrus and ovulation are presented in Table 3. Infrared thermography-detected oestrus events started 49 ± 11 h (range: 36-72 hours) and ended 63 ± 10 h (range: 52-80 hours) after $PGF_{2\alpha}$ treatment. Ovulation occurred 24 to 47 hours after the onset of the IRT oestrus alert in 73% (8/11) of cows. Forty five percent (5/11) of ovulations occurred within the 12- hour period between 12 to 23 hours after the end of IRT oestrus.

Table 3. Intervals from $PGF_{2\alpha}$ treatment to onset and end of IRT oestrus, duration of IRT oestrus, luteolysis to ovulation, onset and end of IRT oestrus to ovulation.*

Intervals (h)	Mean ± SD	Minimum	Maximum	Q1	Q3
Induction of luteolysis to onset of IRT oestrus alert	49.0 ± 11.3	36	72	42	52
Induction of luteolysis to end of IRT oestrus alert	62.6 ± 10.2	52	80	56	70
Induction of luteolysis to ovulation	87.3 ± 19.1	72	124	24	40
Start of IRT oestrus to ovulation	30.7 ± 8.2	16	60	24	40
End of IRT oestrus to ovulation	13.3 ± 7.7	4	64	12	28
Duration of IRT oestrus	12.8 ± 7.0	8	24	8	16

*Times of onset and end of IRT oestrus were defined as the start times of first and last of each sequence of 4-hour periods, respectively (at least three consecutive periods) where temperature increased 1.00 SD in adjustment 2. IRT, infrared thermography; SD, standard deviation.

Factors affecting the IRT oestrus duration and time to ovulation

In the present study, IRT oestrus was significantly ($P < 0.05$) affected by the cow factors body condition score (BCS), parity and locomotion score. Body condition score had significant associations with IRT oestrus start ($P < 0.05$) and end ($P < 0.01$). Cows with a BCS of 2.5 had a longer interval between IRT oestrus alert and hours after $PGF_{2\alpha}$ treatment compared with cows with a BCS of 3.0 or more than 3.0. Primiparous cows

had longer average (8 hours; $P < 0.05$) duration of oestrus compared with multiparous cows and cows that were scored one for locomotion had longer duration ($P < 0.05$) of oestrus compared to cows that were scored 1.5. Milk yield tended to be related to the duration of IRT oestrus. Each 1 kg increase in milk yield was associated with a 0.48 hours average decrease in oestrus duration ($r = 0.57$; $P = 0.09$).

DISCUSSION

Infrared thermography enabled the detection of changes in skin temperatures in relation to the time of ovulation. The aim of the study was to develop and apply an algorithm to detect cows about to ovulate. Algorithms may create the potential to identify true oestrus events; although in some cases, IRT also generated FP alerts.

A significant decrease in muzzle and vulval temperature 48 hours before ovulation followed by a sharp rise 24 hours prior to ovulation was noted in the present study. In dairy cows, a pre-oestrus decrease of body temperature 24 to 48 hours before an oestrus-related increase in body temperature is most likely related to the regression of the CL (Kyle *et al.* 1998). The sharp increase in muzzle and vulval temperature 48 to 24 hours before ovulation observed in the present study is likely to coincide with the timing of oestrus. The significant decrease of vulval temperature noted around ovulation is in agreement with the earlier findings of Suthar *et al.* (2011) and Wrenn *et al.* (1958).

The reported time and extent of body temperature rises during oestrus varies considerably in the literature. Core body temperature rises of 0.3 °C to 1.1 °C (Kyle *et al.* 1998), 0.9 °C to 1.3 °C (Piccione *et al.* 2003) and maximum 0.5 °C (Suthar *et al.* 2011) during oestrus have been reported with durations of elevated temperatures ranging from 7 (Rajamahendran *et al.* 1989; Redden *et al.* 1993) to 11 hours (Mosher *et al.* 1990). Such discrepancies may be explainable by different housing systems (pen housing in the former two studies and tie stall housing in the latter one). Using different baseline temperatures [4-day baseline, (Kyle *et al.* 1998); 5-day preceeding ooestrus, (Piccione *et al.* 2003) or different analytical methods (using five distinct periods to monitor and compare body temperature during induced oestrus (Suthar *et al.* 2011)] to calculate oestrus-related increases in temperature would also likely contribute to the variability reported among the studies.

The cause for changes in vulval temperature may be ascribed to the increased activity at the time of oestrus (Walton and King 1986) or because of a central regulation of body temperature influenced by GnRH (Kyle *et al.* 1998). However, housing animals in tie stalls with restricted movement (Suthar *et al.* 2011) also resulted in a significant increase in body temperature during oestrus, indicating such changes are more likely to be associated with the time of the preovulatory LH surge which preceded ovulation by 24 hours (Rajamahendran *et al.* 1989; Mosher *et al.* 1990; Suthar *et al.* 2011).

In the present study, elevated and lowered vaginal temperatures were noted during the luteal (72 hours before ovulation) and pre-oestrus (48 hours before ovulation) stages, respectively, and may correspond to the high and low levels of plasma P_4. Similar results have been demonstrated by manually measuring vaginal temperature once daily (Lewis and Newman 1984) and using radio telemetry monitoring of temperature (Mosher *et al.* 1990; Redden *et al.* 1993; Kyle *et al.* 1998). Although vulval and muzzle temperatures increased significantly 1 day before ovulation compared to those temperatures at 2 days before ovulation, P_4 concentrations did not show significant differences between these days. It is possible that the rise in body temperature approximately 24 hours before ovulation could have been related to increasing oestradiol concentrations (not measured in this study).

Different thresholds of vulval temperature rise have been used in the present study to detect cows in oestrus. Among all the thresholds tested, 1 SD in adjustment 2 had the highest ability to detect TP cases. The sensitivity of IRT depended on the threshold used, which agrees with previous reports (López-Gatius *et al.* 2005*b*; Roelofs *et al.* 2005*a*) whereby pedometers were used with different thresholds to monitor and generate alerts around the increase in the number of steps around oestrus. Three oestrus detection methods used in this study varied widely in the cost and efficiency. Estrotect, the cheapest aid for oestrus detection, had a greater specificity but lower sensitivity compared to IRT. In the present study, sensitivity reached 100% when IRT was combined with visual observation and Estrotect but resulted in more FP cases. During the experimental period, all treatment cows were kept in the same paddock (separate from the main milking herd). It is recognized that the increased activity in oestrus cows may have influenced the activity of other an-oestrus cows at any one of the monitoring session which may have resulted in an increased rate of FP cases of behavioural oestrus.

Infrared thermography was performed until either ovulation or 128 hours after $PGF_{2\alpha}$ treatment (whichever occurred first). It is possible that some cows may have shown oestrus or ovulated after data collection concluded, however, we did not expect that 40% of cows would fail to ovulate within the data collection period because a follicle greater than 11 mm was present in all cows 24 hours after $PGF_{2\alpha}$ treatment, and all cows had luteal regression 48 hours after $PGF_{2\alpha}$ treatment. In the present study, 33% (4/12) of ovulated cows did not display behavioural signs of oestrus. These cows might be in silent oestrus (i.e. where no expression of oestrus behaviour was observed with an ovulation event). It was observed that 31% (5/16 for IRT) or 11% (1/9 for visual observation) of cows detected in oestrus did not ovulate within 128 hours after $PGF_{2\alpha}$ treatment. This incidence of ovulation failure is considerably greater than the rates of 6.5% (López-Gatius *et al.* 2005*a*) with rates of 12.4% in warm months (> 25°C) and 3.4% in cool months reported in the literature (López-Gatius *et al.* 2005*a*). During the experimental period, the mean ambient temperature and temperature humidity index of the 5-day period was 23 ± 6°C and 68 ± 7, respectively. Aside from the relatively high temperature and temperature humidity index, other physiological factors such as failure in the mechanism triggering ovulation (i.e., no LH surge or insufficient LH secretion) or a lack of response by the dominant follicle to the LH surge are possible causes for an-ovulation (Wiltbank *et al.* 2011).

In the present study, the observed interval (33.6 ± 12.1 hours) between ovulation and onset of IRT oestrus was longer than the interval reported previously by Bloch *et al.* (2006; 28.6 hours) but shorter than the interval reported by Saumande *et al.* (2005; 38.5 hours). In these studies, CIDR-$PGF_{2\alpha}$ protocols were used to synchronize oestrus, and oestrus was defined when a female would stand to be mounted. Walker *et al.* (1996) stated that the interval from oestrus to ovulation did not differ between natural or induced oestrus; therefore, it would be possible to speculate that the synchronization of oestrus in the present study had no influence on the oestrus to ovulation interval.

The intervals of onset of oestrus (ranging between 16 to 60 hours), end of oestrus (ranging between 4 to 64 hours) and time of ovulation were variable in the present study. However, the wide degree of individual variability observed was in agreement with the other studies, in which detection of oestrus was based on behavioural signs or activity profiles (Walker *et al.* 1996; Roelofs *et al.* 2005*a*; Valenza *et al.* 2012). To

achieve the greatest conception rates, insemination should take place between 7 and 18 hours (12-hour window) before ovulation (Trimberger and Davis 1943). Based on these intervals, a predictor for the time of ovulation should preferably determine the time of ovulation with an accuracy of 12 hours and should be able to predict time of ovulation at least 18 hours before ovulation (Roelofs *et al.* 2005*b*).

The mean duration of IRT oestrus (13.6 h) observed in the present study was shorter than that reported by Valenza *et al.* (2012) but longer than the findings of Roelofs *et al.* (2005*a*) which was 16.1 h and 10.0 hours, respectively. However, use of different methods (accelerometer and pedometer respectively) would be expected to create this level of discrepancy. Monitoring frequency versus continuous data would also be a likely contributing factor.

Parity, locomotion score and milk yield were associated with the mean duration of IRT oestrus. Decreased oestradiol during the follicular phase (Wiltbank *et al.* 2006) because of hepatic steroid metabolism (Sangsritavong *et al.* 2002) in lactating cows might explain the trending relationship between milk yield and duration of oestrus. The overall (P = 0.06) shorter duration of oestrus in multiparous cows agrees with the findings of a previous study (Roelofs *et al.* 2005*b*), but is in contrast with another study reporting no effect (Valenza *et al.* 2012) or observed shorter duration in primiparous cows (Walker *et al.* 1996). Reduced physical movement in cows with a locomotion score of 1.5 compared to cows with a locomotion score of 1 may contribute to the lower body temperature, and hence reduced increase in core surface temperature to be detected by IRT, and this may explain the shorter duration of oestrus in the present study.

Vulval and muzzle temperatures recorded by IRT were seen to have a high level of variation both within and between cows. One explanation could be that the device was hand held, so the distance to the skin surface would have had a level of error and slight vibrations and/or movement of the camera could have influenced the accuracy of the measurements (Hoffmann *et al.* 2013). In addition, any faecal contamination of the vulval surface and vulval skin colour could also influence the recorded temperature. A high level of variability was noted for IRT muzzle temperatures compared to IRT vulval temperatures. This may have been the result of changes of muzzle temperature directly caused by drinking from a water trough or as a result of the physiological response of

cows to thermo regulate the body temperature through the muzzle (Hoffmann *et al.* 2013).

Vulval temperature recorded by IRT appears to be a promising body region to monitor surface temperature changes, however the concept of automating the IRT data capture is somewhat challenging with the vulval surface. In particular, the occlusion of the vulva by the hanging tail and potential for faecal contamination of the skin surface are likely to limit the success of such an application. The head, the eye and the back of the ear have all been recently reported to be promising body regions for IRT, because these regions are likely to be less challenging for regular data capture (Hoffmann *et al.* 2013). Consequently, different body regions such as eye and back of the ear should be evaluated to determine their potential to enhance the specificity and positive predictive value of IRT for oestrus detection and prediction of ovulation in dairy cows.

Environmental conditions such as ambient temperature and relative humidity can affect the accuracy of IRT. Discrepancies exist in the literature about the appropriate temperature for performing IRT. Love and Linsted (1976) suggested that an ambient temperature of approximately 20°C is most appropriate for IRT, whereas in another study by Turner *et al.* (1986), 30°C has been reported to be an acceptable ambient temperature for IRT image capture. However, Sykes *et al.* (2012) observed detectable vulval temperature changes between oestrus and di-oestrus groups at ambient temperatures below 10°C. Relative humidity changes the components of the atmosphere which needs to be taken into account during thermography (Kastberger and Stachl 2003). At particular wavelengths, certain components of the atmosphere such as vapour and carbon dioxide absorb IR radiation (Kastberger and Stachl 2003; Stelletta *et al.* 2012). If environmental humidity is not taken into account, the displayed temperature may be overestimated (Kastberger and Stachl 2003). Ambient temperature and humidity are potential barriers to the sensitivity of the surface temperatures monitored and therefore IRT needs to be evaluated in different ambient conditions.

CONCLUSIONS

These results presented here indicate that measuring the surface temperature using IRT may become a useful tool for detecting oestrus in cows, and provide an indication as to when ovulation is likely to occur. A significant reduction of vulval and muzzle

temperatures 48 h before ovulation might be related to CL regression, while an increase of temperatures 24 h before ovulation coincided with the timing of oestrus. Sensitivity and specificity was affected by the threshold and adjustments used in the algorithms developed and presented in this manuscript. Of the threshold and algorithms evaluated, the 1.0 SD threshold of adjustment 2 resulted in the greatest sensitivity. However, the specificities and positive predictive values were lower than desirable. Monitoring surface temperatures of other body areas (including the eyes or ears) may prove to be more practical on farm than monitoring of the vulva and could be evaluated to enhance the specificity and positive predictive value of this technology.

REFERENCES

Alsaaod M, Büscher W (2012) Detection of hoof lesions using digital infrared thermography in dairy cows. *Journal of Dairy Science* **95,** 735-742.

Bloch A, Folman Y, Kaim M, Roth Z, Braw-Tal R, Wolfenson D (2006) Endocrine alterations associated with extended time interval between estrus and ovulation in high-yield dairy cows. *Journal of Dairy Science* **89,** 4694-4702.

Clapper JA, Ottobre JS, Ottobre AC, Zartman DL (1990) Estrual rise in body temperature in the bovine I. Temporal relationships with serum patterns of reproductive hormones. *Animal Reproduction Science* **23,** 89-98.

Colak A, Polat B, Okumus Z, Kaya M, Yanmaz LE, Hayirli A (2008) Short communication: Early detection of mastitis using infrared thermography in dairy cows. *Journal of Dairy Science* **91,** 4244-4248.

Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G (1989) A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* **72,** 68-78.

Fisher AD, Morton R, Dempsey JMA, Henshall JM, Hill JR (2008) Evaluation of a new approach for the estimation of the time of the LH surge in dairy cows using vaginal temperature and electrode less conductivity measurements. *Theriogenology* **70,** 1065-1074.

Hellebrand HJ, Brehme U, Beuche H, Stollberg U, Jacobs H (2003) Application of thermal imaging for cattle management. In 'Proceddings of the First European

Conference on Precision Livestock Farming'. pp. 761-763. (Wageningen Academic Publishers: Wageningen, the Netherlands)

Herlihy MM, Crowe MA, Berry DP, Diskin MG, Butler ST (2013) Factors associated with fertility outcomes in cows treated with protocols to synchronize estrus and ovulation in seasonal-calving, pasture-based dairy production systems. *Journal of Dairy Science* **96,** 1485-1498.

Hockey CD, Morton JM, Norman ST, McGowan MR (2010) Evaluation of a neck mounted 2-hourly activity meter system for detecting cows about to ovulate in two paddock-based Australian dairy herds. *Reproduction in Domestic Animals* **45,** 107-117.

Hoffmann G, Schmidt M, Ammon C, Rose-Meierhöfer S, Burfeind O, Heuwieser W, Berg W (2013) Monitoring the body temperature of cows and calves using video recordings from an infrared thermography camera. *Veterinary Research Communications* **37,** 91-99.

Holman A, Thompson J, Routly JE, Cameron J, Jones DN, Grove-White D, Smith RF, Dobson H (2011) Comparison of oestrus detection methods in dairy cattle. *Veterinary Record* **169,** p. 47.

Jones M, Denson A, Bowers S, Moulton K, Williams E, Graves K, Dos Santos A, Kouba A, Willard S (2005). Thermography of the vulva in Holstein dairy cows: A comparison of estrus vs. diestrus. In 'ASAS/ADSA National Meeting'. (Cincinnati, Ohio)

Kastberger G, Stachl R (2003) Infrared imaging technology and biological applications. *Behavior Research Methods* **35,** 429-439.

Kyle BL, Kennedy AD, Small JA (1998) Measurement of vaginal temperature by radiotelemetry for the prediction of estrus in beef cows. *Theriogenology* **49,** 1437-1449.

Lewis GS, Newman SK (1984) Changes throughout estrous cycles of variables that might indicate estrus in dairy cows. *Journal of Dairy Science* **67,** 146-152.

López-Gatius F, López-Béjar M, Fenech M, Hunter RHF (2005*a*) Ovulation failure and double ovulation in dairy cattle: Risk factors and effects. *Theriogenology* **63,** 1298-1307.

López-Gatius F, Santolaria P, Mundet I, Yániz JL (2005*b*) Walking activity at estrus and subsequent fertility in dairy cows. *Theriogenology* **63,** 1419-1429.

Love TJ, Linsted RD (1976) Theoretical basis for use of skin temperature as a plethysmographic indicator. *American Society of Mechanical Engineering* **6,** 1-4.

Mosher MD, Ottobre JS, Haibel GK, Zartman DL (1990) Estrual rise in body temperature in the bovine II. The temporal relationship with ovulation. *Animal Reproduction Science* **23,** 99-107.

Piccione G, Caola G, Refinetti R (2003) Daily and estrous rhythmicity of body temperature in domestic cattle. *BioMed Central Physiology* **3,** 1- 8.

Rajamahendran R, Robinson J, Desbottes S, Walton JS (1989) Temporal relationships among estrus, body temperature, milk yield, progesterone and luteinizing hormone levels, and ovulation in dairy cows. *Theriogenology* **31,** 1173-1182.

Redden KD, Kennedy AD, Ingalls JR, Gilson TL (1993) Detection of estrus by radiotelemetric monitoring of vaginal and ear skin temperature and pedometer measurements of activity. *Journal of Dairy Science* **76,** 713-721.

Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp B (2005*a*) Pedometer readings for estrous detection and as predictor for time of ovulation in dairy cattle. *Theriogenology* **64,** 1690-1703.

Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp B (2005*b*) Various behavioral signs of estrous and their relationship with time of ovulation in dairy cattle. *Theriogenology* **63,** 1366-1377.

Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC (2002) High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17[beta] in dairy cattle. *Journal of Dairy Science* **85,** 2831-2842.

Saumande J, Humblot P (2005) The variability in the interval between estrus and ovulation in cattle and its determinants. *Animal Reproduction Science* **85,** 171-182.

Scolari SC, Clark SG, Knox RV (2011) Vulvar skin temperature changes significantly during estrus in swine as determined by digital infrared thermography. *Journal of Swine Health and Production* **19,** 151-155.

Stelletta C, Gianesella M, Vencato J, Fiore E, Morgante M (2012) Thermographic applications in veterinary medicine. In: 'Infrared thermography.' (Ed. RV Prakash) pp. 117-140. (InTech: China) Available at http://www.Intechopen.Com/books/infraredthermography/thermographic-applications-in-veterinary-medicine [verified 30 August 2013]

Suthar VS, Burfeind O, Patel JS, Dhami AJ, Heuwieser W (2011) Body temperature around induced estrus in dairy cows. *Journal of Dairy Science* **94,** 2368-2373.

Sykes DJ, Couvillion JS, Cromiak A, Bowers S, Schenck E, Crenshaw M, Ryan PL (2012) The use of digital infrared thermal imaging to detect estrus in gilts. *Theriogenology* **78,** 147-152.

Trimberger GW, Davis HP (1943) Breeding efficiency in dairy cattle bred at various stages of estrus by artificial insemination. *Journal of Dairy Science* **26,** 757-759.

Turner AT, Purohit RC, Fessler JF (1986) Thermography: A review in equine medicine. *Compendium on Continuing Education for the Practising Veterinarian* **8,** 855-861.

Valenza A, Giordano JO, Lopes Jr G, Vincenti L, Amundson MC, Fricke PM (2012) Assessment of an accelerometer system for detection of estrus and treatment with gonadotropin-releasing hormone at the time of insemination in lactating dairy cows. *Journal of Dairy Science* **95,** 7115-7127.

van Eerdenburg FJ, Loeffler HS, van Vliet JH (1996) Detection of oestrus in dairy cows: A new approach to an old problem. *Veterinary Quarterly* **18,** 52–54.

Walker SL, Smith RF, Jones DN, Routly JE, Morris MJ, Dobson H (2010) The effect of a chronic stressor, lameness, on detailed sexual behaviour and hormonal profiles in milk and plasma of dairy cattle. *Reproduction in Domestic Animals* **45,** 109-117.

Walker WL, Nebel RL, McGilliard ML (1996) Time of ovulation relative to mounting activity in dairy cattle. *Journal of Dairy Science* **79,** 1555-1561.

Walton JS, King GJ (1986) Indicators of estrus in Holstein cows housed in tie stalls. *Journal of Dairy Science* **69,** 2966-2973.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A (2006) Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology* **65,** 17-29.

Wiltbank MC, Sartori R, Herlihy MM, Vasconcelos JLM, Nascimento AB, Souza AH, Ayres H, Cunha AP, Keskin A, Guenther JN, Gumen A (2011) Managing the dominant follicle in lactating dairy cows. *Theriogenology* **76,** 1568-1582.

Wrenn TR, Bitman J, Sykes JF (1958) Body temperature variations in dairy cattle during the estrous cycle and pregnancy. *Journal of Dairy Science* **41,** 1071-1076.

CHAPTER 4

Changes in Plasma Oxidative Stress Biomarkers in Dairy Cows after Oestrus Synchronisation with Controlled Internal Drug Release (CIDR) and Prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$)

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OVERVIEW OF CHAPTER 4

Chapter 4 presents findings from a companion study to Chapter 3. The findings of Chapter 3 showed that 40 percent (n = 8) of cows were recorded for an-ovulation after oestrus synchronization treatment. This lead us to hypothesise that decreased concentrations of reactive oxygen species and increased amounts of antioxidants might be associated with the lack of ovulation. It was also hypothesised that ovulated and an-ovulated cows would display different progesterone profiles.

ABSTRACT

This study was designed to evaluate the plasma profiles of oxidative stress biomarkers, progesterone and ovarian follicle diameter in ovulatory versus an-ovulatory cows. Twenty cows were synchronised using controlled internal drug release (CIDR) and prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) protocol. Plasma samples were analysed for progesterone (P_4), oxidative stress (OS) biomarkers; reactive oxygen metabolites (ROMs), biological antioxidant potential (BAP), oxidative stress index (OSI = ROMs/BAP × 100), advanced oxidation protein products, ceruloplasmin and glutathione (GSH). Plasma P_4 concentration was greater in ovulated cows 24 hours after $PGF_{2\alpha}$ treatment but lower 48 hours after $PGF_{2\alpha}$ treatment compared with that of an-ovulated cows at those sampling sessions ($P < 0.05$). Ovulated cows were diagnosed with greater ovarian follicle diameter compared with that of their herd mates not diagnosed for ovulation. Significant interaction of time of $PGF_{2\alpha}$ treatment and ovulation status (ovulatory versus an-ovulatory) with the plasma concentrations of OSI, BAP and GSH were observed. Ovulated cows had significantly lower BAP compared with that of an-ovulated cows ($P < 0.05$) 9, 48, 60 and 128 hours after $PGF_{2\alpha}$ treatment. Plasma concentrations of GSH were lower ($P < 0.05$) in ovulated cows than that of an-ovulated cows 60 and 96 hours after $PGF_{2\alpha}$ treatment. However, OSI was greater ($P < 0.05$) in ovulated cows than that of an-ovulated cows 9, 48, 60 and 128 hours after $PGF_{2\alpha}$ treatment. Significant associations were observed between OS status and sampling time. Oxidative stress status may have important physiological role in facilitating the ovulation process in oestrus synchronised dairy cows.

Keywords: biological advanced potential, glutathione, ovulation, oxidative stress index, progesterone.

INTRODUCTION

Normally reactive oxygen species (ROS) and antioxidants remain in balance, but when this balance is disrupted as a consequence of ROS overproduction or depletion of antioxidants, oxidative stress (OS) occurs (Agarwal *et al*. 2005; Celi 2011*a*). Oxidative stress can affect a variety of physiological functions in the reproductive system, and excessive levels of ROS can disrupt several reproductive events and result in adverse pregnancy outcomes (Agarwal *et al*. 2005; Al-Gubory *et al*. 2010). In ruminants and in the dairy cow in particular, OS has been associated with several pathological conditions, such as retained placenta, udder oedema, and mastitis, which in turn may impair reproductive performance (Miller *et al*. 1993).

The role of OS in the control of female reproduction has not been fully elucidated in ruminants; however, it is plausible that the oxidant/antioxidant balance can influence the reproductive axis at different levels (Celi 2011*a*). It is apparent that OS plays a crucial role in the cause and progression of several reproductive events such as fertilisation and early embryo development (Al-Gubory *et al*. 2010). For example, previous studies have shown that OS is associated with embryonic losses in dairy cows (Celi *et al*. 2011, 2012) and that OS has a role in the pathogenesis of follicular cysts and repeat breeder syndrome in dairy cows (Rizzo *et al*. 2007, 2009). Studies in rodents have also highlighted the involvement of OS in the regulation of ovulation (Sato *et al*. 1992).

As the oxidant/antioxidant balance may play a determinant role in the regulation of reproductive function in dairy cattle, this study was conducted to examine the changes in some plasma OS biomarkers in cows with ovulatory and an-ovulatory response after oestrus synchronisation with controlled internal drug release (CIDR) and prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$).

MATERIALS AND METHODS

All procedures conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and implemented by the Animal Ethics Committee of The University of Sydney (N00/9–2012/1/5829).

Animals and experimental design

Twenty (14 primiparous and 6 multiparous) healthy, lactating, cycling Holstein Friesian dairy cows averaging 65 ± 5 days in milk and producing 27 ± 6 kg (mean ± SD [standard deviation]) of milk per day were enrolled into the study, which was performed during October and November 2012 (Spring) at the University of Sydney's Corstorphine dairy farm, Camden, NSW, Australia. The mean air temperature, maximum air temperature and maximum relative humidity during the experimental period were 19°C, 33°C and 99%, respectively. To conduct intensive measurements during the experimental period, cows were kept in a paddock separate to the main milking herd and fed *ad libitum* lucerne silage plus concentrate (8 kg/cow.day) at milking (0500 hours and 1400 hours).

The synchronisation protocol and experimental design are detailed in Talukder *et al.* (2014*b*). In brief, oestrus was synchronised in all trial cows by inserting a CIDR (Eazi-Breed, Pfizer Animal Health Limited, West Ryde, NSW, Australia) into the vagina for 8 days. After 8 days, CIDR were removed and 2 mL (500 µg) $PGF_{2\alpha}$ synthetic analogue, cloprostenol sodium (Estrumate, Schering-Plough Animal Health Limited, Baulkham Hills, NSW, Australia) was administered to each cow.

Ovarian activity was monitored via transrectal ultrasound scanning (Ibex Pro portable ultrasound; E.I. Medical Imaging, Loveland, CO, USA) three times daily during the period 48–68 hours after $PGF_{2\alpha}$ treatment and six times daily thereafter until either ovulation or 128 hours after $PGF_{2\alpha}$ treatment (whichever occurred first). At the first ultrasound examination, both ovaries were examined and the ovary containing the largest follicle and the size of the follicle was recorded. In each subsequent ultrasound examination, only the ovary containing the largest follicle was examined and the size of the follicle was recorded (Hockey *et al.* 2010). Of the 20 cows enrolled in this study, 12 ovulated, seven did not ovulate and one cow developed cystic ovarian disease. Data from the cystic cow were excluded from the analyses.

All cows were also assessed on the day before commencing the study for body condition score (BCS) and locomotion score. All recruited cows had an average BCS of 3.0 ± 0.5 (mean ± SD) and an average locomotion score of 1.2 ± 0.2 (mean ± SD).

Blood sampling and assays

Blood samples were collected into lithium heparinised (10 mL) vacutainers (BD, North Ryde, NSW, Australia) for analysis of plasma progesterone (P_4) concentration according to the following schedule: twice daily 24 hours before and 32 hours after $PGF_{2\alpha}$ treatment, six times daily (at 0200, 0600, 1000, 1400, 1800, and 2200 hours) 36 hours after $PGF_{2\alpha}$ treatment until confirmation of ovulation or 128 hours after $PGF_{2\alpha}$ treatment. Samples were stored on ice during sampling and transport before centrifugation at 1075*g* and 4°C for 15 min. Plasma was separated and stored at –20°C until laboratory analyses were conducted.

Plasma P_4 was determined by enzyme-linked immunosorbent assay using Progesterone EIA Kit (Cayman Chemical Co., Ann Arbour, MI, USA). The mean intra-assay and inter-assay coefficients of variation were 3.64% and 4.55%, respectively. The detection limit of the assay was 0.01 ng/mL.

The amount of free oxygen radicals in plasma samples was determined by measuring the concentrations of reactive oxygen metabolites (d-ROMs Test; Diacron, GR, Italy), while the concentrations of antioxidants were measured using the biological antioxidant potential (BAP) test according to kit instructions (Diacron, GR, Italy); ROMs and BAP concentrations were determined in a dedicated spectrophotometer (FREE system, Diacron International, GR, Italy). The extent of OS was expressed as an Oxidative Stress Index (OSI), which was estimated using the ratio of ROMs/BAP × 100, as the combination of ROMs and BAP results are deemed to provide an accurate representation of OS status (Celi 2011*b*). Plasma glutathione (GSH) concentration were measured by an enzymatic recycling method (chemicals sourced from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia) adapted for a microtitre plate reader (Baker *et al*. 1990). Advanced oxidation protein products (AOPP) were measured according to the methods of Witko-Sarsat *et al.* (1998). Ceruloplasmin concentrations were determined according to the methods described by Sunderman and Nomoto (1970) except that absorbance was read at 510 nm (FLUROstar Optima, BMG Labtech, Mornington, VIC, Australia). Ceruloplasmin concentration was calculated as follows:

Ceruloplasmin (g/L) = $0.752\ (A_R - A_B)$,

where A_R is the absorbance of sample R, and A_B is the absorbance of sample B.

Statistical analyses

The raw data was initially assessed using descriptive statistics and then tested for normal distribution. Logarithmic transformations were performed for the data (P_4, ROMs, BAP, AOPP, ceruloplasmin and GSH) that had skewed distributions and the means were back transformed for presentation of results (Talukder *et al.* 2014*a*). A linear mixed model (LMM) was used to estimate the effect of time (hours before and after $PGF_{2\alpha}$ treatment), group (ovulatory vs an-ovulatory cycle) and the time by group interaction (GENSTAT 14th Edition, VSN International, Hertfordshire, UK). The model was as follows:

$$Y_{ijk} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + R\varepsilon_{ijk}$$

where, Y_{ijk} = response variate according to group i (i = ovulatory vs an-ovulatory cows) at time j (j = –24, –9, 0, 9, 24, … 128 hours) by cow k (k = 1–19), μ = mean effect of group, β_i = effect of group, γ_j = effect of time j, $(\beta\gamma)_{ij}$ = effect of group by time interaction, $R\varepsilon_{ijk}$ = random residual error within cow k at time j.

The 7 days of data was pooled for statistical analyses. The relationship between time of sampling (at 0200, 0600, 1000, 1400, 1800, and 2200 hours) and the oxidants/antioxidants parameters were tested by LMM. Ambient temperature and humidity were included as covariates in the model. A series of separate LMM was fitted to each of the oxidants/antioxidants parameters specifying fixed effects of parity (1 or >1), BCS (1–5), 7 days average milk yield (≤28 kg or >28 kg), and locomotion score (1–2), with 'cow' included as a random effect in each of these models. Each explanatory variable was evaluated by univariable analyses using REML. All means are presented as model-based mean ± standard error (s.e.).

RESULTS

Plasma P_4 concentration and ovarian follicular diameter

Plasma P_4 concentration was 2-fold greater 24 hours after $PGF_{2\alpha}$ treatment in ovulated cows than that of an-ovulated cows. However, 48 hours after $PGF_{2\alpha}$ treatment P_4 concentration was lower in ovulated cows compared with the P_4 concentration in an-ovulated cows, which was 0.6 ng/mL and 1.3 ng/mL, respectively (Figure 1A). Ovarian

follicle diameter was higher (P = 0.04) from 72-128 hours after $PGF_{2\alpha}$ treatment in ovulated cows than in cows that did not ovulate (Figure 1B).

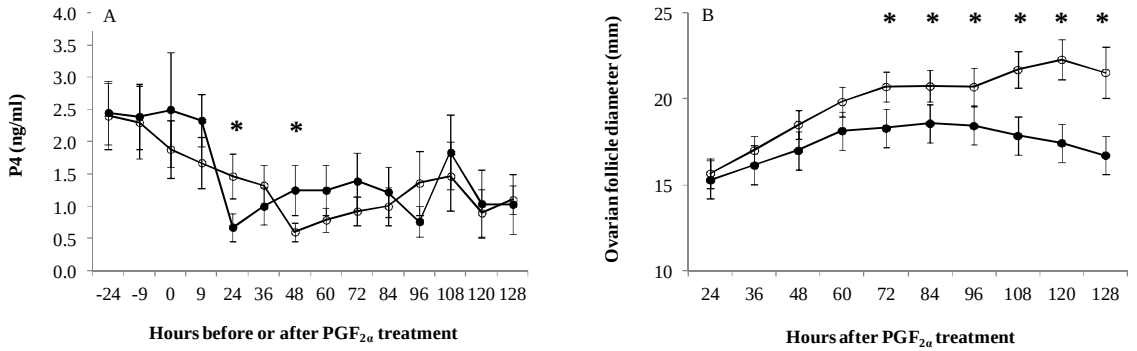

Figure 1. A) Plasma progesterone concentration and B) follicle diameter (means ± s.e.) of cows with ovulatory (○) and an-ovulatory (●) event in relation to time of $PGF_{2\alpha}$ treatment. *indicates significant differences (P < 0.05) between cows with ovulatory and an-ovulatory event at the same time point.

Biomarkers of OS in ovulatory and an-ovulatory cows

Plasma ROMs concentration did not differ significantly between ovulated and an-ovulated cows (Figure 2A). Significant effects of time of $PGF_{2\alpha}$ treatment, group and the time by group interaction was observed for BAP concentration. Plasma concentrations of BAP were significantly lower (P = 0.04) in ovulated cows compared with an-ovulated cows 9, 48, 60 and 128 hours after $PGF_{2\alpha}$ treatment (Figure 2B). However, OSI were significantly greater in ovulated cows 9, 48, 60 and 128 hours after $PGF_{2\alpha}$ treatment than those of an-ovulated cows (P = 0.03 Figure 2C). Plasma GSH decreased significantly (P < 0.05) in ovulated cows compared with that of an-ovulated cows 60 and 96 hours after $PGF_{2\alpha}$ treatment (Figure 2D). No significant differences in ceruloplasmin and AOPP concentrations were observed after $PGF_{2\alpha}$ treatment between ovulatory and an-ovulatory cycles (Figure 2E, F).

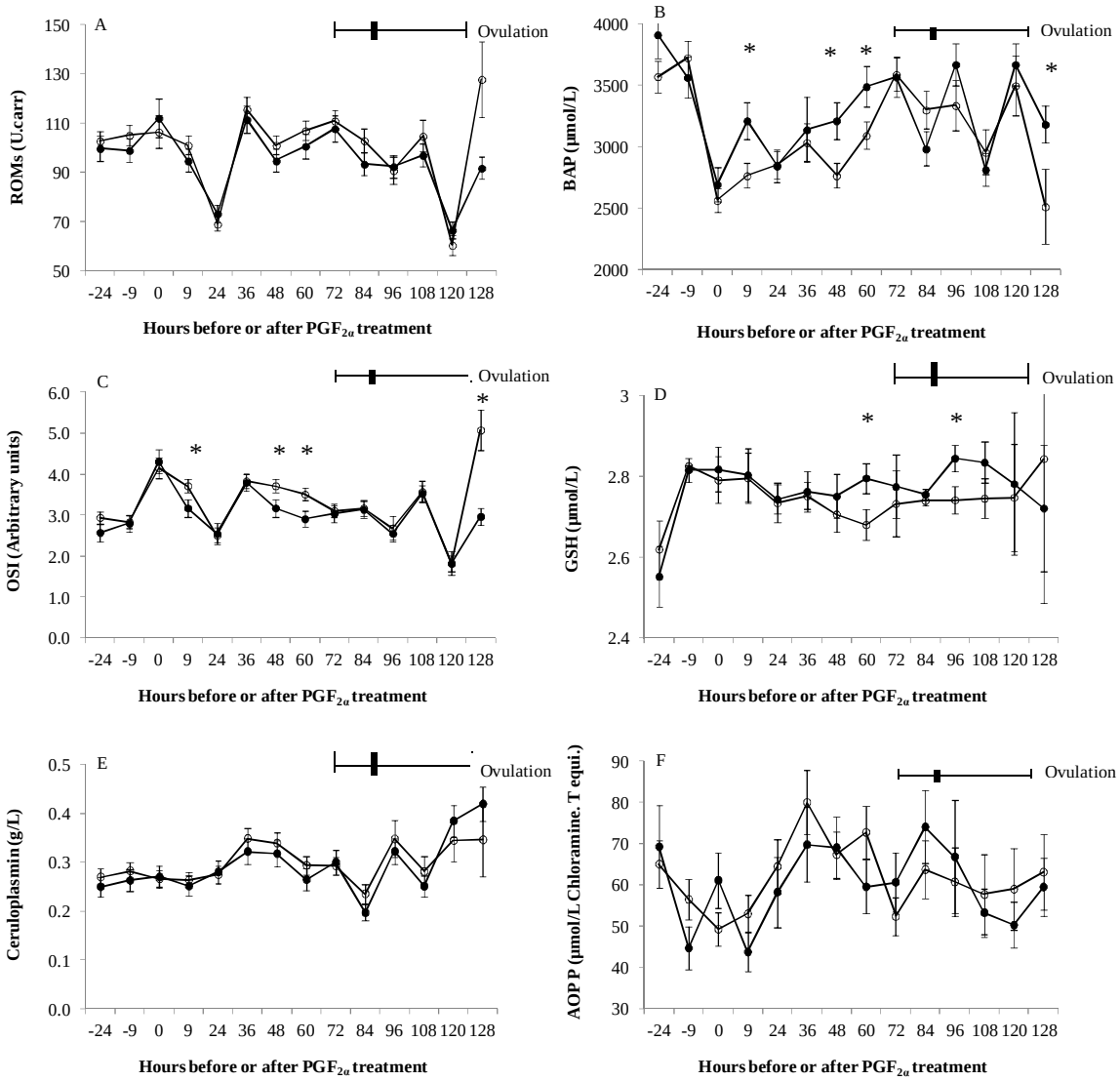

Figure 2. Plasma concentration (means ± s.e.) of oxidative stress markers in cows with ovulatory (○) and an-ovulatory (●) event in relation to time of $PGF_{2\alpha}$ treatment. A) Reactive oxygen metabolites (ROMs), B) biological antioxidant potential (BAP), C) oxidative stress index (ROMs/BAP), D) glutathione (GSH), E) ceruloplasmin and F) advanced oxidation protein products (AOPP). * indicates significant ($P < 0.05$) differences between cows with ovulatory and an-ovulatory event at the same time point. The horizontal bar indicates the range of time of ovulation while the thick vertical bar within the line of horizontal bar indicates the average time of ovulation.

Circadian changes in OS biomarkers

Circadian changes in plasma concentrations of ROMs, BAP, OSI, GSH, AOPP and ceruloplasmin are shown in Figure 3. The highest values of ROMs were observed at 1800 hours ($P < 0.001$; Figure 3A) and the lowest during the night (2200 hours) and early morning (0200 hours and 0600 hours); whereas BAP was at its lowest levels

during 1800 hours and at its highest at 1000 hours (P = 0.017; Figure 3B). As a result OSI values were similar during most time of the day with the exception of 1800 hours when it reached its highest value (P = 0.036; Figure 3C). Plasma GSH concentration was observed at its lowest (P < 0.001) at 0200 hours and 0600 hours, and at its highest at 1400 hours (Figure 3D). The highest (P = 0.001) concentration of ceruloplasmin was observed at 0600 hours and 1000 hours; ceruloplasmin concentration decreased in the afternoon and then its values slowly increased overnight (Figure 3E). Plasma AOPP concentration was relatively stable throughout the day with its lowest values (Figure 3F; P = 0.001) observed in the afternoon (1400 hours).

Factors affecting the OS biomarkers

In the present study, plasma concentration of BAP and ceruloplasmin had a significant (P < 0.05) association with locomotion score. Cows with a locomotion score of 1.0 had a greater BAP and ceruloplasmin concentration than cows with a locomotion score of 1.5 (BAP: 3348 ± 46.7 vs 3150 ± 68.2 µmol/L; ceruloplasmin: 0.31 ± 0.02 vs 0.29 ± 0.01 g/L, respectively). Primiparous cows had higher ROMs compared with their multiparous herd mates (101.6 ± 2.6 vs 92.6 ± 2.3 U.Carr; P < 0.05). No effect of milk yield was noted for any of the biomarkers of OS measured in this study.

DISCUSSION

The present study reports differences in the antioxidant status between ovulated and an-ovulated oestrous cycles in lactating dairy cows. The data presented indicates that changes in antioxidant status observed during the preovulatory stage may have an essential role in the response to $PGF_{2\alpha}$ leading to an ovulatory event. A relatively higher concentration of P_4 was observed in an-ovulated cows compared with the ovulated herd mates between 48 and 72 hours after $PGF_{2\alpha}$. Higher concentration of P_4 in an-ovulated cows during 48 and 72 hours after $PGF_{2\alpha}$ might be related to the greater level of BAP compared with ovulated cows. This finding supports the result of Hayashi *et al*. (2003) who reported a delay in the decrease in P_4 release after the infusion of free radical scavengers in ewes and failure of luteinising hormone to induce P_4 secretion when the follicles were previously exposed to antioxidants (Shkolnik *et al*. 2011).

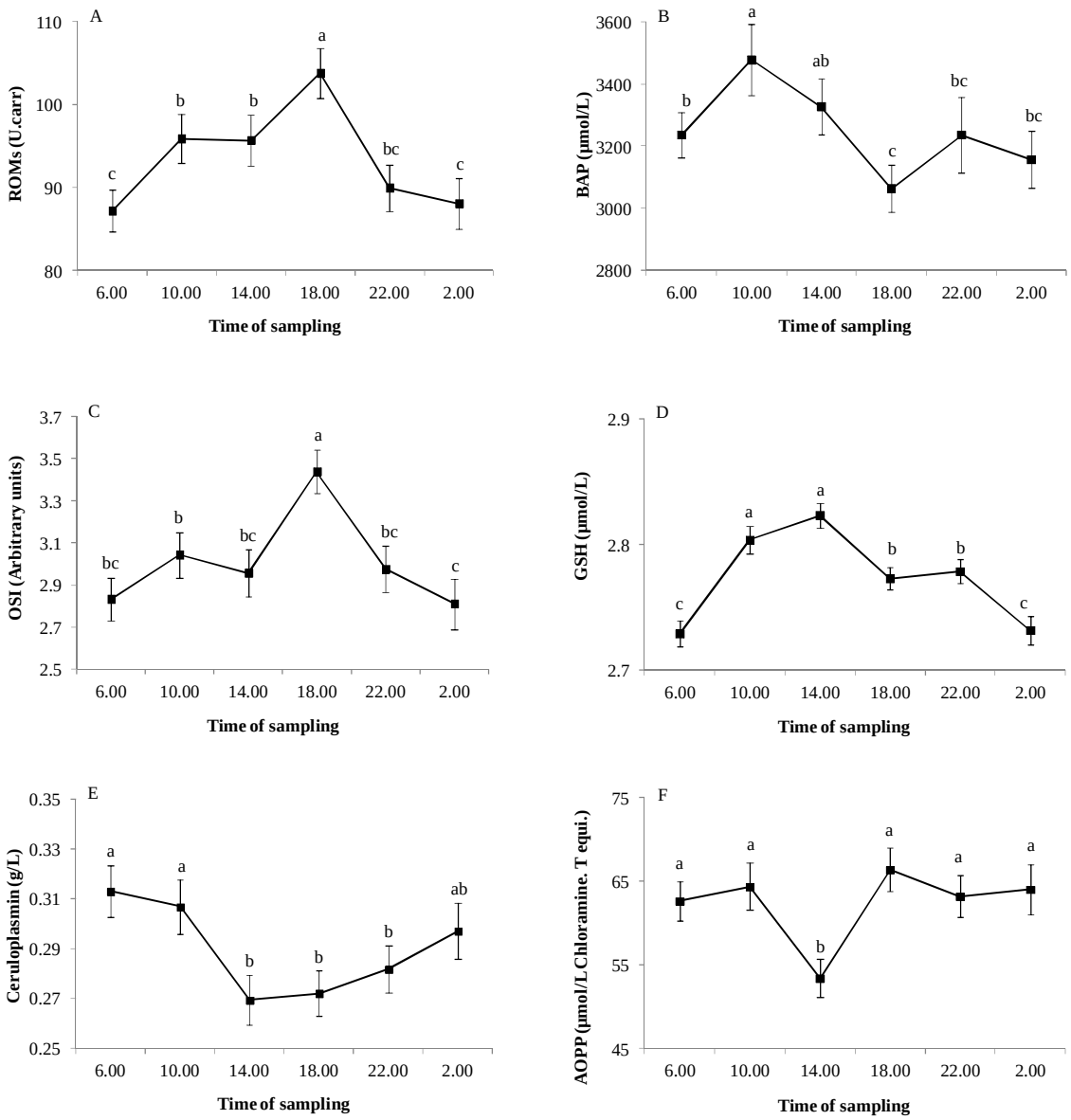

Figure 3. Plasma concentrations (means ± s.e.) of oxidative stress parameters at different times of day. A) Biological antioxidant potential (BAP), B) oxidative stress index (ROMs/BAP), C) reactive oxygen metabolites (ROMs), D) glutathione (GSH), E) ceruloplasmin and F) advanced oxidation protein products (AOPP). Ambient temperature and humidity were included as covariates in the model. The same lowercase letter indicates that there is no significantly difference between the time points (at P = 0.05).

As complete functional luteolysis is usually achieved 18–40 hours after $PGF_{2\alpha}$ treatment (Martins *et al*. 2011), ovulated cows displayed a P_4 profile consistent with the expected biological activity of this drug. Therefore, it is possible to speculate that complete luteal regression was achieved 48 h after $PGF_{2\alpha}$ treatment in all ovulated cows. Differences in P_4 concentrations between ovulated and an-ovulated cows were significant only at 24

and 48 hours after $PGF_{2\alpha}$ treatment, and this likely reflects a different pattern in luteal regression. However, differences in follicular diameters could be noted from 48 hours and became significant from 72 hours onward. This observation suggests that the differences in follicular development were a consequence of the different response of an-ovulated cows to the treatment. Possibly, the resulting incomplete or delayed luteolysis prevented ovulation in these animals. An-ovulated cows did not respond properly to the CIDR and $PGF_{2\alpha}$ treatment for several reasons or possibilities; however, this study was not designed to investigate the mechanism of ovulation failure in an-ovulated cows after CIDR and $PGF_{2\alpha}$ treatment.

It has been reported that $PGF_{2\alpha}$ induces an increase of ROMs in luteolysis in ewe (Hayashi *et al*. 2003) and a decrease in the serum P_4 concentrations early in the luteolysis process in rats (Sato *et al*. 1992). The inhibitory effect of $PGF_{2\alpha}$ on P_4 production by corpus luteum is partly mediated through the increased production of free radicals (Sugino 2005) though not reflected by changes in ROMs concentration in the present study; however, other free radicals might be responsible at an ovarian level. It is possible that an increase in ROMs might have occurred at a local level and not detectable in peripheral blood because of the scavenging action of antioxidant (BAP and GSH), which decreased in this study. Indeed, changes in the components of antioxidant systems are often not the cause but the consequence of the OS induced by higher free radical activity.

After synchronisation of the oestrous cycle, cows are expected to actively display behavioural sign of oestrus within 36 to 48 hours after $PGF_{2\alpha}$ treatment. A significant increase of BAP concentration in an-ovulated cows during 48 and 60 hours after $PGF_{2\alpha}$ treatment compared with their ovulated herd mates is likely due to a change in the physiological process. Skierska *et al*. (2008) also reported higher total antioxidant levels in an-ovulatory compared with ovulatory women. It could be presumed that the elevated level of plasma BAP in an-ovulatory cows between 48 and 60 hours after $PGF_{2\alpha}$ treatment indicate more antioxidant protection or a compensatory response to OS as measured by OSI in the present study. It can be hypothesised that $PGF_{2\alpha}$ treatment was unable to generate adequate amounts of free radicals in the an-ovulated cows and consequently resulted to ovulation failure. We observed a significant increase of OSI 48 and 60 hours after $PGF_{2\alpha}$ treatment for ovulated cows compared with an-ovulated cows.

The rapid increase of OSI from 48 to 60 hours onward might be related to the luteal regression. In this study, cows received exogenous P_4 for 1 week before $PGF_{2\alpha}$ treatment and it is possible that exogenous P_4 exerted an inhibitory action on the endogenous P_4 production by the CL, which may partially explain the low P_4 concentrations observed before $PGF_{2\alpha}$ treatment. Prospective studies may be warranted if an in-depth understanding of the changes in OS markers throughout a natural reproductive cycle in dairy cows is to be generated. Moreover, further studies comparing the oxidant and antioxidant status in ovarian fluids and utero-ovarian circulation would be required to allow a full understanding of the physiological role of OS biomarkers on the ovulatory process.

Plasma concentration of GSH was lower in ovulated cows compared with that of an-ovulated cows 60, 96 and 108 hours after $PGF_{2\alpha}$ treatment. Glutathione is the major non-protein sulfhydryl compound in mammalian cells and is generally considered as a good indicator of the blood scavenging capacity (Gabai *et al*. 2004). High OS might have contributed to the plasma GSH pattern in ovulated cows in the present study. Though it can be speculated that the depletion of systemic GSH levels could be a direct response to OS, an increase of plasma GSH in response to chronic OS has also been reported (Forman *et al*. 1997). Jérôme and Jean-François (2003) analysed the mRNA expression and enzymatic activities of the major antioxidant glutathione peroxidase (GSH-Px) in the bovine oviduct throughout the oestrous cycle and observed the highest levels of GSH and enzymatic activities for GSH-Px at the middle (10–12 days) and end (18–20 days) of the oestrous cycle.

Although in this study, ceruloplasmin concentrations did not differ significantly between ovulated and an-ovulated cows, the values were within the range reported in heifers (Golder *et al*. 2013, 2014). Despite the contribution of ceruloplasmin in iron homeostasis, it acts as a very effective antioxidant. Skierska *et al*. (2008) evaluated plasma ceruloplasmin ferroxidase activity in women with ovulatory and an-ovulatory cycles and reported a higher activity of ceruloplasmin ferroxidase in an-ovulating compared with the ovulating subjects. Plasma concentrations of AOPP reported in this study were within the range previously reported in cows (Celi *et al*. 2011). No differences of plasma AOPP and ceruloplasmin suggest that plasma concentrations of

these two biomarkers did not reflect physiological changes in the ovary and therefore no inflammatory events were present.

Plasma concentrations of OS markers were significantly associated with the time of sampling in the present study. A diurnal pattern of oxidative markers with acrophase in the middle of the photophase (1100 hours) has been reported in dry cows (Giannetto *et al*. 2010). The observed circadian changes in OSI might largely be attributed to the temporal pattern of ROMs concentration. In the present study, concentrate feeding during the time of milking may have contributed to increasing trend in ROMs concentration between early morning (0600 hours) and evening (1800 hours).

In the present study, the greater level of BAP in cows with a locomotion score of 1.0 supports our previous findings of higher concentration of BAP in healthy rams compared with rams with lesions in hooves (Talukder and Celi 2013). Primiparous cows had higher levels of plasma ROMs compared with multiparous cows. To the best of our knowledge there are no reports on the effect of parity on OS in dairy cows, however it is reasonable to assume that this observation could be due to the extensively reported metabolic challenge associated with initiation of lactation, growth and mammary gland development experienced by primiparous cows.

CONCLUSIONS

Our results suggest that the decrease in antioxidant status present during the peri-ovulatoty stage may be an essential event preceding the ovulatory response. The observed decrease in BAP and GSH may well be the consequence of OS caused by the luteolytic action of $PGF_{2\alpha}$ and the subsequent ovulatory event. The results also showed the circadian changes of OS markers with nocturnal acrophase in ROMs production and diurnal acrophase for antioxidant production.

REFERENCES

Agarwal A, Gupta S, Sharma R (2005) Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology* **3**, 1-21.

Al-Gubory KH, Fowler PA, Garrel C (2010) The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *The International Journal of Biochemistry and Cell Biology* **42**, 1634-1650.

Baker MA, Cerniglia GJ, Zaman A (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry* **190**, 360-365.

Celi P (2011*a*) Oxidative Stress in Ruminants. In 'Studies on Veterinary Medicine. Vol. 5.' (Eds L Mandelker, P Vajdovich.) pp. 191-231. (Humana Press, Totowa, NJ)

Celi P (2011*b*) Biomarkers of oxidative stress in ruminant medicine. *Immunopharmacology and Immunotoxicology* **33,** 233-240.

Celi P, Merlo M, Barbato O, Gabai G (2012) Relationship between oxidative stress and the success of artificial insemination in dairy cows in a pasture-based system. *The Veterinary Journal* **193**, 498-502.

Celi P, Merlo M, Da Dalt L, Stefani A, Barbato O, Gabai G (2011) Relationship between late embryonic mortality and the increase in plasma advanced oxidised protein products (AOPP) in dairy cows. *Reproduction, Fertility and Development* **23**, 527-533.

Forman HJ, Tian L, Liu RM (1997) Glutathione cycling in oxidative stress. In 'Lung biology in health and disease. Vol. 105. Oxygen, gene expression, and cellular function.' (Eds L Biadasz, L Clerch, DJ Massaro) pp. 99–121. (Marcel Dekker: New York)

Gabai G, Testoni S, Piccinini R, Marinelli L, Stradaioli G (2004) Oxidative stress in primiparous cows in relation to dietary starch and the progress of lactation. *Animal Science* **79**, 99-108.

Giannetto C, Fazio F, Assenza A, Caola G, Pennisi P, Piccione G (2010) Circadian rhythms of redox states and total locomotor activity in dairy cattle. *Czech Journal of Animal Science* **55**, 183-189.

Golder HM, Celi P, Rabiee AR, Lean IJ (2014) Effects of feed additives on rumen and blood profiles during a starch and fructose challenge. *Journal of Dairy Science* **97**, 985-1004.

Golder HM, Lean IJ, Rabiee AR, King R, Celi P (2013) Effects of grain, fructose, and histidine feeding on endotoxin and oxidative stress measures in dairy heifers. *Journal of Dairy Science* **96**, 7881-7891.

Hayashi K, Miyamoto A, Konari A, Ohtani M, Fukui Y (2003) Effect of local interaction of reactive oxygen species with prostaglandin F2α on the release of progesterone in ovine corpora lutea in vivo. *Theriogenology* **59**, 1335-1344.

Hockey CD, Morton JM, Norman ST, Mcgowan MR (2010) Improved prediction of ovulation time may increase pregnancy rates to artificial insemination in lactating dairy cattle. *Reproduction in Domestic Animals* **45**, e239-e248.

Jérôme L, Jean-François B (2003) Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. *Biology of Reproduction* **68**, 1157-1164.

Martins JPN, Policelli RK, Pursley JR (2011) Luteolytic effects of cloprostenol sodium in lactating dairy cows treated with G6G/Ovsynch. *Journal of Dairy Science* **94**, 2806-2814.

Miller JK, Brzezinska-Slebodzinska E, Madsen FC (1993) Oxidative stress, antioxidants, and animal function. *Journal of Dairy Science* **76**, 2812-2823.

Rizzo A, Minoia G, Trisolini C, Manca R, Sciorsci RL (2007) Concentrations of free radicals and beta-endorphins in repeat breeder cows. *Animal Reproduction Science* **100**, 257-263.

Rizzo A, Minoia G, Trisolini C, Mutinati M, Spedicato M, Jirillo F, Sciorsci RL (2009) Reactive Oxygen Species (ROS): Involvement in bovine follicular cysts etiopathogenesis. *Immunopharmacology and Immunotoxicology* **31**, 631-635.

Sato EF, Kobuchi H, Edashige K, Takahashi M, Yoshioka T, Utsumi K, Inoue M (1992) Dynamic aspects of ovarian superoxide dismutase isozymes during the ovulatory process in the rat. *FEBS Letters* **303**, 121-125.

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N (2011) Reactive oxygen species are indispensable in ovulation. *Proceedings of the National Academy of Sciences* **108**, 1462-1467.

Skierska E, Kęska A, Lutoslawska G (2008) Plasma hormones, ceruloplasmin ferroxidase activity and total antioxidant status (TAS) concentration in regularly menstruating women of reproductive age with ovulatory and anovulatory menstrual cycles. *Medicina Sportiva* **12**, 14-19.

Sugino N (2005) Reactive oxygen species in ovarian physiology. *Reproductive Medicine and Biology* **4**, 31-44.

Sunderman FWJ, Nomoto S (1970) Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. *Clinical Chemistry* **16**, 903-910.

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2014*a*) Factors affecting reproductive performance of dairy cows in a pasture-based, automatic milking system research farm: a retrospective, single-cohort study. *Animal Production Science* **55,** 31-41.

Talukder S, Celi P (2013) Use of digital infrared thermography and oxidative stress biomarkers as a diagnostic tool to diagnose interdigital dermatitis in sheep. *Journal of Dairy Science*, **9**6, E-Suppl. 1, P 382.

Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P (2014*b*) Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture based system. *Theriogenology* **81**, 925-935.

Witko-Sarsat V, Friedlander M, Khoa TN, Capeillère-Blandin C, Nguyen AT, Canteloup S, Dayer J-M, Jungers P, Drüeke T, Descamps-Latscha B (1998) Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure1, 2. *The Journal of Immunology* **161**, 2524-2532.

CHAPTER 5

Evaluation of Infrared Thermography (IRT) Body Temperature and Collar Mounted Accelerometer and Acoustic Technology for Predicting Time of Ovulation of Cows in a Pasture-Based System

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OVERVIEW OF CHAPTER 5

In Chapter 3, results presented suggested that IRT showed some potential as an oestrus detection aid with acceptable levels of sensitivity reported. However, the specificities and positive predictive values were lower than desirable. This chapter tested the hypothesis that the specificity of IRT in detecting an ovulation event could be improved if body parts that were less likely to be contaminated by faecal matter or affected by moisture were monitored. In addition, another oestrus detection aid, accelerometer (monitors activity and rumination level) was also evaluated.

ABSTRACT

This study was conducted to test the hypothesis that the specificity of infrared thermography (IRT) in detecting cows about to ovulate could be improved by using different body parts that are less likely to be contaminated by faecal matter. In addition, the combined activity and rumination data captured by accelerometers were evaluated to provide a more accurate indication of ovulation than the activity and rumination data alone. Thermal images of 30 cows were captured for different body areas (eye, ear, muzzle and vulva) twice daily after am and pm milking sessions during the entire experimental period. Milk progesterone data and insemination records were used to determine the date of ovulation. Cows were fitted SCR heat and rumination long distance tags (SCR HR LD) for 1 month. Activity and rumination-based oestrus alerts were initially identified using default threshold values set by the manufacturer; however, a range of thresholds was also created and tested for both activity and rumination to determine the potential for higher levels of accuracy of ovulation detection. Visual assessment of mounting indicators resulted in 75% sensitivity (Se), 100% specificity (Sp) and 100% positive predictive value (PPV). Overall, IRT showed poor performance for detecting cows about to ovulate. Vulval temperature resulted in the greatest (80%) Sp but the poorest (21%) Se compared to the IRT temperatures of other body areas. The SCR HR LD tags default threshold value resulted in 78% Se, 57% Sp and 70% PPV. Lowering the activity threshold from the default value, improved the sensitivity but created a large number of false positives, which resulted in a decrease in specificity. Lowering the activity threshold to 20, resulted in a detection performance of 80% Se, 94% Sp and 67% PPV while the rumination levels achieved 35% Se, 69% Sp and 14% PPV. The area under the curve for the activity level, rumination level and the combined measures of activity and rumination levels were 0.82, 0.54 and 0.75 respectively. Alerts generated by SCR HR LD tags based on a lower activity threshold level had high sensitivity and may be able to detect a high proportion of cows in ovulatory periods in pasture-based system; however, the specificities and positive predictive value were lower than the visual assessment of mounting indicators.

Keywords: automated oestrus detection, SCR HR LD tags, infrared camera, dairy cow.

INTRODUCTION

Detection of oestrus is a significant component of farm profitability in artificially bred dairy herds (Saint-Dizier and Chastant-Maillard 2011) but it is gradually being recognized as a major limiting factor of reproductive performance in modern dairy herds Lucy 2007; Holman *et al.* 2011). The traditional method of detecting cows in oestrus is visual observation of the behavioural signs for example, standing to be mounted by herd mate(s) (Diskin and Sreenan 2000). Visual observation is typically aided by mounting detectors attached to the tail head. The friction generated or pressure applied when the cow is mounted by herd mate(s) reveals a visual change to the detection aid which can be detected by the herdsperson (Holman *et al.* 2011). Achieving efficient oestrus detection by visual observation depends on the timing, duration and frequency of observation (Roelofs *et al.* 2010). In addition, discrete behavioural signs of oestrus, absence of standing mounts for up to 60% of ovulations and the shorter duration of oestrus in modern, high-yielding dairy cows make visual detection of oestrus more difficult (Holman *et al.* 2011; Saint-Dizier and Chastant-Maillard 2011; Kamphuis *et al.* 2012). This, combined with the increase of herd size and the need to improve labour efficiency, has increased the reliance on automated oestrus detection tools to reduce dependence on humans and increase oestrus detection rates.

An increase in locomotive activity is a behavioural change which commonly occurs when a cow is in oestrus (Roelofs *et al.* 2010). In addition, cows in or approaching oestrus spend less time in feeding (Diskin and Sreenan 2000) which may result in reduced dry matter intake and hence reduced rumination levels. Numerous studies have reported the use of activity meters (using pedometer or accelerometer technology) for detecting cows in oestrus automatically in either indoor systems (de Mol *et al.* 1997; At-Taras and Spahr 2001; Roelofs *et al.* 2005) or in pasture-based systems (Kamphuis *et al.* 2012). Using a commercially available technology (Hi Tag, SCR Engineers Ltd., Netanya, Israel) it is possible to measure the activity level (AL) and rumination level (RL) of an individual animal. A previous study (Kamphuis *et al.* 2012) reported on the use of a similar accelerometer technology for oestrus detection in a pasture-based seasonally-calved dairy herd, however, this study evaluated the AL rather than analyzing the AL and RL as a combined oestrus detection method. In addition, whilst the accelerometer technology may be similar, the interpretation of the data through this

technology may differ substantially between studies. Combined activity and rumination data generated by the SCR heat and rumination (HR) long distance (LD) tags may be well suited to oestrus detection for grazing cows.

Body temperature changes during the oestrus cycle (Kyle *et al.* 1998); decreasing approximately 2 days before oestrus and then increasing at the time of the luteinizing hormone (LH) peak (Fisher *et al.* 2008). The temperature rhythms during the oestrous cycle have been measured by rectal (Wrenn *et al.* 1958) and vaginal thermometry (Clapper *et al.* 1990; Mosher *et al.* 1990) and have been shown to be associated with the LH surge and ovulation. However, these methods are time consuming and disruptive for the animal (Hoffmann *et al.* 2013). Whilst they are also invasive, the use of ingested boluses (to measure temperature in the reticulum) and vaginal devices can monitor the core body temperature continuously (Saint-Dizier and Chastant-Maillard 2011). Non-invasive diagnostic tools such as infrared thermography (IRT) may provide an opportunity to measure body surface temperatures and may also be suited to automation if they prove to be useful oestrus detection aids. Talukder *et al.* (2014*b*) reported that IRT vulval temperature resulted in higher sensitivity but lower specificity for predicting a cow about to ovulate compared to visual observation and Estrotect® (Heat Detector patch; Rockway Inc., Spring Valley, WI). Due to occlusion of vulva by the hanging tail, monitoring of vulval temperatures may limit the practical application of IRT on farm. Head, and especially the eye and back of the ear have been reported to be promising body regions for IRT because these regions experience less visual obstruction compared to the vulva which may render them more useful for automated IRT oestrus detection (Hoffmann *et al.* 2013).

The present study tested the hypothesis that the specificity of IRT in detecting an ovulation event could be improved if body parts that are less likely to be contaminated by faecal matter are monitored. An additional hypothesis tested in this investigation was that the combined activity and rumination data captured by the SCR HR LD tags would provide a more accurate indication of ovulation than the activity and rumination data alone.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee (The University of Sydney, NSW, Australia, approval number: N00/9-2012/1/5829).

Animals, experimental design and data collection

This study was conducted on 30 (11 primiparous and 19 multiparous), healthy (based on farm records) lactating, cycling Holstein Friesian dairy cows averaging 60 ± 17 days in milk (DIM) and producing 33 ± 6 kg (mean ± SD [standard deviation]) of milk per day (during the week prior to the study commencement). The study was performed during October and November 2013 (Spring) for 1 month at the University of Sydney's Corstorphine dairy farm, Camden, NSW, Australia. The mean air temperature, maximum air temperature and maximum relative humidity during the experimental period were 18°C, 37°C and 99%, respectively. All cows grazed kikuyu grass (*Pennisetum clandestinum*), over sown with short rotation ryegrass (*Lolium multiflorum*), and perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The cows had access to pasture between the two milkings and were grazed in accordance with the best practice of using pasture on offer and leaf stage as the criterion to flag time to graze (Fulkerson and Donaghy 2001). On the day before the study commencement, transrectal ultrasound scanning was performed to confirm the presence of follicle(s) and absence of any abnormal structures (cysts) and gynaecological abnormalities using a portable scanner (Ibex Pro portable ultrasound; E.I. Medical Imaging, Loveland, Colorado, USA).

Oestrus was monitored by visual observation of the mounting indicators (i.e. the degree of removal of colour in Estrotect® during each am and pm milking. Inseminations were performed at two time periods per day soon after the milkings (am and pm). If a cow was identified as displaying the complete activation of Estrotect®, she was considered to be in oestrus and was inseminated at the next insemination period.

Cows were assessed for body condition score (BCS), using a scoring system from 1 to 5 incorporating 0.5 scores (Edmonson *et al.* 1989) and for locomotion score (LS) using 1 to 5 system (Sprecher *et al.* 1997) on a weekly basis. Lactation number, daily milk production, days in milk (DIM), and body weight were captured for each animal using

the electronic data support software, DelPro (Tumba, Sweden). Vaginal temperature was recorded with a digital thermometer (Microlife AG, 9443 Widnau, Switzerland) at each IRT data capture session.

The variables possibly affecting the accuracy of ovulation detection are listed in Table 1. Continuous predictor variables were tested for linear association with ovulation detection method; if there was no linear effect, continuous predictor variables were then categorised based on the median values for easier interpretation.

Table 1. Factors assessed for their effects on the performance of oestrus detection method (n = 1412).

Factors	Classes	Class description
Days in milk	2	≤ 68 (Low) > 68 (High)
Body condition score	2	≤ 2 (Low) > 2 (Acceptable)
Locomotion score	2	1 (Healthy) > 1 (Lame)
Lactation number	Continuous	-
Daily milk production (kg)	2	≤ 14 (Low) > 14 (High)
Milk protein %	2	≤ 2.88 (Low) > 2.88 (High)
Milk fat %	2	≤ 4.03 (Low) > 4.03 (High)

Collection of activity and rumination data measured by SCR HR LD tags

During the experimental period, all cows were fitted with SCR HR LD tags (Hi Tag, SCR Engineers Ltd., Netanya, Israel). These collars consisted of an accelerometer to quantify activity and a microphone to monitor rumination in 2 hour (h) time blocks, as validated by Schirmann *et al.* (2009). The neck collars were fitted 7 days before the start of the trial to establish baseline thresholds for AL and RL. The device continuously monitored the individual cow AL and RL in 2 h time blocks, with data downloaded

automatically to the support software on a computer located at the dairy. A base unit receiver located above the entrance of the milking parlour retrieved the data (via radio communication) into a control unit. Using a mathematical algorithm, a weighted index of AL and RL was calculated by the software that expressed the momentary deviation of the AL and RL from the average activity and rumination respectively in the same time period during the past 7 days (Kamphuis *et al.* 2012). System alerts were automatically generated based on the weighted AL and RL index. In addition, if the weighted activity in any of the 2 h time blocks exceeded a user-defined threshold value (factory default activity threshold value was 30) an activity alert was reported for that cow at the next milking. The peak weighted AL (the greatest weighted AL in 2 h time blocks within 24 h) and the minimum weighted RL (the lowest weighted RL in 2 h time blocks within 24 h) were used to test the sensitivity (Se), specificity (Sp) and positive predictive value (PPV) of different thresholds by comparison with the system-generated alerts. Ranges of threshold levels were created using 10 unit increments with values ranging from 0 to 100 (<0, <10, <20, <30, <40, <50, <60, <70, <80, <90 and <100). The threshold <10 means the values from zero to 9; the similar approach was applicable for the other thresholds. The generated threshold values (of both AL and RL) were then analyzed to determine their effect on ovulation detection performance.

Infrared thermography of different body areas

Thermal scanning of cows was performed using an infrared camera (FLIR, 620 series, FLIR Systems Co. Ltd., St Leonards, NSW, Australia) twice daily after am and pm milking sessions during the entire experimental period. The vulva, eyes, ears and muzzle surface temperatures were measured at each IRT scanning session. Seven days of IRT temperatures were used as a baseline temperature for each of the body areas. Before the thermal scanning of the vulva, the vulva was gently wiped with dry paper towels to remove faecal matter and the tail was held aside. Thermal imaging was performed from a fixed distance of approximately 1 metre from the animal as described in our previous study (Talukder *et al.* 2014*b*). Before each thermal scanning session, the emissivity value was set to 0.98 and thermograph resolution was calibrated to ambient temperature and humidity as per manufacturer's recommendation using a solar weather station (Oregon Scientific International Ltd., Los Angeles, USA).

Images were stored in a memory card and then transferred to a laptop for analysis using ThermaCAM Researcher Professional 2.9. The software allowed the user to determine the surface temperature in a user-defined field of interest on the image and calculated the minimum, maximum, and average temperatures and standard deviation (through software recognition of each pixel within the defined area) for each of these 'fields'. Maximum IRT temperatures of body surfaces were used in line with previous studies (Schaefer *et al.* 2004; Talukder *et al.* 2014*b*). In addition, we observed better results using the maximum IRT temperature compared to average or minimum IRT temperature. Vulval and muzzle images were analyzed by adopting the approach mentioned in Talukder *et al.* (2014*b*) while free hand drawn geometrical polygonal shapes covering the entire ear was used for the calculation of ear temperature. The eye area was oval shaped and encompassed the entire eye, eyelid and a narrow band of skin around the eye (Figure 1). A total of 1140, 1115, 1113, 1114, 1114 and 1112 images of vulva, left eye, right eye, left ear, right ear and muzzle respectively were captured and included in the data analysis.

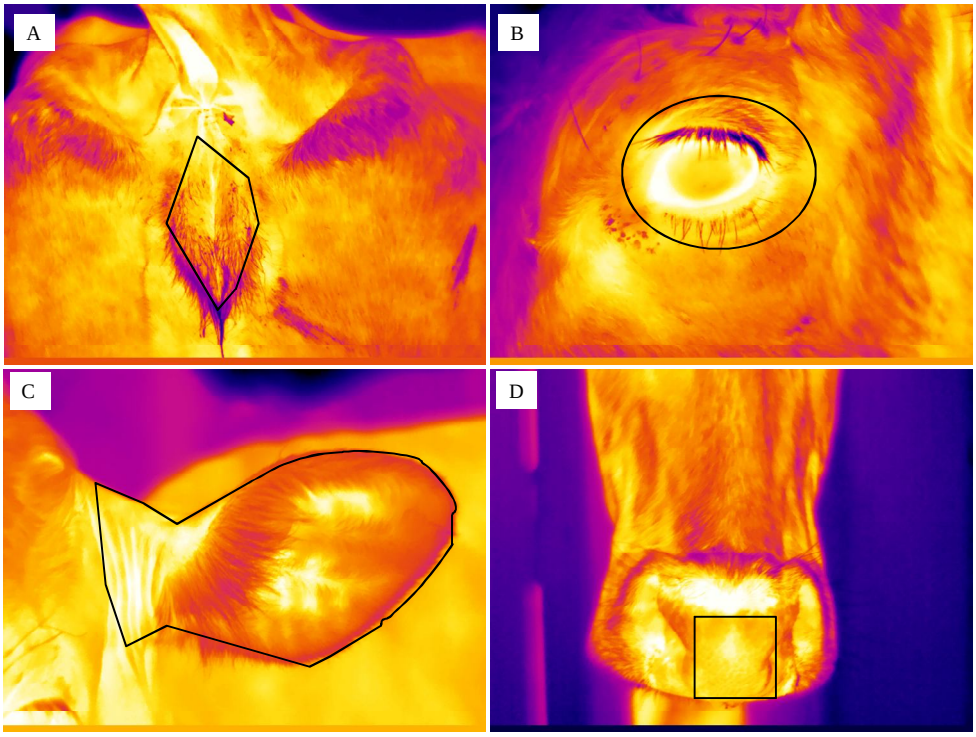

Figure 1. Hand drawn area of vulva (A), eye (B), ear (C) and muzzle (D) which defined the temperature data area used by the support software.

Milk sampling, herd testing and hormonal analysis

Composite milk samples were collected into a 5 mL glass vial and a 15 mL plastic sample bottle thrice weekly on Monday, Wednesday and Friday during pm milking. The 5 mL glass vials were stored at 4°C until sent to a laboratory for herd-testing (compositional analyses) while the 15 mL plastic sample bottles were frozen at -20°C pending analysis of progesterone (P_4). Herd test results (milk fat % and protein %) of collected milk samples were obtained from Dairy Express (University of New England, Armidale, NSW).

Milk samples were analyzed for P_4 concentration using a solid phase RIA (Coat-A-Count Progesterone; Siemens Medical Solutions Diagnostics, Los Angeles, CA) validated for milk samples (Srikandakumar *et al.* 1986). The mean intra-assay and inter-assay coefficients of variation were 2.88% and 5.75% respectively. The detection limit of the assay was 0.05 ng/mL.

Gold standard definition of ovulation

To identify when cows were likely to have ovulated, individual milk P_4 profiles were used to confirm the time between ovulations and the associated timing of oestrus (Aungier *et al.* 2012). A trough-like curve in these P_4 profiles was assumed to indicate a periovulatory event (luteolysis followed by ovulation with subsequent development of the new functional corpus luteum) and was initially identified as evidence of an ovulation event (Figure 2). The following approach reported by Kamphius *et al.* (2012) was used to determine the ovulation date: 1) the day of AI; 2) the centre of the trough like curve of P_4 profiles in the absence of an AI date (Figure 2).

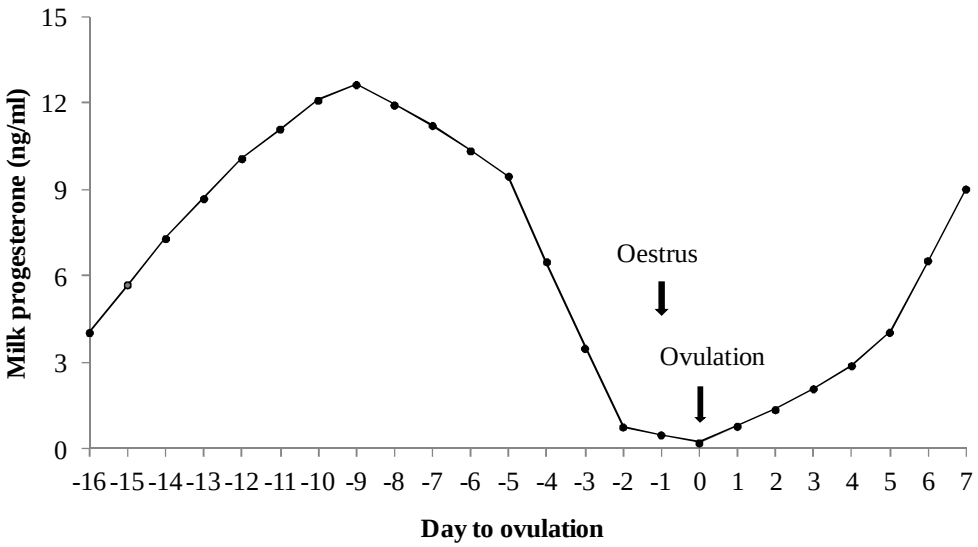

Figure 2. Example of a cow with ovulation event (day 0) based on milk progesterone concentration.

Algorithm and steps for detection and description of IRT oestrus temperature

Diurnal variation in ambient temperature and humidity during the am and pm milking sessions necessitated adjustment of vulval maximum temperatures as described in Talukder *et al.*(2014*b*). In brief, the following steps were followed:

Step 1: Fitting linear mixed model

A linear mixed model was fitted to the vulval maximum temperature data using a restricted maximum likelihood (REML) procedure. Along with the ambient temperature and humidity, a list of variables, namely days in milk, milking session (am and pm), body weight, BCS, LS, and parity, were used for the adjustment in the same model. Any variable having a *P*-value > 0.05 was excluded from the model in one step and refitted using a REML procedure with the following form:

$$\text{VulvaT} = \text{constant} + \beta_A\,(\text{AmbT} - \overline{\text{AmbT}}) + \beta_H\,(\text{Hum} - \overline{\text{Hum}}) + \sum_k \beta_k\,(\text{Cov}_k - \overline{\text{Cov}_k}) + \text{Cow} + \varepsilon$$

where VulvaT = vulval temperature; constant = overall mean vulval temperature (averaged over the entire data set); AmbT = ambient temperature (covariate); Hum = humidity (covariate); Cov_k = days in milk, milking session (am and pm), body weight, BCS, LS, parity (covariates); Cow = random effect of cow and ε = random error. The

terms $\overline{\text{AmbT}}$, $\overline{\text{Hum}}$, and $\overline{\text{Cov}_k}$ represent the averages of these values, i.e. the covariates are expressed as deviations from their means in the model. This model, as well as subsequent models, was fitted using GENSTAT 16th edition (VSN International, Hertfordshire, UK).

Step 2: Adjustment of vulval temperature using regression coefficients

Vulval temperatures (of individual cow observations) were adjusted to the mean ambient temperature, mean humidity, and mean of other covariates, over the study period as follows:

$$\text{VulvaT}_{adj1} = \text{VulvaT} - b_A(\text{AmbT} - \overline{\text{AmbT}}) - b_H(\text{Hum} - \overline{\text{Hum}}) - \sum_k b_k(\text{Cov}_k - \overline{\text{Cov}_k})$$

where, b_A, b_H and b_k are the estimated regression coefficients from the REML procedure (step 1).

Step 3: Adjustment for baseline temperature

The empirical baseline temperature data was used as an adjustment with the following form.

$$\text{VulvaT}_{adj2} = \text{VulvaT}_{adj1} - \text{VulvaT}_B$$

where VulvaT_B is the average of the raw baseline vulval temperatures for a cow during the first 7 days. VulvaT_{adj2} is used for testing different thresholds and calculation of sensitivity and specificity.

Other body surfaces (left eye, right eye, left ear, right ear and muzzle) maximum temperatures were adjusted similarly as described above for vulval temperature.

Algorithm optimization

Different thresholds of 1, 1.25, and 1.50 SD above the baseline temperature were set in body surfaces temperature adjustment 2 to determine the oestrus-related increase of IRT temperature. A period when the adjusted vulval temperature exceeds the threshold for any observation time was considered as an indication of an oestrus-related increase of temperature and defined as an IRT oestrus alert.

Simultaneous use of multiple IRT measures

In order to determine if the simultaneous use of multiple IRT measures of different body areas provided an improved oestrous test procedure compared to individual measures, a logistic generalized linear mixed model (GLMM) was fitted to the binary oestrous data, with the following form:

$$\log_e\left(\frac{\pi}{1-\pi}\right) = \text{constant} + \beta_V \text{VulvaT} + \beta_{LEye}\text{LEyeT} + \beta_{REye}\text{REyeT} + \beta_{LEar}\text{LEarT} + \beta_{REar}\text{REarT} + \beta_M \text{MuzzleT} + \text{Cow}$$

where π was the probability of the cow being in oestrus, VulvaT, LEyeT, REyeT, LEarT, REarT, and MuzzleT were the results from adjustment 2 of vulval, left eye, right eye, left ear, right ear, and muzzle temperatures, respectively, and where Cow was a random cow effect. Having fitted the model, different logit (=$\log_e[\pi/(1-\pi)]$) thresholds (2, 2.5 and 3) of fitted values were examined for sensitivity and specificity and positive predictive value.

A similar approach was used for combined measures of AL and RL measured by SCR HR LD tags.

Definition of true positive, true negative, false positive and false negative

Ovulated cows (with or without increased body surface temperature at different thresholds) at any observation session were considered as true positive (TP) and false negative (FN) respectively. An-ovulated cows (with or without increased temperature) for any observation session were considered as false positive (FP) and true negative (TN), respectively.

Test status (TP, TN, FP, FN) of SCR HR LD tags as defined earlier were calculated using the same method described for calculation of IRT body surface temperature.

Calculation of sensitivity, specificity and positive predictive value

Sensitivity (Se) was defined as the proportion of true ovulation events in which at least one IRT oestrus alert occurred (as defined by the binary classification) and was calculated by the formula, TP/(TP+FN). Specificity (Sp) was defined as the proportion of true anovulation events in which no IRT oestrus alert occurred and was calculated by

the formula, TN/(TN+FP). Positive predictive value (PPV) was calculated as TP/(TP+FP).

Sensitivity, specificity and positive predictive value of SCR HR LD tags and Estrotect® were calculated using the same method described for calculation of IRT oestrus alert.

Receiver operator characteristic (ROC) curve analysis for IRT temperature and SCR HR LD tags

The sensitivity and specificity were calculated at several thresholds and the overall utility of the method was assessed through construction of a receiver operator characteristic (ROC) curve (Hanley and McNeil 1982), whereby the sensitivity vs (1 – specificity) was plotted at each threshold. After the points were connected with lines, the area under the curve (AUC) was calculated using a simple trapezium method. The area under the curve (AUC) ($0 < AUC < 1$) was used as a measure of test performance, with $AUC = 0.5$ corresponding to a test performance no better than chance. More specifically, the AUC was used as a measure of the probability that a randomly selected ovulated cow had a test result indicating greater probability (of being in ovulation) than that for a randomly chosen an-ovulated cow.

Statistical analysis of factors associated with ovulation detection performance

A multivariable logistic GLMM was used to identify factors (listed in Table 1) associated with an oestrous event (binary outcome variable). Cow identification number was considered as a random factor. Animal factors (lactation number, BCS, LS, DIM and body weight) and production factors (milk production, milk fat %, and milk protein %) were considered as fixed effects. A backwards, stepwise model-building strategy was used i.e. a full model was built and then each variable was removed in turn based on the Wald's test ($P < 0.05$). After building the final model, the interaction effect between the factors were tested as described in Talukder *et al.* (2014*a*)

RESULTS

Of the 30 cows enrolled in this study, 20 cows were found to have an ovulation event during the study period according to the P_4 profile combined with AI date. Of the 20 ovulated cows, visual appraisal of the Estrotect® assisted detection of the oestrus event occurred in 75% (n = 15) of cows. The descriptive statistics of daily milk production, milk composition, AL and RL are presented in Table 2.

Descriptive analyses of IRT temperature of different body areas, vaginal temperature and their correlations

Of the IRT temperature of the various body areas evaluated in the present study, the eyes recorded the highest mean temperature (left eye: 36.3 ± 2.1°C; right eye: 36.2 ± 2.1°C) while the vulva had the second highest (35.0 ± 2.4°C) mean temperature (Table 2). Vaginal temperature (measured by thermometer) was higher than all of the IRT temperatures (Table 2). Strong correlations were reported for all IRT temperatures of the various body areas (r > 0.8; P < 0.001; Table 3). Infrared thermography temperatures were positively correlated with the ambient temperature but negatively correlated with the relative humidity (P < 0.001). Vaginal temperature had a weak but positive correlation with IRT temperature and ambient conditions (P < 0.001; Table 3).

Sensitivity, specificity and positive predictive value of IRT oestrus alert

Figure 3 shows the test results for the IRT traits presented in a ROC curve. It shows the results from each body area, as well the results from all body area using the logistic GLMM. Sensitivity and specificity of IRT was influenced by the thresholds of temperature rise used to define the occurrence of an oestrus alert. As the thresholds were increased, a decrease in sensitivity and an increase in specificity were observed. Vulval IRT temperature resulted in the highest specificity but lowest sensitivity compared to those of other body areas. At a threshold of 1 SD, muzzle temperature had the greatest sensitivity of the measured individual body parts (56.8%; Figure 3). The combination of all IRT measures resulted in the greatest sensitivity (93.7%) but the poorest specificity (6.7%) (Figure 3).

Table 2. Descriptive statistics of IRT temperature (°C) of different body areas, activity level, rumination level, daily milk production and milk composition.

Parameters	*n*	Mean	Median	SD	Lower quartile (Q1)	Upper quartile (Q3)
Vaginal temp. (°C)	1142	38.5	38.5	0.5	38.2	38.8
Vulval temp. (°C)	1140	35.0	35.3	2.4	33.9	36.6
Left eye temp. (°C)	1115	36.3	36.5	2.2	35.6	37.7
Right eye temp. (°C)	1113	36.2	36.4	2.1	35.6	37.4
Left ear temp. (°C)	1114	34.8	34.5	4.4	32.3	37.2
Right ear temp. (°C)	1114	34.6	34.7	3.3	32.9	36.6
Muzzle temp. (°C)	1112	32.8	33.0	3.1	31.0	34.9
Daily activity level	14990	835.2	826.0	162.4	735.0	924.0
Daily rumination level (minutes)	14990	502.0	513.5	99.9	437.0	569.0
Daily milk production (kg)	665	28.7	28.9	6.2	24.9	32.9
Milk protein %	665	2.7	2.7	0.2	2.6	2.9
Milk fat %	665	3.9	3.9	0.90	3.3	4.6

Table 3. Pearson correlation coefficients among ambient temperature, humidity, vaginal temperature measured by thermometer and IRT temperature of different body areas.

Parameters	Ambient temp.	Ambient humidity	Vaginal temp.	IRT vulval temp.	IRT left eye temp.	IRT right eye temp.	IRT left ear temp.	IRT right ear temp.	IRT muzzle temp.
Ambient temp.	-								
Ambient humidity	-0.68*	-							
Vaginal temp.	0.39*	-0.27*	-						
IRT vulval temp.	0.59*	-0.49*	0.40*	-					
IRT left eye temp.	0.60*	-0.57*	0.41*	0.87*	-				
IRT right eye temp.	0.58*	-0.57*	0.39*	0.87*	0.97*	-			
IRT left ear temp.	0.65*	-0.55*	0.44*	0.82*	0.89*	0.86*	-		
IRT right ear temp.	0.64*	-0.56*	0.46*	0.85*	0.92*	0.90*	0.91*	-	
IRT muzzle temp.	0.65*	-0.50*	0.46*	0.82*	0.89*	0.87*	0.91*	0.91*	-

*****Indicates significance at $P < 0.001$.

Sensitivity, specificity and positive predictive value of SCR HR LD tags

The SCR default threshold value resulted in 78% Se, 57% Sp and 70% PPV. The performance of SCR activity and rumination levels for detecting cows in oestrus at different thresholds is depicted in Figure 4. An increase in threshold was accompanied with a decrease in sensitivity and an increase in specificity and positive predictive value. At threshold < 20 (0-19), AL had a detection performance of 80.0% Se, 94.3% Sp and 66.7% PPV while threshold < 30 (20-29) resulted in 65.0% Se, 97.1% Sp and 76.5% PPV. When the threshold was lowered to < 10 (0-9) minutes, the RL presented 75% sensitivity but the specificity decreased dramatically to 36% (Figure 4).

As the performance of AL and RL were different, further results for these level types are reported separately using ROC curves (Figure 5). The ROC curve demonstrates the 'trade–off' between sensitivity and specificity. An increase in sensitivity resulted in an accompanying increase in FP cases, which corresponds to a decrease in specificity. At a fixed FP rate of 1% (specificity of 99%), sensitivity levels were 40% and 0% for AL and RL respectively (Figure 5). The AUC for the AL, RL and the combined measures of AL and RL were 0.82, 0.54 and 0.75 respectively, indicating that the AL generated more accurate alerts for true oestrus events than the RL or an alert which combined the measures of both the RL and AL.

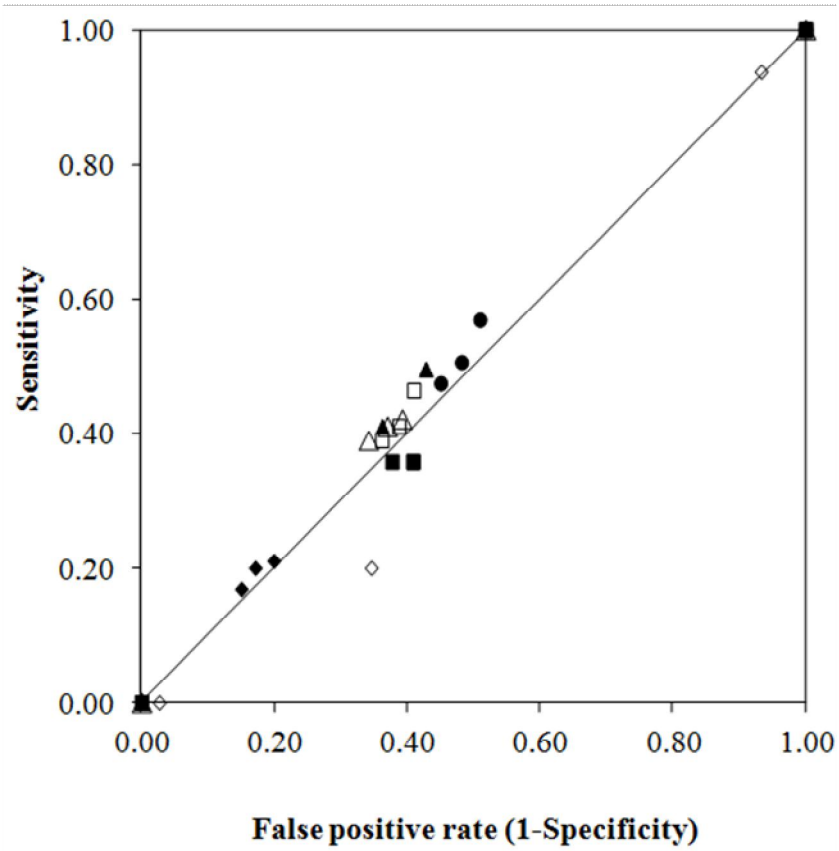

Figure 3. Receiver operating characteristic (ROC) curves (restricted to a maximum false positive rate of 1% which is equivalent to a minimum specificity of 99%) for IRT. The area under the curve (AUC) for vulva (♦), left eye (□), right eye (■), left ear (Δ), right ear (▲), muzzle (●) and combined measures of all body areas (◊) are 0.51, 0.52, 0.48, 0.52, 0.53, 0.53 and 0.43 respectively.

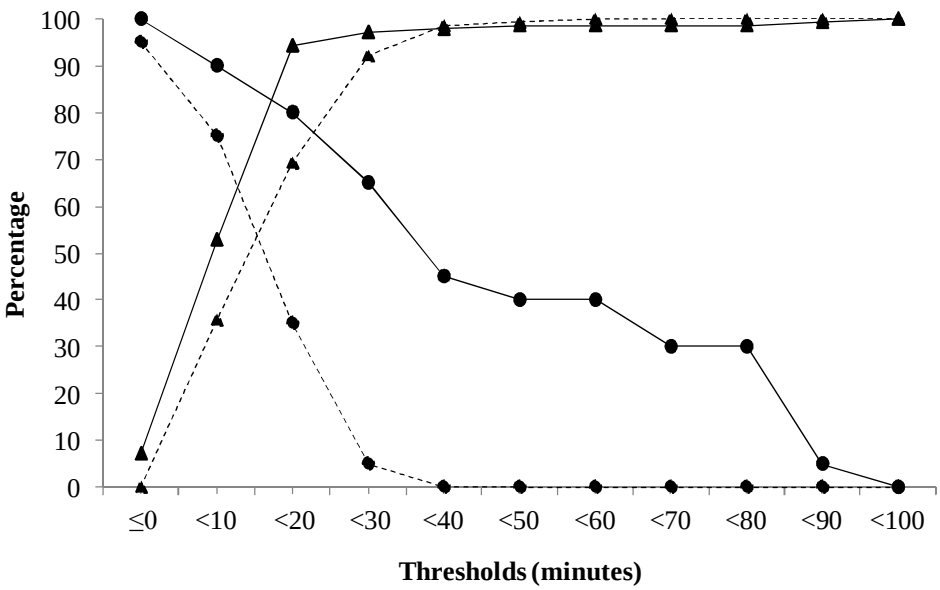

Figure 4. Effect of changing thresholds on sensitivity (●) and specificity (▲) for SCR tags measuring activity (solid line) and rumination level (dashed line).

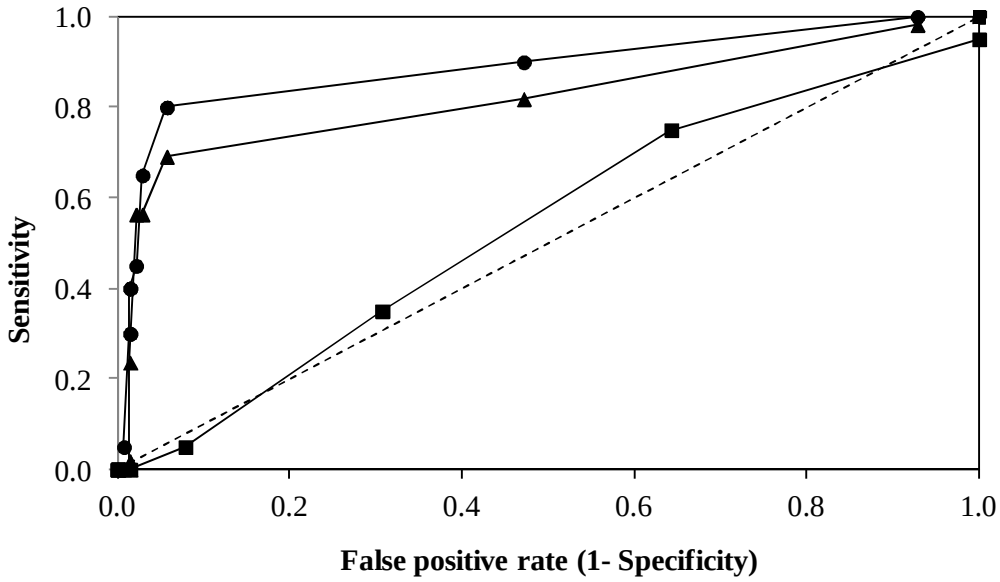

Figure 5. Receiver operating characteristic (ROC) curves (restricted to a maximum false positive rate of 1% which is equivalent to a minimum specificity of 99%) for SCR tags. The area under the curve (AUC) for the activity level (●), rumination level (■) and the combined measures of activity and rumination level (▲) are 0.82, 0.54 and 0.75, respectively.

Factors affecting the performance of ovulation detection methods evaluated by logistic regression modelling

Of the six predictor variables (listed in Table 1) tested, locomotion score and milk protein % were the factors that had a significant ($P < 0.05$) effect on the probability of a true oestrus event being detected by the SCR HR LD tags using the default manufacturer settings; however, no significant effect ($P = 0.54$) of their interaction was noted. The odds ratio (OR) of a true oestrus event having an alert generated by the SCR HR LD tags was significantly lower for cows which were scored to be at least slightly lame (LS: > 1) compared to non-lame (LS: ≤ 1) cows [OR = 2.35; 95% confidence interval (CI) 1.52 to 3.63; $P < 0.001$], and in cows with low versus high milk protein % [OR = 4.95; 95% CI 1.34 to 18.2]; $P < 0.05$]. Since few true positive oestrus events were detected by IRT, multivariable regression was not conducted.

DISCUSSION

The hypothesis tested in this study was rejected. The findings of the present study indicated that IRT showed poor performance for detecting cows in oestrus. Although the muzzle, eye and ear temperatures resulted in improved specificities, the sensitivities were lower compared to vulval temperature. Activity measured by accelerometer in 2 h time blocks resulted in detection of a higher proportion of cows in oestrus in a pasture-based system than the rumination or the combined measures of activity and rumination.

In the present study, IRT eye temperature was higher than IRT vulval temperature which contrasts with the findings of Hoffmann *et al.* (2013). The collection of data for 2 days, cubicle housing system and use of IRT video image to measure the body surface temperature in that study (Hoffmann *et al.* 2013) may explain the discrepancy. In the present study, ambient temperature and humidity were moderately correlated with IRT temperatures. These findings are consistent with the previous studies reporting the positive correlation of ambient temperature with udder temperature (Berry *et al.* 2003) and foot temperature (Rainwater-Lovett *et al.* 2009). The moderate correlation between IRT temperature and ambient temperature may reflect the mild environmental conditions during the experimental period. The strong correlations between vulval and muzzle temperature observed in the present study are consistent with the previous study (Talukder *et al.* 2014*b*) which was 0.75.

This study is the first to evaluate the potential of IRT technology in different body areas (eye, ear, muzzle and vulva) for the detection of cows in oestrus. Irrespective of the different body areas explored for IRT image capture, the accuracy of oestrus detection was relatively poor. However, eye temperature has been reported as the earliest and the most consistent indicator of body temperature in diagnosis of febrile ponies (Johnson *et al.* 2011) and bovine viral diarrhoea virus infection in calves (Schaefer *et al.* 2004). In those two studies, experimental animals were challenged to equine herpes virus (Johnson *et al.* 2011) and bovine viral diarrhoea virus infection (Schaefer *et al.* 2004). The detection of disease through increased body temperature with the aid of IRT may be more successful than the detection of oestrus with high levels of sensitivity (91.5%) and specificity (84.6%) reported by Johnson *et al.* (2011).

Among the threshold tested for IRT in the present study, a threshold of 1.5 SD resulted in the highest specificity but the poorest sensitivity which was in contrast to the findings of Talukder *et al.* (2014*b*). However, in that study (Talukder *et al.* 2014*b*), oestrus was synchronized in the cows and IRT data capture was conducted at four hour intervals which may explain the discrepancies of findings with the present study. Whilst it is possible, it is not expected that the synchrony of oestrus impacted on the oestrus detection accuracy achieved in that study (Talukder *et al.* 2014*b*). However, performing IRT twice daily in the present study may have resulted in oestrus cases going undetected when thresholds were set at levels that resulted in a large number of FP cases. It may be possible to reduce the number of false positives by performing IRT continuously rather than twice daily although such a practice would have limited application potential (particularly in pastured cows).

Various environmental conditions and extraneous factors can affect the accuracy of IRT. In a recent porcine study, Sykes *et al.* (2012) reported the greatest differences of vulval temperature at ambient temperature < 10°C. On the other hand, Love and Linsted (1976) reported that an ambient temperature of approximately 20°C is ideal for capturing IRT images while in another study by Turner *et al.* (1986), an ambient temperature < 20°C was reported to be acceptable for performing IRT. Infrared thermography eye temperature was reported to be influenced by sunlight and distance from camera to eye, suggesting the need to ensure these two factors are managed with regard to consistency (i.e. capture images from a set distance and conduct the monitoring in a shaded

environment (Johnson *et al.* 2011). Additional factors, for example vapour, carbon dioxide, angle of measurement, wind velocity, drafts, foreign material on the hair coat and eye injury/disease may also have a significant influence (Stelletta *et al.* 2012; Hoffmann *et al.* 2013).

A large variation (both within and between cows) of IRT temperatures of different body areas was observed in the present study. Aside from the potential for operator influence (exactness of distance between camera and body part), faecal contamination (most relevant for vulval images), skin colour, moisture (particularly on vulva and muzzle) and hair density/length (particularly for ear measurements) may have influenced the variability of the IRT data. It is recognised that all of the above mentioned factors would be difficult to address in a practical application.

In the present study, the estimates of sensitivity, specificity and positive predictive value of AL for detection of cows in oestrus were within the range of those reported in previous studies using an accelerometer (Kamphuis *et al.* 2012). Results presented here indicated that AL resulted in more accurate oestrus detection performance than either RL alone or the combination of RL and AL.

The value of the RL may be significantly influenced by the nutritional status and the rumen environment of cows, regardless of their feeding/management system. Whilst true indicators of these factors were not captured in the present study, it is possible that the diet composition negatively impacted on the daily RL across the herd and therefore the ability to detect significant changes in RL as an indicator of an oestrus event. In the present study, the average daily rumination was 502.0 ± 99.93 minutes which was within the range reported in the previous study (Bar and Solomon 2010).

The sensitivity and specificity of ovulation detection using accelerometer and activity meters are known to vary with the threshold or algorithm set by the manufacturer and the defined reference period of previous activity (baseline) reported in the earlier studies (de Mol *et al.* 1997; At-Taras and Spahr 2001; Roelofs *et al.* 2005; Saint-Dizier and Chastant-Maillard 2011), although animals were managed in similar housing systems (free stall barns) reported in those studies. Using a reference period of seven days and adopting different thresholds to define increased activity in the present study, created a range in both sensitivity and specificity (from 0% to 100% and 7% to 100%

respectively). Adopting a reference period of 10 days and a variety of standard deviation ranges to determine the change in activity resulted in sensitivity ranging from 86.7% to 94.1% and specificity ranging from 90.4% to 98.2% in a herd managed in pasture-based system (Hockey *et al.* 2010). Hockey's study (Hockey *et al.* 2010) was conducted on a larger number of animals (n = 400) in a seasonally-calved herd where most of the calving occurred from May to July (late autumn/early winter) and a different activity meter (Rescounter II®, Westfalia-Surge, Bonen, Germany) was mounted on a neck collar which may explain the achieved higher sensitivities and specificities compared to the present study. The performance of any activity meter is likely to be impacted by the housing and the herd management system (Aungier *et al.* 2012). Due to the pasture rotation, cows walk different distances from one paddock to another which can result in a significant level of variation in activity in pasture-based systems (Saint-Dizier and Chastant-Maillard 2011). Future studies may be conducted including the different walking distances as a variable for adjusting the algorithm and to evaluate its effect on activity based oestrus alerts. These studies should also include daily or am-pm herd average activity level as a covariate to adjust for the variation in walking distance from different paddocks to the dairy. Within each farm, the threshold value can be set based on the management system (pasture allocation, calving system) which may reduce the number of false positives but may also incur the risk of missing some events, thereby reducing the sensitivity of such a method (Holman *et al.* 2011). In addition, incorporating the most recent oestrus event date in the algorithm may reduce the number of FP (Saint-Dizier and Chastant-Maillard 2011).

Using the gold standard of milk P_4 profiles combined with AI dates, SCR HR LD tags detected five true oestrus events (16%) that were not detected through the visual assessment of mounting indicators (Estrotect®). However, four 13% (n = 4) true oestrus events that were detected by visual assessment of the Estrotect® devices did not generate an alert via the SCR HR LD tags. Whilst the detection of the additional five oestrus alerts is encouraging, the FN would indicate that the SCR HR LD tags could be used to enhance oestrus detection via visual observation but certainly would not be recommended as a replacement for visual detection. Poor sensitivity/specificity is likely to be a major limitation in the adaptation of a new technology by the dairy farmers (Hockey *et al.* 2010) as farmers generally expect a technology to enhance the performance of current methods. In the present study, different threshold resulted in

different sensitivity and specificity. Although the activity threshold < 50 resulted in 99% specificity, it resulted in the poorest sensitivity. This is because the activity threshold < 50 is reliant on a significant increase in activity for an alert to be generated which results in a higher than desirable proportion of true oestrus events not being associated with an alert. Prospective studies regarding the pattern and the cause of FP alerts may help to identify possible ways to improve the sensitivity of the system-generated activity alerts. However, it is most likely that incorporation of additional data and/or calculation into the algorithms would be required to generate alerts with the highest levels of sensitivity and manageable levels of FP.

The potential factors evaluated in the present study, lameness was shown to be negatively associated with the probability of a true oestrus event being detected by the SCR HR LD tags using the default manufacturer settings in the present study which agrees with the findings of previous studies (López-Gatius *et al.* 2005; Walker *et al.* 2008). This may be the consequence of reduced oestrus intensity/expression in lame cows (López-Gatius *et al.* 2005; Walker *et al.* 2008). Cows with high milk protein % were more likely to be detected in oestrus by SCR HR LD tags compared to cows having low milk protein % which agrees with the findings of a previous study (Fahey *et al.* 2003). The relationship between reproductive performance and milk protein % may be driven by energy balance (Fahey *et al.* 2003) which has a strong positive effect on resumption of oestrus cycles. Though the energy balance status has not been evaluated in the present study, it may be speculated that cows with high milk protein % had a more desirable energy balance status and hence had a higher probability of being detected by SCR HR LD tags compared to their herd mates with low milk protein %.

CONCLUSIONS

The findings of the present study indicated that the SCR HR LD tags give a good indication of when ovulation is likely to occur. Although the specificities estimated in this study were deemed acceptable, the sensitivities and positive predictive values using different thresholds were considerably lower than the desirable 100% detection rate. The oestrus detection performance of IRT can be improved by performing IRT continuously rather than twice in a day (which may reduce the prevalence of false positives) although its feasibility in terms of farm practice particularly in pasture-based dairy farms needs to be evaluated.

REFERENCES

At-Taras EE, Spahr SL (2001) Detection and characterization of estrus in dairy cattle with an electronic heat mount detector and an electronic activity tag. *Journal of Dairy Science* **84,** 792-798.

Aungier SPM, Roche JF, Sheehy M, Crowe MA (2012) Effects of management and health on the use of activity monitoring for estrus detection in dairy cows. *Journal of Dairy Science* **95,** 2452-2466.

Bar D, Solomon R (2010) Rumination collars: What can they tell us. In 'Proceedings of the First North American Conference.' pp. 214–215. (Precision Dairy Management, Toronto, Canada)

Berry RJ, Kennedy AD, Scott SL, Kyle BL, Schaefer AL (2003) Daily variation in the udder surface temperature of dairy cows measured by infrared thermography: Potential for mastitis detection. *Canadian Journal of Animal Science* **83,** 687-693.

Clapper JA, Ottobre JS, Ottobre AC, Zartman DL (1990) Estrual rise in body temperature in the bovine I. Temporal relationships with serum patterns of reproductive hormones. *Animal Reproduction Science* **23,** 89-98.

de Mol RM, Kroeze GH, Achten JM, Maatje K, Rossing W (1997) Results of a multivariate approach to automated oestrus and mastitis detection. *Livestock Production Science* **48,** 219-227.

Diskin MG, Sreenan JM (2000) Expression and detection of oestrus in cattle. *Reproduction, Nutrition, Development* **40,** 481-491.

Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G (1989) A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* **72,** 68-78.

Fahey J, Morton JM, Macmillan KL (2003) Associations between milk protein percentage and reproductive performance in Holstein-Friesian cows in seasonal-calving dairy herds. In 'Proceedings of the Fifteenth Conference.' pp. 68-71. (Association for the Advancement of Animal Breeding and Genetics: 50 years of DNA, Melbourne, Australia)

Fisher AD, Morton R, Dempsey JMA, Henshall JM, Hill JR (2008) Evaluation of a new approach for the estimation of the time of the LH surge in dairy cows using vaginal temperature and electrode less conductivity measurements. *Theriogenology* **70,** 1065-1074.

Fulkerson WJ, Donaghy DJ (2001) Plant-soluble carbohydrate reserves and senescence - key criteria for developing an effective grazing management system for ryegrass-based pastures: A review. *Australian Journal of Experimental Agriculture* **41,** 261-275.

Hanley JA, McNeil BJ (1982) The meaning and use of the area under a receiver operating characteristic (roc) curve. *Radiology* **143,** 29-36.

Hockey CD, Morton JM, Norman ST, McGowan MR (2010) Evaluation of a neck mounted 2-hourly activity meter system for detecting cows about to ovulate in two paddock-based Australian dairy herds. *Reproduction in Domestic Animals* **45,** 107-117.

Hoffmann G, Schmidt M, Ammon C, Rose-Meierhöfer S, Burfeind O, Heuwieser W, Berg W (2013) Monitoring the body temperature of cows and calves using video recordings from an infrared thermography camera. *Veterinary Research Communications* **37,** 91-99.

Holman A, Thompson J, Routly JE, Cameron J, Jones DN, Grove-White D, Smith RF, Dobson H (2011) Comparison of oestrus detection methods in dairy cattle. *Veterinary Record* **169,** p 47.

Johnson SR, Rao S, Hussey SB, Morley PS, Traub-Dargatz JL (2011) Thermographic eye temperature as an index to body temperature in ponies. *Journal of Equine Veterinary Science* **31,** 63-66.

Kamphuis C, DelaRue B, Burke CR, Jago J (2012) Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. *Journal of Dairy Science* **95,** 3045-3056.

Kyle BL, Kennedy AD, Small JA (1998) Measurement of vaginal temperature by radiotelemetry for the prediction of estrus in beef cows. *Theriogenology* **49,** 1437-1449.

López-Gatius F, Santolaria P, Mundet I, Yániz JL (2005) Walking activity at estrus and subsequent fertility in dairy cows. *Theriogenology* **63,** 1419-1429.

Love TJ, Linsted RD (1976) Theoretical basis for use of skin temperature as a plethysmographic indicator. *American Society of Mechanical Engineering* **6,** 1-4.

Lucy MC (2007) Fertility in high-producing dairy cows: Reasons for decline and corrective strategies for sustainable improvement. *Journal of Reproduction and Fertility Supplement* **64,** 237–254.

Mosher MD, Ottobre JS, Haibel GK, Zartman DL (1990) Estrual rise in body temperature in the bovine II. The temporal relationship with ovulation. *Animal Reproduction Science* **23,** 99-107.

Rainwater-Lovett K, Pacheco JM, Packer C, Rodriguez LL (2009) Detection of foot-and-mouth disease virus infected cattle using infrared thermography. *The Veterinary Journal* **180,** 317-324.

Roelofs J, López-Gatius F, Hunter RHF, van Eerdenburg FJCM, Hanzen C (2010) When is a cow in estrus? Clinical and practical aspects. *Theriogenology* **74,** 327-344.

Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp B (2005) Pedometer readings for estrous detection and as predictor for time of ovulation in dairy cattle. *Theriogenology* **64,** 1690-1703.

Saint-Dizier M, Chastant-Maillard S (2011) Towards an automated detection of oestrus in dairy cattle. *Reproduction in Domestic Animals* **47,** 1056–1061.

Schaefer AL, Cook N, Tessaro SV, Deregt D, Desroches G, Dubeski PL, Tong AKW, Godson DL (2004) Early detection and prediction of infection using infrared thermography. *Canadian Journal of Animal Science* **84,** 73-80.

Schirmann K, von Keyserlingk MAG, Weary DM, Veira DM, Heuwieser W (2009) Technical note: Validation of a system for monitoring rumination in dairy cows. *Journal of Dairy Science* **92,** 6052-6055.

Sprecher DJ, Hostetler DE, Kaneene JB (1997) A lameness scoring system that uses posture and gait to predict dairy cattle reproductive performance. *Theriogenology* **47,** 1179-1187.

Srikandakumar A, Ingraham RH, Ellsworth M, Archbald LF, Liao A, Godke RA (1986) Comparison of a solid-phase, no-extraction radioimmunoassay for progesterone with an extraction assay for monitoring luteal function in the mare, bitch, and cow. *Theriogenology* **26,** 779-793.

Stelletta C, Gianesella M, Vencato J, Fiore E, Morgante M (2012) Thermographic applications in veterinary medicine. In: 'Infrared thermography.' (Ed. RV Prakash) pp. 117-140. (InTech: China) Available at http://www.Intechopen.Com/books/infraredthermography/thermographic-applications-in-veterinary-medicine [verified 30 August 2013]

Sykes DJ, Couvillion JS, Cromiak A, Bowers S, Schenck E, Crenshaw M, Ryan PL (2012) The use of digital infrared thermal imaging to detect estrus in gilts. *Theriogenology* **78,** 147-152.

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2014*a*) Factors affecting reproductive performance of dairy cows in a pasture-based, automatic milking system research farm: A retrospective, single-cohort study. *Animal Production Science* **55,** 31-41.

Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P (2014*b*) Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture-based system. *Theriogenology* **81,** 925-935.

Turner AT, Purohit RC, Fessler JF (1986) Thermography: A review in equine medicine. *Compendium on Continuing Education for the Practising Veterinarian* **8,** 855-861.

Walker SL, Smith RF, Jones DN, Routly JE, Dobson H (2008) Chronic stress, hormone profiles and estrus intensity in dairy cattle. *Hormones and Behavior* **53,** 493-501.

Wrenn TR, Bitman J, Sykes JF (1958) Body temperature variations in dairy cattle during the estrous cycle and pregnancy. *Journal of Dairy Science* **41,** 1071-1076.

CHAPTER 6

Changes in Milk Oxidative Stress Biomarkers in Lactating Dairy Cows with Ovulatory and An-Ovulatory Oestrous Cycles

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OVERVIEW OF CHAPTER 6

Plasma oxidative stress biomarkers were shown to differ significantly between ovulated and an-ovulated cows in Chapter 4. Whilst this was somewhat of a breakthrough, it was deemed that the real value would be realised if similar findings could be achieved with biomarkers in milk. In-line sensors and automated milk sampling technology already exists for some milk components and the monitoring of biomarkers in milk (if successful) may allow for the development of fully automated and accurate oestrus detection on farm with the advantages of timeliness and integration that would likely result. As a result, in chapter 6 it was hypothesized that oxidative stress biomarkers measured in milk would be able to provide a reliable indication of an ovulation event.

ABSTRACT

This study was conducted to evaluate changes in milk profiles of oxidative stress (OS) biomarkers in dairy cows with ovulatory and an-ovulatory oestrous cycles. Thirty healthy, cycling Holstein cows averaging 60 ± 17 days in milk, and producing 33 ± 6 kg of milk per day (the week before commencing the study) were enrolled in this study. Composite milk samples were collected thrice weekly and assayed for the following OS biomarkers: lipoperoxides (LPO), biological advanced potential, superoxide dismutase (SOD), advanced oxidation protein products (AOPP), ceruloplasmin, glutathione (GSH), β-carotene and glutathione peroxidase (GSH-Px). Milk samples were also tested for fat and protein composition and the fat: protein ratio (FPR) was categorised as low (≤1.31), medium (1.32-1.56) and high (>1.57) to evaluate their main effect and the interaction effect of FPR and the week of study on OS using linear mixed models with cow identification being a random factor. Cows with ovulatory oestrous cycles (n = 20) presented significantly greater SOD levels compared to cows that did not ovulate (n = 10; $P < 0.05$). On the other hand, LPO, GSH-Px and GSH concentrations were lower in ovulated cows compared to the an-ovulated cows ($P< 0.05$). The highest level of LPO and AOPP were noted at prooestrus phase while β-carotene presented the lowest value at that phase of oestrous cycle. It could be postulated that the elevated level of milk SOD and the observed lower level of LPO, GSH-Px and GSH in ovulating cows may be an essential event preceding the ovulatory response.

Keywords: oxidative stress biomarker, ovulation, milk, dairy cow, progesterone.

INTRODUCTION

The characterisation of the oxidant/antioxidant balance is attracting a high level of interest in ruminant physiology (Celi, 2011) as it may play an important role in the regulation of several physiological functions including reproduction (Agarwal *et al.* 2006; Celi 2011). Oxidants and antioxidants act as signalling molecules for various physiological functions in the female reproductive tract (Agarwal *et al.* 2006). For example, superoxide dismutase (SOD) is the first enzymatic antioxidant in the ovary that plays a vital protective role by catalysing the conversion of superoxide radicals into hydrogen peroxide (H_2O_2) while glutathione peroxidise (GSH-Px) detoxifies H_2O_2 to H_2O (Al-Gubory *et al.* 2005). Moreover, it seems that these antioxidants act within growing follicles, granulosa cells of Graafian follicles, the endometrium and corpus luteum (CL) to regulate important functions like, ovulation, fertilisation and embryo development (Rizzo *et al.* 2012; Sugino 2005).

Oxidative stress (OS) has been reported to have an important role in the normal functioning of the female reproductive system and in the pathogenesis of female infertility (Agarwal *et al.* 2005). In ruminants and in the dairy cow in particular, OS has been associated with several pathological conditions, such as retained placenta (Kankofer *et al.* 2010), mastitis (Ranjan *et al.* 2005) and embryo mortality (Celi *et al.* 2012; Celi *et al.* 2011) which impair reproductive performance.

While there is evidence of the presence of antioxidative factors (SOD, GSH-Px, β-carotene) in colostrum and milk of dairy cows (Lipko-Przybylska *et al.* 2010; Lipko-Przybylska *et al.* 2012), there is currently no conclusive evidence as to whether or not OS biomarkers measured in milk provide a reliable indication of reproductive status.

Considering that changes in plasma OS biomarkers seem to be related to the ovulatory status of dairy cows (Talukder *et al.* 2014*c*), if a similar relationship was to be found in milk samples, it could allow the development of an on-farm automated technology that could monitor the ovulatory status of individual cows during milking. Therefore, this study was conducted to examine the naturally occurring changes in milk OS biomarkers in ovulatory and an-ovulatory lactating dairy cows. Differences in OS biomarkers amongst the different phases of oestrous cycles were also evaluated.

MATERIALS AND METHODS

All procedures conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and implemented by the Animal Ethics Committee of The University of Sydney (N00/9-2012/1/5829).

Animals and feeding system

Thirty (11 primiparous and 19 multiparous; parity: 2.1 ± 1.1) healthy (according to on-farm records), lactating Holstein cows averaging 60 ± 17 days in milk and producing 33 ± 6 kg (mean ± SD) of milk per day were used in this study. The study was conducted between October and November 2013 (Spring) for 1 month at the University of Sydney's Corstorphine dairy farm, Camden, NSW, Australia. The mean air temperature, maximum air temperature and maximum relative humidity during the experimental period were 18°C, 37°C and 99%, respectively. Before the commencement of the study, a transrectal ultrasound scan was performed on all cows using a portable scanner (Ibex Pro portable ultrasound; E.I. Medical Imaging, Loveland, CO, USA) to confirm the presence of ovarian follicle(s).

All cows were grazed on kikuyu grass (*Pennisetum clandestinum*), over sown with short rotation ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The cows had access to pasture between the two milkings and were grazed in accordance with the best practice using pasture on offer and leaf stage as the criterion to flag pasture as 'grazeable' (Fulkerson *et al.*2001). Cows received their target concentrate (7.5 ± 0.8 kg/cow.day; Elite Plus, Weston Animal Nutrition, Enfield, NSW, Australia) allocation twice daily at milking. Cows were also supplemented (as a group) with lucerne silage (16.9 ± 0.4 kg/cow.day) on a feed pad after the afternoon milking. The chemical composition of the silage and concentrate pellets were analysed by near infrared spectroscopy and wet chemistry in MC Franklin lab, University of Sydney (Sydney, NSW, Australia) (Table 1).

Table1. Chemical composition of lucerne silage and concentrate pellet fed during the experimental period. Values are means obtained from near infrared and wet chemistry.

Item (% dry matter)	Silage	Concentrate
Dry matter	51.3	98.0
Neutral detergent fibre	59.8	20.0
Acid detergent fibre	39.2	6.4
Organic matter	92.0	-
Water soluble carbohydrate	14.5	-
Crude protein	9.3	16.0
Nitrogen	1.5	2.6
Dry matter digestibility	58.7	88.0
Metabolizable energy	8.8	13.0

Data collection

Cows were assessed weekly for body condition score (BCS), using a scoring system from 1 to 5 incorporating 0.5 scores (Edmonson *et al.* 1989) and for locomotion score (LS) using 1 to 5 system (Sprecher *et al.* 1997). Daily milk production, and body weight (BW) was captured for each animal using the electronic data support software, AlPro (Tumba, Sweden). Composite milk samples were collected into two 15 mL plastic sample bottles thrice weekly (Monday, Wednesday, and Friday) during the pm milking. One bottle was frozen at -20°C pending progesterone (P_4) analyses, while the other bottle was used for analysis of somatic cell count, fat and true protein concentrations (Foss 303 Milko Scan, Denmark). Fat:protein ratio (FPR) was calculated to provide an indication of energy balance (Grieve *et al.* 1986; Heuer *et al.* 1999), as FPR >1.5 is indicative of a greater rate of fat synthesis in the mammary gland and therefore of increased mobilization of body fat reserves (Grieve *et al.* 1986; Heuer *et al.* 1999). The variables listed in Table 2 were assessed to evaluate their association with biomarkers of OS.

Ambient temperature and humidity were recorded twice daily (6 am and 1 pm) solar weather station (Oregon Scientific International Ltd., Los Angeles, USA). Temperature humidity index (THI) was calculated for each IRT scanning session using the equation

reported by Kendall *et al.* (2008): THI = [(1.8 × T + 32) – {(0.55 − 0.0055 × RH) × (1.8 × T−26)}].

Table 2. Factors assessed for their effects on the biomarkers of oxidative stress.

Factors	Classes	Class description
Days in milk	2	≤68 (Low) >68 (High)
Body condition score	3	≤2.5 (Low) 3.0 (medium) ≥3.5 (Acceptable)
Locomotion score	2	1 (Healthy) >1(Lame)
Parity	2	Primiparous Multiparous
Fat corrected milk yield (Kg)	3	≤30 (Low) 31-38 (Medium) >38 (High)
Milk fat:protein ratio	3	≤1.31 (Low) 1.32-1.56 (Medium) >1.57 (High)
Body weight (Kg)	3	≤540 (Low) 541-578 (Medium) >578 (High)

Progesterone (P_4) assay and defining phases of oestrous cycle

Milk samples were analysed for P_4 concentration using a solid phase radio immuno assay (Coat-A-Count Progesterone; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) validated for milk samples (Srikandakumar *et al.* 1986). The mean intra-assay and inter-assay coefficients of variation were 2.88% and 5.75% respectively. The detection limit of the assay was 0.05 ng/mL.

Oestrus was monitored by visual observation of the mounting indicators Estrotect® Heat Detector patch (Genetics Australia Co-operative Ltd, Bacchus Marsh, VIC, Australia) during the am and pm milking. Inseminations were performed at two time periods per

day soon after the milkings (am and pm). If a cow was identified as displaying the complete activation of Estrotect^{TM}, she was considered to be in oestrus and was inseminated at the next insemination period. Individual milk P_4 profiles were constructed to identify when cows were likely to be in oestrus and the associated timing of ovulation (Aungier *et al.* 2012). A trough-like curve in these P_4 profiles was assumed to indicate a periovulatory event (luteolysis followed by ovulation with subsequent development of the new functional CL) and was initially identified as evidence of ovulation (Figure 1A). The day of ovulation was identified as either the date of performing AI where this resulted in a confirmed positive pregnancy outcome based on farm record, regardless of the shape of the progesterone profile; (2) the day of an unsuccessful AI when the insemination was coincident with the trough like curve in progesterone concentrations; (3) if a trough like curve occurred without a corresponding day of AI, the ovulation date was set preferentially being at the centre of the trough-like curve in progesterone concentrations (Talukder et al., 2015; Kamphuis et al., 2012). Cows that met the above criteria were deemed to have ovulated while those that did not meet the criteria were considered as an-ovulated cows (Figure 1B). Of the 30 cows enrolled in this study, 20 ovulated, while 10 did not ovulate. Day of ovulation was considered as day of oestrus (day 0). Only one oestrus was recorded for each cow. Only the first oestrus was considered for data analysis for any cows that had more than one oestrus event during the study period. Phases of the oestrous cycle were defined as prooestrus (day 17-20), oestrus (day 0), metoestrus (day 2-4) and dioestrus (day 5-17) (Ireland *et al.* 1980).

Separation of skim milk, and whey protein

Ten mL of milk sample were defatted (centrifugation at 5000 g for 15 minutes at 4°C) and skim milk was collected. Subsequently, casein was removed and whey protein was obtained from half of the collected skim milk by adding 8% acetic acid to skim milk followed by centrifugation at 5000*g* for 15 min and collection of the supernatant. Skim milk and whey protein samples were stored at −20°C until pending analyses. Whole milk samples were analysed for biological antioxidant potential (BAP) and beta(β)-carotene assay; skim milk samples were assayed for lipoperoxides (LPO), SOD, glutathione (GSH), GSH-Px and ceruloplasmin while whey samples were assayed for advanced oxidation protein products (AOPP).

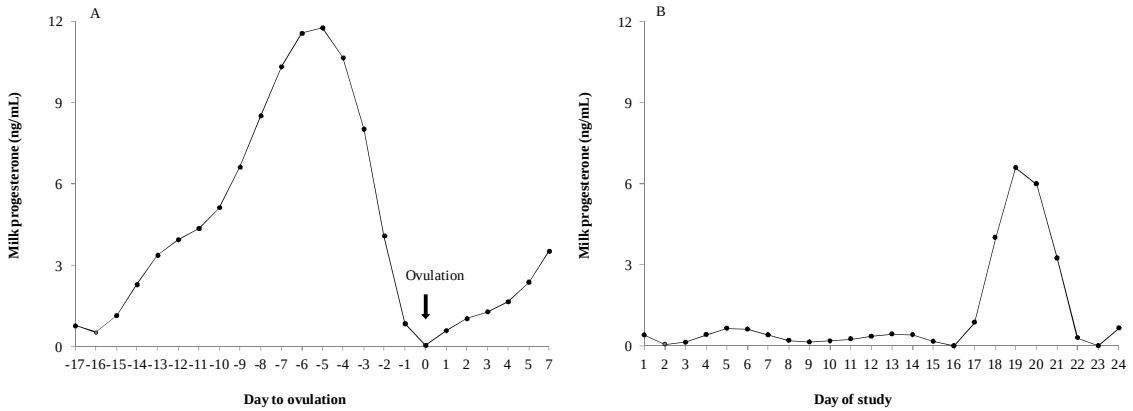

Figure 1. Example of progesterone profile of an ovulated (A; Cow 1142) and anovulated (B; Cow 1215) cow. The vertical arrow indicates the day of ovulation (Day 0).

Milk OS assay

The levels of LPO, as markers of oxidative damage on lipids, were assayed in skim milk using the Lipocell test (FREE system, Diacron International, GR, Italy). The concentrations of BAP were measured in a dedicated spectrophotometer (FREE Carpe Diem, Diacron International, Grosseto, Italy) according to kit instructions (BAP test, Diacron, GR, Italy). Beta-carotene levels were measured by a hand held photometer (iCheck®, BioAnalyt GmbH, Teltow, BR, Germany) as described by the manufacturer. Exactly 400µl of milk was injected into the extraction and measuring vials (iCheck®, BioAnalyt GmbH, Teltow, Germany). The sample was shaken vigorously for 10 seconds and then left to rest for 5 minutes to achieve complete phase separation. Thereafter, the vials were inserted into the portable photometer and read at 450 nm. Results were given as mg/L. Commercial kits were used for the determination of GSH-Px (Cayman, Glutathione Peroxidase Assay Kit, Sapphire, NSW, Australia) and SOD activity (Cayman, Superoxide Dismutase Assay Kit, Sapphire, NSW, Australia). Glutathione concentration was measured by an enzymatic recycling method (chemicals sourced from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia) adapted for a micro titre plate reader (Baker *et al.* 1990). Ceruloplasmin concentrations were determined according to the methods described by Sunderman *et al.* (1970) except that absorbance was read at 510 nm (FLUROstar Optima, BMG Labtech, Mornington, VIC, Australia). Ceruloplasmin concentration was calculated as follows:

Ceruloplasmin (g/L) = 0.752 ($A_R - A_B$),

where A_R is the absorbance of sample R, and A_B is the absorbance of sample B.

Advanced oxidation protein products were measured according to the methods of Witko-Sarsat *et al.* (1998). Concentrations of AOPP, GSH, ceruloplasmin, GSH-Px and SOD activities were determined in a spectrophotometer (FLUOstarOptima, BMG Labtech, Mornington, VIC, Australia).

Statistical analyses

Raw data was initially assessed using descriptive statistics and then tested for normal distribution. Linear mixed models (LMM) were used to estimate the differences of OS indicators between cows deemed to have ovulatory vs an-ovulatory oestrous cycle using GENSTAT 14th Edition, VSN International, Hertfordshire, UK) and adding somatic cell count as a covariate. The model was as follows:

$Y_{ijk} = \mu + \beta_i + R\varepsilon_{ij}$

Where, Y_{ij}= response variate according to group i (i = ovulatory vs an-ovulatory cows) by cow j (j = 1 to 30), μ = mean effect of group, β_i= effect of group, $R\varepsilon_{ij}$= random residual error within cow j.

To further evaluate the relationship between OS, energy balance and milk production, cows were further categorised based on their fat:protein ratio (FPR) as low (LFPR: ≤1.31); medium (MFPR: 1.32-1.57) or high (HFPR: >1.57) FPR, and on their fat corrected milk yield (FCMY) as low (LFCMY: ≤30 Kg/cow/day), medium (MFCMY: 31-38Kg/cow/day) or high (HFCMY: >38 Kg/cow/day). A series of separate linear mixed models were fitted for each of the OS parameters specifying fixed effects and the interaction effect of explanatory variables listed in Table 2 with 'cow' included as a random effect in each of these models following the approach decsribed in Talukder *et al.* (2014*a*). All means are presented as model-based mean ± SEM.

RESULTS

Of the 30 cows enrolled in this study, 20 cows were found to have an ovulation event during the study period according to the P_4 profile combined with AI date. Of the 20 ovulated cows, visual appraisal of the Estrotect® assisted detection of the oestrus event in 75% (n = 15) of the cows. The descriptive statistics of days in milk, body condition score, locomotion score, parity, fat corrected milk yield, milk fat protein ratio, daily body weight between ovulated (n = 20) and an-ovulated (n = 10) are presented in Table 3.

Table 3. Mean (± SEM) of days in milk, body condition score, locomotion score, parity, fat corrected milk yield, milk fat protein ratio, daily body weight between ovulated (n = 20) and an-ovulated (n = 10) cows.

Factors	Ovulated	An-ovulated
Days in milk	74.8 ± 3.7	63.9 ± 5.2
Body condition score	3.1 ± 0.3	3.1 ± 0.2
Locomotion score	1.2 ± 0.3	1.1 ± 0.2
Parity	2.3 ± 1.2	1.9 ± 0.9
Fat corrected milk yield (Kg)	32.2 ± 0.7	30.0 ± 0.9
Milk fat:protein ratio	1.5 ± 0.04	1.4 ± 0.06
Body weight (Kg)	566.3 ± 8.7	557.2 ± 12.3

Biomarkers of milk OS in ovulatory and an-ovulatory cows

Differences in milk OS biomarkers between ovulated and an-ovulated cows are shown in Figure 2. Ovulated cows had greater SOD activity compared to that of an-ovulated cows ($P < 0.05$). On the other hand, LPO, GSH-Px and GSH activities were lower in ovulated cows than an-ovulated cows ($P < 0.05$). Overall, no differences were observed in milk AOPP, β-carotene, ceruloplasmin, and BAP level between ovulated and an-ovulated cows (Figure 2).

Milk P_4 and biomarkers of OS at different phases of oestrous cycle

Milk concentration of LPO, AOPP, β-carotene and GSH-Px differed significantly in relation to the four phases of oestrus cycle ($P < 0.05$; Figure 3). Lipoperoxides and AOPP were greater during prooestrus compared to the other phases of the oestrous cycle. On the other hand, β-carotene presented its lowest value during the prooestrus phase. Milk GSH-Px peaked during the dioestrus phase and was at its lowest during the oestrus phase (35.3 ± 1.6 nmol/min/mL and 31.3 ± 2.3 nmol/min/mL respectively; $P < 0.05$). The concentrations of SOD, GSH, ceruloplasmin and BAP did not significantly differ among the different phases of the oestrous cycle (Figure 3).

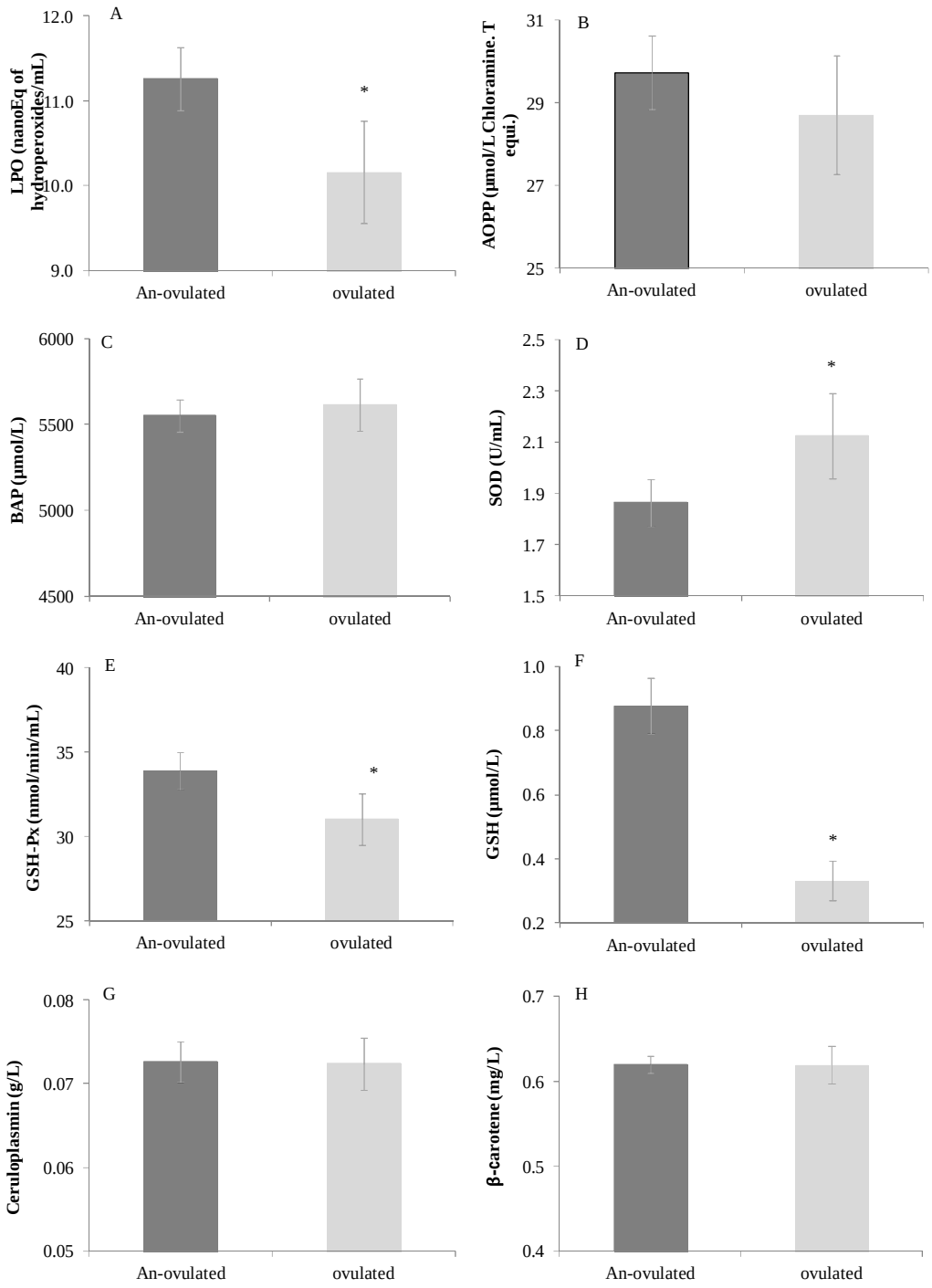

Figure 2. Milk biomarkers of oxidative stress (means ± SEM) in cows with ovulatory (white bar; $n = 20$) and an-ovulatory (black bar; $n = 10$) event. * indicates the significant differences ($P < 0.05$) between cows with ovulatory and an-ovulatory event.

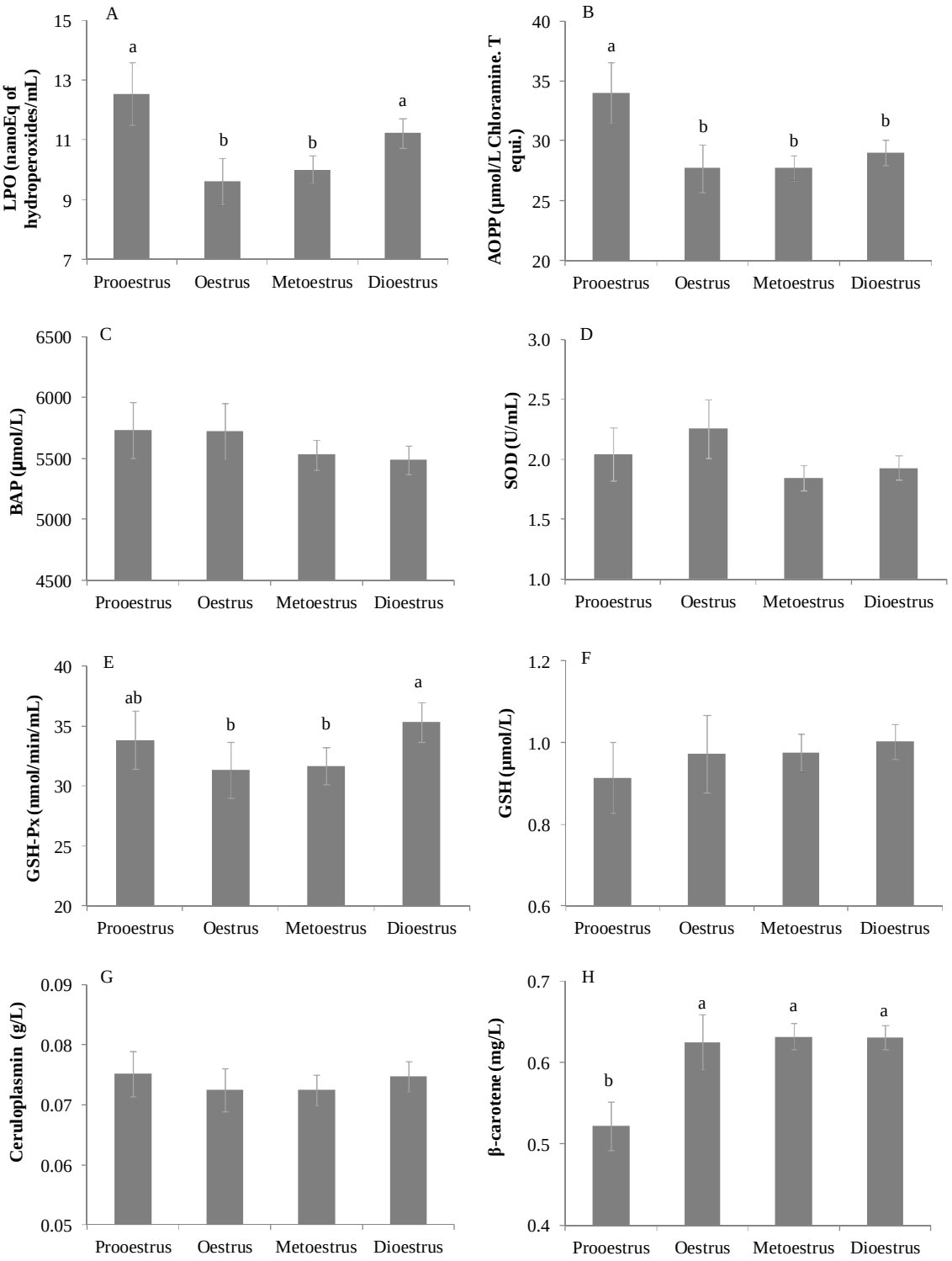

Figure 3. Milk biomarkers of oxidative stress (means ± SEM) in ovulated cows (n = 20) at different phases of oestrous cycle. Lowercase letters indicate significant difference (P < 0.05) among the different phases of the oestrous cycle.

Factors affecting the OS biomarkers

In the present study, the concentration of BAP, SOD, GSH-Px and ceruloplasmin had significant ($P < 0.05$) associations with locomotion score. Cows with a locomotion score of >1 had a lower BAP, SOD, GSH-Px and ceruloplasmin concentration compared to cows with a locomotion score of <1 (BAP: 5266 ± 110 µmol/L vs 5682 ± 96 µmol/L; SOD: 1.6 ± 0.1 U/mL vs 2.0 ± 0.1 U/mL; GSH-Px: 31.5 ± 0.9 nmol/min/mL vs 34.5 ± 0.7 nmol/min/mL and ceruloplasmin: 1.07 ± 0.002 g/L vs 1.08 ± 0.002 g/L respectively). Primiparous cows had lower ceruloplasmin compared to their multiparous herd mates (0.07 ± 0.002 g/L vs 0.08 ± 0.002 g/L; $P < 0.05$). Stage of lactation had also a significant effect of the OS biomarkers measured in this study, cows with <68 DIM presenting greater BAP, SOD, GSH-Px, GSH and ceruloplasmin concentration than those of cows with >68 DIM ($P < 0.05$). Body condition score was significantly ($P < 0.001$) associated with GSH-Px, GSH and ceruloplasmin. Cows having BCS 2.5 had the highest GSH-Px and ceruloplasmin while cows BCS 3.5 had the lowest GSH-Px and ceruloplasmin (GSH-Px: 38.9 ± 2.5 nmol/min/mL vs 30.1 ± 1.2 nmol/min/mL, ceruloplasmin: 0.08 ± 0.004 g/L vs 0.07 ± 0.002 g/L respectively). On the other hand, GSH concentration was lowest in cows with BCS 2.5 and highest in cows having BCS 3.5 (0.72 ± 0.06 µmol/L vs 1.06 ± 0.06 µmol/L, respectively). Fat corrected milk yield was significantly associated with milk LPO, BAP and SOD concentration. Cows in LFCM showed the highest concentration of BAP and LPO but the lowest SOD value compared to cows in MFCM and HFCM ($P < 0.05$). Body weight was not significantly associated with any of the OS biomarkers measured in the present study.

Fat corrected milk yield, FPR, body weight and BCS across the duration of the study are presented in Table 4. The main effect of time (week of study), Fat protein ratio category and their interaction on OS biomarkers are illustrated in Figure 4. In week 1, MFPR cows had the lowest concentration of LPO compared to HFPR and LFPR cows while in week 4 HFPR cows had the lowest level of LPO. In Week 2, GSH concentration was greater ($P < 0.05$) in MFPR cows compared to that of LFPR cows, while in week 4, HFPR cows presented greater GSH value than that of LFPR or MFPR cows. In weeks 1 and 2, HFPR cows presented the greatest β-carotene level while in week 4 the greatest β-carotene level were observed in the LFPR cows ($P < 0.05$). Low FPR cows had significantly greater ceruloplasmin concentration than the MFPR ones on week 1 and 2;

however, on week 3 the LFPR group presented the lowest ceruloplasmin value ($P < 0.05$). Neither the main effect of time or FPR, nor their interaction effect was significantly associated with AOPP level in the present study. Superoxide dismutase and GSH-Px levels differed significantly with time ($P < 0.05$) but they were not affected by FPR categories or the time*FPR interaction. The highest and the lowest SOD level was observed on week 1 (2.2 ± 0.1 U/mL) and week 3 (1.5 ± 0.1 U/mL) respectively. Glutathione peroxidase activity decreased from week 1 to week 4 ($P < 0.05$). The concentration of BAP was inversely associated with FPR which was 5727.3 ± 108.8 umol/L in LFPR and 5415.4 ± 102.8 umol/L in HFPR. No significant effect of time time*FPR interaction were observed on BAP concentration.

Table 4. Mean (± SEM) fat corrected milk yield, fat protein ratio, body weight and body condition score across the weeks of study.

Week	Fat corrected milk yield (Kg/cow.day)	Fat protein ratio	Body weight (Kg)	Body condition score
Week 1	31.3 ± 6.0	1.5 ± 0.04	557 ± 7	3.0 ± 0.04
Week 2	31.4 ± 6.3	1.6 ± 0.04	563 ± 7	3.0 ± 0.04
Week 3	31.6 ± 6.4	1.5 ± 0.04	573 ± 7	3.1 ± 0.04
Week 4	30.2 ± 5.7	1.4 ± 0.05	557 ± 8	3.1 ± 0.04

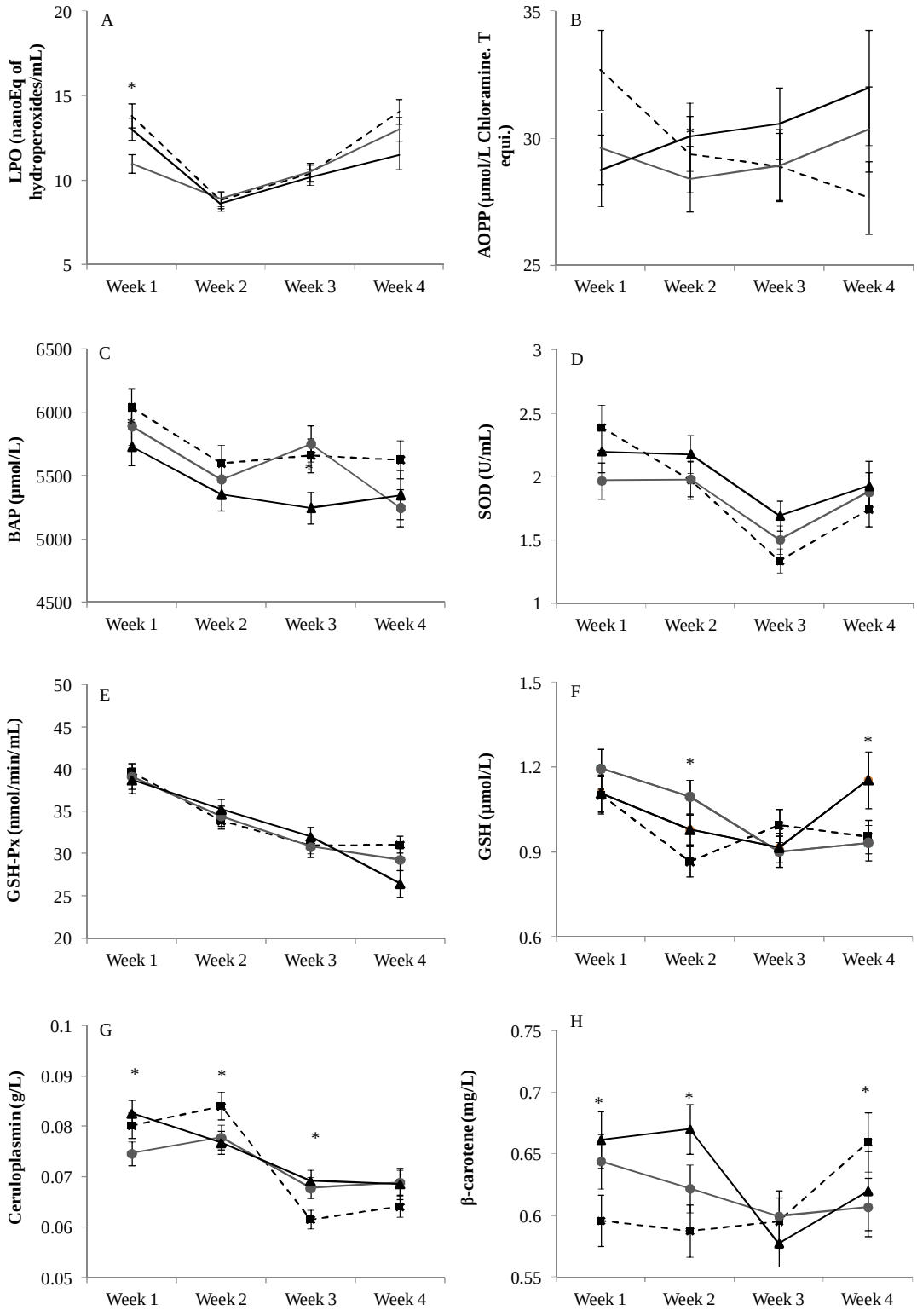

Figure 4. Graphs illustrating the effects of fat protein ratio [categorised as low ≤1.31 (■), medium 1.32-1.57 (●) and high >1.57 (▲)] and the weeks of study on the oxidative stress biomarkers. * indicates the significant interaction effect of fat protein ratio and week on the oxidative stress biomarkers.

DISCUSSION

The data presented in this study indicates that milk oxidative markers (LPO, SOD, GSH-Px and GSH) differed significantly between cows with ovulatory and an-ovulatory oestrus cycles. Milk concentration of LPO, AOPP, β-carotene and GSH-Px differed significantly in relation to the four phases of oestrus cycle. The level of BAP, SOD, GSH-Px and ceruloplasmin had significant ($P < 0.05$) associations with locomotion score, stage of lactation and BCS.

Milk SOD activity was greater in ovulated cows while the concentrations of LPO, GSH-Px and GSH were lower in ovulated cows compared to those of an-ovulated cows. Considering the physiological function of reactive oxygen species as important mediators of inflammatory reaction necessary for the ovulation event, we would have expected a greater concentration of LPO in ovulated cows compared to that of an-ovulated cows. However, the observed lower concentration of LPO in ovulated cows in the present study may be explained by the greater level of SOD in ovulated cows, which may act as a buffer and reduce the level of LPO. In the present study, greater SOD value in ovulated cows supports the findings of Tamate *et al.* (1995) suggesting the role of superoxide radical and SOD as a mediator for ovulation in humans. The observed increase in SOD activity in ovulated cows may due to self-protective antioxidant mechanism against the increased peroxide production required for ovulation which is thought to be associated with inflammatory like changes (Agarwal *et al.* 2005). Since SOD activity increases H_2O_2 production through the dismutation of superoxide (O_2^-) to H_2O_2, protection from ROS would only be given by a simultaneous increase in GSH-Px concentration and availability of GSH (Celi 2010; Kehrer *et al.* 1994). The observed lower GSH-Px and GSH in ovulated cows might have been the consequence of trying to keep LPO at a relatively lower level.

Milk concentration of GSH was lower in ovulated cows compared with that of an-ovulated cows in the present study. Glutathione is the major non-protein sulfydryl compound in mammalian cells and is generally considered as a good indicator of the blood scavenging capacity (Gabai *et al.* 2004; Talukder *et al.* 2014*b*). The observed lower GSH pattern in ovulated cows may be a resultant of increased utilization of GSH (which is the major exogenous source of redox potential in the ovary) to protect the ovarian tissue against the excessive production of free radicals which supports the

observed lower level of LPO in the present study. In addition, it is possible to speculate that the production of free radicals exceed the scavenging capacity of SOD in ovulated cows, and therefore in this situation, GSH might repair oxidized and damaged molecules using NADPH as an original electron source (Fujii *et al.* 2005) supporting the lower availability of GSH in ovulated cows observed in the present study.

No differences in ceruloplasmin and AOPP was noted between ovulated and an-ovulated cows which is consistent with our previous findings (Talukder *et al.* 2014*c*) suggesting the possible failure of these biomarkers to reflect the physiological changes in the ovary. Similarly, no changes in β-carotene and BAP were observed between ovulated and an-ovulated cows. In line with our previous observations where plasma BAP was decreased in ovulated cows, we would have expected to observe a similar trend in milk. It is likely that the mammary gland utilize some of the constituents of BAP for milk protein synthesis as observed in utilizing the constituent amino acid of GSH for milk synthesis (Mantovani *et al.* 2010).

Among the antioxidants examined, GSH-Px levels increased significantly during the dioestrous phase supporting the findings of Rapoport *et al.* (1998). It is possible to speculate that the high level of GSH-Px during dioestrus may have reduced the accumulation of free radicals and maintained the P_4 secretion during this phase (Al-Gubory *et al.* 2005). One possible explanation for the observed lower GSH-Px activity during oestrus and metoestrus is that cows may have experienced some degree of lipid peroxidation during the previous phase (prooestrus) and hence OS during these phases reflected by the observed consequential antioxidant depletion (Venditti *et al.* 2006). The differences of GSH-Px during the oestrous cycle might also be related to prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) which has an antisteroidogenic effect and has been reported to rise 1-4 days before oestrus, except during the first oestrus after calving (Kindahl *et al.* 1976). From all these findings, one can speculate that after the initial functional luteolysis during prooestrus, the rise in secretion of $PGF_{2\alpha}$ (although not measured in the present study) and a rapid decline in P_4 concentration might be attributed to a decrease in the level of GSH-Px at oestrus. The value of OS biomarkers measured in the present study (over the different phases of the oestrous cycle) may be associated with many environmental, managerial, and cow related factors which need to be evaluated through further studies. It is necessary to translate the information (changes of OS biomarkers

during the oestrous cycle) into a commercial application that could be adopted by industry.

Beta-carotene in addition to being an antioxidant serves as a precursor of retinol which has been reported to stimulate P_4 secretion by porcine luteal cells in vitro (Rapoport *et al.* 1998; Talavera *et al.* 1988). High levels of β-carotene in corpora lutea may protect against damage due to oxygen free radicals generated in the course of P_4 synthesis (Rapoport *et al.* 1998) supporting at least in part, the observed lower concentration of milk β-carotene during the prooestrus phase of the oestrous cycle in the present study when both LPO and AOPP concentration was greater. In the present study, LPO levels were greater during the prooestrus phase indicating the possible increased release of inflammatory mediators and vasoactive agents which is a prerequisite event at the preovulatory stage of follicle rupture (Agarwal *et al.* 2005).

Advanced oxidation protein products (AOPP) are markers of protein oxidation generated by the reaction between plasma proteins and myeloperoxidase derived chlorinated oxidants produced by activated neutrophils (Bordignon *et al.* 2014; Witko-Sarsat *et al.* 1998). It is possible to speculate that AOPP can be formed within the mammary gland following mild polymorphonuclear neutrophil leukocytes activation during prooestrus (Shirai *et al.* 2002).

In the present study, the greater level of BAP in cows with a locomotion score of 1.0 supports our previous findings of greater concentration of BAP in healthy rams compared to rams with lesions in hooves (Talukder and Celi, 2013). Primiparous cows had lower levels of ceruloplasmin compared to multiparous cows. Our previous study (Talukder *et al.* 2014*c*) reported that primiparous cows had greater reactive oxygen metabolites compared to their multiparous herd mates and suggested that this observation could be due to the extensively reported metabolic challenge associated with initiation of lactation and the growth and mammary gland development experienced by primiparous cows. In the present study, among the an-ovulated cows 60% (n = 6) were primiparous which partly explain the effect of increased metabolic challenge on ovulation failure in primiparous cows. Increased utilization of ceruloplasmin as a buffering action against free radicals may explain the lower concentration of ceruloplasmin in primiparous cows. The observed greater level of antioxidants in early lactation cows (<68 DIM) agrees with the earlier findings of

Wachter et al. (1999) who reported a progressive decline in blood antioxidant activity as lactation progresses which might be attributable to the depletion of antioxidants by milk. In the present study, oestrous cycles were observed during early lactation, a period of high metabolic activity and hence high oxidative stress in dairy cows.

Cows producing milk with different FPR had similar milk yield, which may indicate that cows in producing greater FPR milk may have enhanced body reserve mobilization and may have been experiencing some degree of metabolic stress. Among the oxidative markers examined in the present study, BAP, SOD and GSH-PX decreased significantly according to week of study. Silage is known for its poor content in antioxidants (Ballet *et al.* 2000) and grain based concentrates have been proven to increase the degradation of some antioxidants at the ruminal level (Weiss *et al.* 1995) thereby exposing cows to OS. In addition, grain based concentrate supplementation with the diet has been reported to reduce pasture intake (Bargo *et al.* 2002). It can be hypothesized that the observed decrease in all the antioxidants measured in this study might have been caused by a shift in diet composition or even intake volumes despite targeted consistency of allocation. Besides the nutritional factors, ambient temperature may also contribute to changes of OS markers across the time of study. However, we did not observe any significant association between ambient temperature or temperature humidity index and OS markers in the present study.

The lowest BAP in the HFPR group could be ascribed to the increased OS resulting from fat mobilization (Pedernera *et al.* 2010) while the greater BAP in the LFPR group suggests an improved antioxidant availability due to lower metabolic stress levels. Bernabucci *et al.* (2005) observed changes of oxidative status and relationships between oxidative and metabolic status and reported that cows in high negative energy balance are more sensitive to oxidative stress.

CONCLUSIONS

Milk concentration of GSH was lower in ovulated cows compared with that of an-ovulated cows in the present study which is consistent with our previous findings for plasma GSH (Talukder *et al.* 2014*c*). This finding is quite important particularly as differences in OS between ovulated and an-ovulated cows were observed in absence of changes in BW, BCS, milk yield and FPR. The decrease in antioxidant status present

during the peri-ovulatoty stage may be an important event preceding the ovulatory response. The observed increase in SOD and a decrease in GSH may well be the consequence of the ovulation process. The results also showed that the concentration of AOPP and LPO increased at prooestrus when the β-carotene level was at minimal level suggesting the possible involvement of inflammatory mediators at that phase. Veterinarians and research scientists can use different diagnostic measures for reproductive status; however, there would be considerable value in the development of a cow-side test to allow implementation of adequate interventions aimed at improving on farm reproductive management.

REFERENCES

Agarwal A, Gupta S, Sharma R (2005) Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology* **3,** 1-21.

Agarwal A, Gupta S, Sikka S (2006) The role of free radicals and antioxidants in reproduction. *Current Opinion in Obstetrics and Gynecology* **18,** 325-332.

Al-Gubory KH, Ceballos-Picot I, Nicole A, Bolifraud P, Germain G, Michaud M, Mayeur C, Blachier F (2005) Changes in activities of superoxide dismutase, nitric oxide synthase, glutathione-dependent enzymes and the incidence of apoptosis in sheep corpus luteum during the estrous cycle. *Biochimica et Biophysica Acta* **1725,** 348-357.

Aungier SPM, Roche JF, Sheehy M, Crowe MA (2012) Effects of management and health on the use of activity monitoring for estrus detection in dairy cows. *Journal of Dairy Science* **95,** 2452-2466.

Baker MA, Cerniglia GJ, Zaman A (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry* **190,** 360-365.

Ballet N, Robert JC, Williams PEV (2000). Vitamins in forages. In 'Forage evaluation in ruminant nutrition, Vol 19.' (Eds DI Givens, E Owen, HM Omed, RE Axford) pp. 399-432. (Oxon, UK, CABI Publishing)

Bargo F, Muller LD, Delahoy JE, Cassidy TW (2002) Milk response to concentrate supplementation of high producing dairy cows grazing at two pasture allowances. *Journal of Dairy Science* **85,** 1777-1792.

Bernabucci U, Ronchi B, Lacetera N, Nardone A (2005) Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. *Journal of Dairy Science* **88,** 2017-2026.

Bordignon M, Da Dalt L, Marinelli L, Gabai G (2014) Advanced oxidation protein products are generated by bovine neutrophils and inhibit free radical production in vitro. *The Veterinary Journal* **199,** 162-168.

Celi P (2010) The role of oxidative stress in small ruminants' health and production. *Revista Brasileira de Zootecnia* **39,** 348-363.

Celi P (2011) Oxidative Stress in Ruminants. In 'Studies on Veterinary Medicine. Vol. 5.' (Eds L Mandelker, P Vajdovich.) pp. 191-231. (Humana Press, Totowa, NJ)

Celi P, Merlo M, Barbato O, Gabai G (2012) Relationship between oxidative stress at mating and pregnancy outcome in dairy cows in pasture-based system. *The Veterinary Journal* **193,** 498–502.

Celi P, Merlo M, Da Dalt L, Stefani A, Barbato O, Gabai G (2011) Relationship between late embryonic mortality and the increase in plasma advanced oxidised protein products (AOPP) in dairy cows. *Reproduction, Fertility and Development* **23,** 527-533.

Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G (1989) A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* **72,** 68-78.

Fujii J, Iuchi Y, Okada F (2005) Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reproductive Biology and Endocrinology* **3,** 1-10.

Fulkerson WJ, Donaghy DJ (2001) Plant-soluble carbohydrate reserves and senescence - key criteria for developing an effective grazing management system for ryegrass-

based pastures: A review. *Australian Journal of Experimental Agriculture* **41,** 261-275.

Gabai G, Testoni S, Piccinini R, Marinelli L, Stradaioli G (2004) Oxidative stress in primiparous cows in relation to dietary starch and the progress of lactation. *Animal Science* **79,** 99-108.

Grieve DG, Korver S, Rijpkema YS, Hof G (1986) Relationship between milk composition and some nutritional parameters in early lactation. *Livestock Production Science* **14,** 239-254.

Heuer C, Schukken YH, Dobbelaar P (1999) Postpartum body condition score and results from the first test day milk as predictors of disease, fertility, yield, and culling in commercial dairy herds. *Journal of Dairy Science* **82,** 295-304.

Ireland JJ, Murphee RL, Coulson PB (1980) Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *Journal of Dairy Science* **63,** 155-160.

Kamphuis C, DelaRue B, Burke CR, Jago J (2012) Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. *Journal of Dairy Science* **95,** 3045-3056.

Kankofer M, Albera E, Feldman M, Gundling N, Hoedemaker M (2010) Comparison of antioxidative/oxidative profiles in blood plasma of cows with and without retained fetal placental membranes. *Theriogenology* **74,** 1385-1395.

Kehrer JP, Lund LG (1994) Cellular reducing equivalents and oxidative stress. *Free Radical Biology and Medicine* **17,** 65-75.

Kendall PE, Tucker CB, Dalley DE, Clark DA, Webster JR (2008) Milking frequency affects the circadian body temperature rhythm in dairy cows. *Livestock Science* **117,** 130-138.

Kindahl H, Edqvist L, Bane A, Granström E (1976) Blood levels of progesterone and 15-keto-13,14-dihydro-prostaglandinF2α during the normal oestrous cycle and early pregnancy in heifers. *Acta Endocrinologica* **82,** 134-149.

Lipko-Przybylska J, Albera E, Kankofer M (2010) Comparison of antioxidant defence parameters in colostrum and milk between Berrichon du Cher ewes and Uhrusk ewes. *Journal of Dairy Research* **77,** 117-122.

Lipko-Przybylska J, Kankofer M (2012) Antioxidant defence of colostrum and milk in consecutive lactations in sows. *Irish Veterinary Journal* **65,** 2-8.

Mantovani R, Sgorlon S, Marinelli L, Bailoni L, Bittante G, Gabai G (2010) Oxidative stress indicators and metabolic adaptations in response to the omission of the dry period in dairy cows. *Journal of Dairy Research* **77,** 273-279.

Pedernera M, Celi P, García SC, Salvin HE, Barchia I, Fulkerson WJ (2010) Effect of diet, energy balance and milk production on oxidative stress in early-lactating dairy cows grazing pasture. *The Veterinary Journal* **186,** 352-357.

Ranjan R, Swarup D, Naresh R, Patra RC (2005) Enhanced erythrocytic lipid peroxides and reduced plasma ascorbic acid, and alteration in blood trace elements level in dairy cows with mastitis. *Veterinary Research Communications* **29,** 27-34.

Rapoport R, Sklan D, Wolfenson D, Shaham-Albalancy A, Hanukoglu I (1998) Antioxidant capacity is correlated with steroidogenic status of the corpus luteum during the bovine estrous cycle. *Biochimica et Biophysica Acta* **1380,** 133-140.

Rizzo A, Roscino MT, Binetti F, Sciorsci RL (2012) Roles of reactive oxygen species in female reproduction. *Reproduction in Domestic Animals* **47,** 344-352.

Shirai F, Kawaguchi M, Yutsudo M, Dohi Y (2002) Human peripheral blood polymorphonuclear leukocytes at the ovulatory period are in an activated state. *Molecular and Cellular Endocrinology* **196,** 21-28.

Sprecher DJ, Hostetler DE, Kaneene JB (1997) A lameness scoring system that uses posture and gait to predict dairy cattle reproductive performance. *Theriogenology* **47,** 1179-1187.

Srikandakumar A, Ingraham RH, Ellsworth M, Archbald LF, Liao A, Godke RA (1986) Comparison of a solid-phase, no-extraction radioimmunoassay for progesterone

with an extraction assay for monitoring luteal function in the mare, bitch, and cow. *Theriogenology* **26,** 779-793.

Sugino N (2005) Reactive oxygen species in ovarian physiology. *Reproductive Medicine and Biology* **4,** 31-44.

Sunderman FWJ, Nomoto S (1970) Measurement of human serum ceruloplasmin by its *p*-phenylenediamine oxidase activity. *Clinical Chemistry* **16,** 903-910.

Talavera F, Chew BP (1988) Comparative role of retinol, retinoic acid and β-carotene on progesterone secretion by pig corpus luteum in vitro. *Journal of Reproduction and Fertility* **82,** 611-615.

Talukder S, Celi P (2013) Use of digital infrared thermography and oxidative stress biomarkers as a diagnostic tool to diagnose interdigital dermatitis in sheep. *Journal of Dairy Science* **96,** E-Suppl. 1, p. 382.

Talukder S, Celi P, Kerrisk KL, Garcia SC, Dhand NK (2014*a*) Factors affecting reproductive performance of dairy cows in a pasture-based, automatic milking system research farm: A retrospective, single-cohort study. *Animal Production Science* **55,** 31-41.

Talukder S, Ingenhoff L, Kerrisk KL, Celi P (2014*b*) Plasma oxidative stress biomarkers and progesterone profiles in a dairy cow diagnosed with an ovarian follicular cyst. *Veterinary Quarterly* 3**4,** 113-117.

Talukder S, Kerrisk KL, Ingenhoff L, Gabai G, Garcia SC, Celi P (2014*c*) Changes in plasma oxidative stress biomarkers in dairy cows after oestrus synchronization with controlled internal drug release (CIDRs) and prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) *Animal Production Science* **54,** 1490–1496.

Talukder S, Thomson PC, Kerrisk KL, Clark CL, Celi P (2015) Evaluation of infrared thermography (IRT) body temperature and collar mounted accelerometer and acoustic technology for predicting time of ovulation of cows in a pasture-based system. *Theriogenology* **83,** 739-748.

Tamate K, Sengoku K, Ishikawa M (1995) The role of superoxide dismutase in the human ovary and fallopian tube. *Journal of Obstetrics and Gynaecology* **21,** 401-409.

Venditti P, Meo SD (2006) Thyroid hormone-induced oxidative stress. *Cellular and Molecular Life Sciences CMLS* **63,** 414-434.

Wachter CM, McDaniel BT, Whitlow LW, Pettyjohn S (1999) Genetics of antioxidant activity in Holsteins and Jerseys: Associations with various traits. *Journal of Dairy Science* **82,** E-Suppl. 1, p. 31.

Weiss WP, Smith KL, Hogan JS, Steiner TE (1995) Effect of forage to concentrate ratio on disappearance of vitamins a and e during in vitro ruminal fermentation. *Journal of Dairy Science* **78,** 1837-1842.

Witko-Sarsat V, Friedlander M, Khoa TN, Capeillère-Blandin C, Nguyen AT, Canteloup S, Dayer J-M, Jungers P, Drüeke T, Descamps-Latscha B (1998) Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure 1, 2. *The Journal of Immunology* **161,** 2524-2532.

CHAPTER 7

Plasma Oxidative Stress Biomarkers and Progesterone Profiles in a Dairy Cow Diagnosed With an Ovarian Follicular Cyst

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OVERVIEW OF CHAPTER 7

In Chapter 3, a cow was diagnosed with cystic ovarian disease after oestrus synchrony. In this chapter, the findings are presented as a case report. The ovarian follicle diameter, hormonal profiles and oxidative stress biomarkers were tracked throughout the study period and are reported here in comparison to the ovulated cows in the same cohort.

ABSTRACT

This study was conducted to examine the oxidative stress biomarkers in a cow diagnosed with a follicular cyst in her left ovary. Progesterone (P_4) and plasma oxidative stress status was measured in 13 Holstein cows after synchronization of oestrus with controlled internal drug release (CIDR) and prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) protocol. The presence and size of ovarian structures were monitored by transrectal ultrasound at 4 hourly intervals. Of the 13 cows, 12 were monitored until ovulation was detected and recorded, whereas one cow failed to ovulate and developed a follicular cyst. Oxidative stress biomarkers; reactive oxygen metabolites (ROMs), biological antioxidant potential (BAP), oxidative stress index (OSI), glutathione (GSH), ceruloplasmin and advanced oxidation protein products (AOPP) were measured in the cystic cow and compared to those of the 12 ovulated cows and are referred to as higher or lower if they are outside the mean ± standard error of mean of those of ovulated cows. The cystic cow had lower ROMs and OSI between 36 h and 84 h after $PGF_{2\alpha}$ injection and at 9 h, from 36 h to 60 h after $PGF_{2\alpha}$ injection respectively. On the other hand, antioxidant (BAP and GSH) was higher in the cystic cow compared to her ovulated herd mates. The observed unbalance between oxidant and antioxidant might have disrupted the physiological events for ovulation to occur leading to cystic ovarian disease.

Keywords: follicular cyst, dairy cow, oxidative stress, progesterone.

INTRODUCTION

Ovarian follicular cyst is a common reproductive disorder in dairy cattle (Silvia *et al.* 2002). The typical incidence for follicular cysts in dairy cows is between 1% and 5% (Beam and Butler 1998). It is characterized by a deviation, growth and establishment of the dominant follicle, with a failure to ovulate to become a persistent follicular structure in the absence of a functional corpus luteum (Peter *et al.* 2009). The occurrence of ovarian follicular cysts has been associated with an extended calving interval (Borsberry and Dobson 1989).

Reactive oxygen species (ROS) and antioxidants remain in balance to maintain the cellular homeostasis, but when this balance is altered as a result of antioxidant depletion or increase in ROS production, oxidative stress (OS) occurs (Celi 2011*b*). Oxidative stress can affect a variety of physiological functions in the reproductive system; for example, follicular fluid environment, folliculogenesis and steroidogenesis and generally high levels of ROS can disrupt several reproductive events that may result in adverse pregnancy outcomes (Agarwal *et al.* 2005; Al-Gubory *et al.* 2010). It is plausible that OS plays a crucial role in the cause and progression of a number of reproductive events and can influence the reproductive axis at different levels. For example, previous studies have shown that OS is associated with embryonic loss (Celi *et al.* 2012; Celi *et al.* 2011) and that OS has a role in the pathogenesis of follicular cystic ovarian disease (Rizzo *et al.* 2009). Therefore, the objective of this study was to examine the plasma concentration of progesterone (P_4) and OS biomarkers that were measured in a cow diagnosed with an ovarian follicular cyst.

CASE DESCRIPTION

A 43-month-old lactating Holstein cow (Identification No. 1285) was diagnosed with a follicular cyst in her left ovary by a registered veterinarian using a transrectal ultrasound probe (Ibex Pro portable ultrasound, E.I. Medical Imaging, Loveland, CO, USA) by the presence of a black fluid filled cavity 30 mm in diameter that persisted throughout the monitoring period (70 - 76 days in milk) and failed to ovulate (Figure 1).

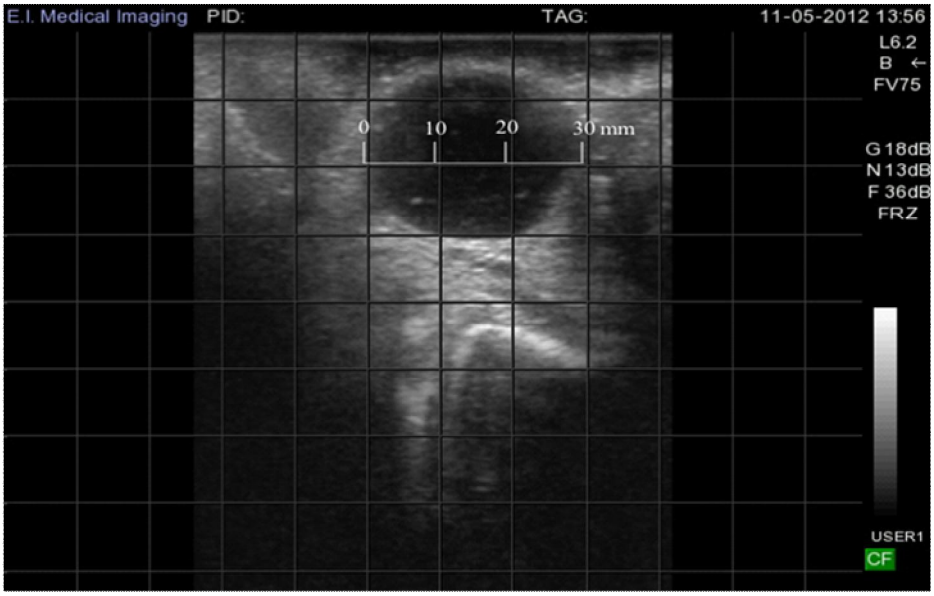

Figure 1. Ovarian ultrasound images of cow 1258. The cyst was diagnosed as a follicular cyst by the presence of a black fluid-filled cavity 30 mm in diameter that persisted throughout the monitoring period and failed to ovulate according to the criteria detailed by Leslie and Bosu (1983).

The structure was accompanied by the display of anoestrus according to the criteria detailed by Leslie and Bosu (1983). Cow 1285 was one of 20 cows enrolled in a study that was conducted to evaluate the potential of infrared thermography temperature monitoring for oestrus detection and prediction of time of ovulation in dairy cows; for more details and findings of the study, the reader is referred to Talukder *et al.* (2014*b*) for details about the nutritional and reproductive management of the cows. Of the 20 cows enrolled in this study, 12 ovulated, 7 did not ovulate, and 1 cow developed cystic ovarian disease. All experimental procedures were approved by The University of Sydney Animal Ethics Committee (N00/9-2012/1/5829).

Oestrus was synchronized by inserting a controlled internal drug release (CIDR; Eazi-Breed®, Pfizer Animal Health Limited, West Ryde, NSW, Australia) into the vagina. After eight days, CIDR's were removed and 2 mL (500 µg) of synthetic prostaglandin analogue ($PGF_{2\alpha}$), cloprostenol sodium (Estrumate®, Schering-Plough Animal Health Limited, Baulkham Hills, NSW, Australia) were injected intramuscularly to all cows at 0600 hours. Cows were monitored from 36 hours after $PGF_{2\alpha}$ injection six times daily until either ovulation or 128 hours after $PGF_{2\alpha}$ injection for oestrus signs as described in Talukder *et al.* (2014*b*).

Blood sampling was conducted according to the following schedule: twice daily from 24 hours before and 24 hours after $PGF_{2\alpha}$ injection, six times daily (at 0200, 0600, 1000, 1400, 1800, and 2200 hours) from 36 hours after $PGF_{2\alpha}$ injection until confirmation of ovulation or 128 hours after $PGF_{2\alpha}$ injection.

Plasma P_4 was determined by enzyme-linked immunosorbent assay (ELISA) using Progesterone EIA Kit (Cayman Chemical Co., Ann Arbour, MI, USA). The mean intra-assay and inter-assay coefficients of variation were 3.64% and 4.55%, respectively. The detection limit of the assay was 0.01 ng/mL.

The amount of free oxygen radicals in plasma samples was determined by measuring the concentration of reactive oxygen metabolites (d-ROMs Test; Diacron, GR, Italy), while the concentration of antioxidants was measured using the biological antioxidant potential (BAP) test according to kit instructions (Diacron, GR, Italy); ROMs and BAP concentrations were determined in a dedicated spectrophotometer (FREE system, Diacron International, GR, Italy). The extent of oxidative stress (OS) was expressed as an oxidative stress index (OSI) which was estimated using the ratio of ROMs/BAP × 100, as the combination of ROMs and BAP results provide a more accurate representation of OS status than ROMs and BAP alone (Celi 2011*a*). Plasma glutathione (GSH) concentration was measured by an enzymatic recycling method adapted for micro titre plate reader (Baker *et al.* 1990). All chemicals used for total plasma GSH were obtained from Sigma Aldrich (Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia). Advanced oxidation protein products (AOPP) were measured according to the methods of Witko-Sarsat *et al.* (1998). Plasma ceruloplasmin concentrations were determined according to the method described earlier (Sunderman and Nomoto 1970) with the exception that a 96-well plate was used for spectrophotometer reading and the absorbance was read at 510 nm (FLUROstar Optima, BMG Labtech, Mornington, VIC, Australia). Ceruloplasmin concentration was calculated as follows:

Ceruloplasmin (g/L) = 0.752 (A_R – A_B), where A_R is the absorbance of sample R (reaction), and A_B is the absorbance of sample B (blank).

All data related to P_4 profiles and OS status of cow 1258 were compared to those of the 12 ovulated cows and are referred to as higher or lower if they are outside the mean ± standard error of mean of the ovulated cows.

Cow 1258 presented lower plasma concentration of ROMs between 36 and 84 hours after $PGF_{2\alpha}$ injection compared to the ovulated cows (Figure 2A). On the other hand, plasma concentrations of BAP were greater in cow 1258 between 9 and 60 hours after $PGF_{2\alpha}$ injection compared to the ovulated cows (Figure 2B). Consequently, cow 1258 had lower OSI values at 9, 36, 48, 60 and 128 hours after $PGF_{2\alpha}$ injection compared to the ovulated cows (Figure 2C). Plasma concentrations of GSH were higher in cow 1258 compared to the ovulated cows at -24, 9, from 36 to 60 hours, and from 84 to 120 hours after $PGF_{2\alpha}$ injection (Figure 2D). Cow 1258 had greater concentrations of plasma ceruloplasmin at 0, 9, 72, 120 and 128 hours but lower concentrations between 24 and 48 hours after $PGF_{2\alpha}$ injection compared to the ovulated cows (Figure 2E). Greater AOPP levels were observed at 0, 9, 72, 108 and 128 hours in cow 1258 while AOPP values were lower at 48 and 60 hours after $PGF_{2\alpha}$ injection compared to her ovulating herd mates at those sampling sessions, respectively (Figure 2F).

Cow 1258 did not display any behavioural signs of oestrus during the study period and presented lower P_4 concentrations 9, 24, 36, 84, 96, 120 and 128 hours after $PGF_{2\alpha}$ injection compared to those of ovulated cows (Figure 3A). The diameters of the largest ovarian follicular structure were greater in cow 1258 from 24 hours after $PGF_{2\alpha}$ injection to 128 hours after $PGF_{2\alpha}$ injection compared to her ovulated herd mates are presented in Figure 3B.

Overall, the results gathered in this study suggest that antioxidant/oxidant balance was likely to be disrupted in cow 1258 compared to her ovulated herd mates.

Cow 1258 had relatively lower plasma ROMs concentration after $PGF_{2\alpha}$ injection compared to the ovulated cows which is consistent with the findings of Rizzo *et al.* (2009) where a lower concentration of ROMs has been reported in the follicular fluid of cystic cows. Reactive oxygen species and their metabolites play important roles in the regulation of ovarian metabolism, leading to breakdown of follicular walls, a prerequisite step to ovulation (Celi 2011*a*; Rizzo *et al.* 2012). It could be argued that the $PGF_{2\alpha}$ treatment was unable to generate adequate amounts of free radicals in cow 1258 and that the lower plasma ROMs concentrations might have been too low to induce the breakdown of the follicular wall, leading to the transformation of the preovulatory follicle into a follicular cyst.

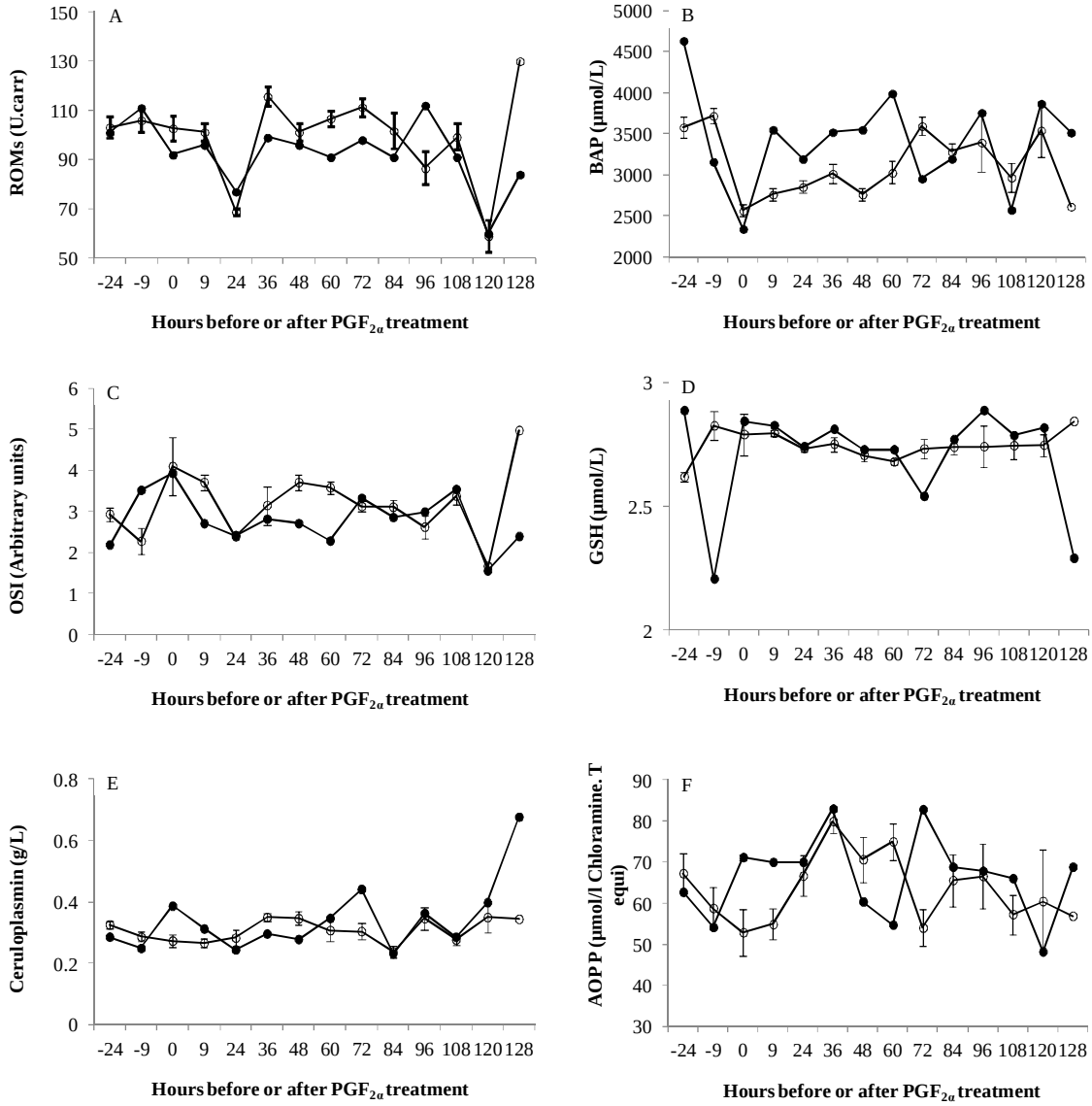

Figure 2. Plasma concentrations of A) reactive oxygen metabolites (ROMs), B) biological antioxidant potential (BAP), C) oxidative stress index (ROMs/BAP), D) glutathione (GSH), E) ceruloplasmin and F) advanced oxidation protein products (AOPP) in cow 1258 (●) that was diagnosed with a follicular cyst and the mean (± SEM) of 12 ovulated cows (○).

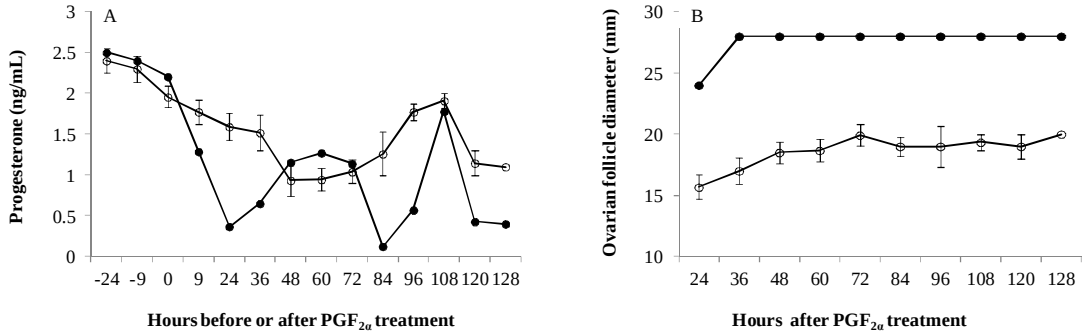

Figure 3. Plasma progesterone concentration A) and follicle diameter B) (means ± SEM) of cow 1258 (●) that was diagnosed with a follicular cyst and the mean (± SEM) of 12 ovulated cows (○).

Cow 1258 had higher concentration of BAP between 9 hours to 60 hours after $PGF_{2\alpha}$ injection compared to the ovulated cows. The BAP test provides a measurement of many antioxidants, including uric acid, ascorbic acid, proteins, α-tocopherol and bilirubin, all of which are indicative of short-term changes of the antioxidant system. The observed higher BAP concentration might be attributed to the concomitant lower concentration of ROMs. In our previous study (Talukder *et al.* 2014*a*), it was reported that ovulated cows had significantly lower plasma antioxidant and higher OSI compared to that of an-ovulated cows which might be ascribed to an increase in antioxidant consumption for the ovulation event. Therefore, it can be hypothesized that the observed higher concentration of BAP may have interfered with the physiological role of ROMs on ovulation (Riley and Behrman 1991).

Glutathione is the major non-protein sulphydryl compound in mammalian cells and is considered as a good indicator of the free radical scavenging capacity of blood (Gabai *et al.* 2004). In the present study, the lower ROMs concentration after $PGF_{2\alpha}$ injection might have contributed to the observed plasma GSH pattern. The higher GSH and BAP levels may have contributed to the observed decreased OSI in the cystic cow. Increased protection of GSH against free radicals and other oxidants might be speculated to be associated with decreased OS resulting in the development of an ovarian cyst in cow 1258.

In the present study, cow 1258 had lower ROMs and OSI after $PGF_{2\alpha}$ injection suggesting that the antioxidant/oxidant balance may have been disrupted. It is possible

that the disruption between oxidant and antioxidant balance prevented the necessary physiological responses involved in inflammatory reactions which trigger the follicle rupture and hence ovulation. The lower OS reported here may either hinder the ovulation event or alter the steroidogenesis of the dominant follicle resulting in the development of ovarian cyst in cow 1258 (Rizzo *et al.* 2009).

Plasma ceruloplasmin concentrations observed in this case study were within the range reported in heifers (Golder *et al.* 2014). Ceruloplasmin is a serum ferroxidase that contains greater than 95% of the copper found in plasma (Hussein *et al.* 2012) and is involved in cellular pro-oxidant and antioxidant processes (Ehrenwald and Fox 1996).

Advanced oxidation protein products (AOPP) are the markers of protein oxidation generated by the reaction between plasma proteins and myeloperoxidase-derived chlorinated oxidants produced by activated neutrophils (Witko-Sarsat *et al.* 1998). In humans, AOPP increase has been associated with several immuno-inflammatory markers and they have been proposed as an exquisite marker of OS correlating with monocyte activation (Witko-Sarsat *et al.* 1998). The prolonged half-life of AOPP allows an indirect reflection of the intensity of the OS (Santulli *et al.* 2013) and has been reported to be elevated in dairy cows with embryonic mortality (Celi *et al.* 2011). The AOPP level was elevated at 9 hours and this may have been associated with the development of the cyst in cow 1258; however, this cannot be concluded definitively from the results of this study.

The clinical signs of anoestrus and the presence of an enlarged persistent follicular structure in cow 1258 were consistent with the diagnosis of a follicular cyst (Mwaanga and Janowski 2000). Low P_4 concentration (<1 ng/ml) in plasma has been also reported as one of the characteristics of follicular cysts (Mwaanga and Janowski 2000). The low P_4 concentration may be the direct result of an absence of any luteal tissue being present in the ovaries of cows with cystic ovarian disease.

The data of this study suggest that inadequate ROMs concentration may be unable to induce ovulation in cow 1258. Insufficient concentrations of ROMs might have resulted in the observed higher levels of BAP and GSH that may have inhibited the oxidative reactions involved in the breakdown of the follicular wall and the resultant transformation of the preovulatory follicle into a follicular cyst. Further studies

investigating the oxidant and antioxidant status in ovarian fluids and utero-ovarian circulation of preovulatory follicles compared to the fluid from cystic follicles would be required to allow a full understanding of the role of OS biomarkers in the development of an ovarian follicular cyst. The findings presented in this manuscript provide sufficient justification to warrant targeted research into the true value that the oxidative status of individual animals might provide as an indicator of cystic ovaries or any other reproductive conditions.

REFERENCES

Agarwal A, Gupta S, Sharma R (2005) Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology* **3,** 1-21.

Al-Gubory KH, Fowler PA, Garrel C (2010) The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *International Journal of Biochemistry and Cell Biology* **42,** 1634-1650.

Baker MA, Cerniglia GJ, Zaman A (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry* **190,** 360-365.

Beam S, Butler W (1998) Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *Journal of Reproduction Fertility Supplement* **54,** 411-424.

Borsberry S, Dobson H (1989) Periparturient diseases and their effect on reproductive performance in five dairy herds. *Veterinary Record* **124,** 217-219.

Celi P (2011*a*) Biomarkers of oxidative stress in ruminant medicine. *Immunopharmacology and Immunotoxicology* **33** 233-240.

Celi P (2011*b*) Oxidative Stress in Ruminants. In 'Studies on Veterinary Medicine. Vol. 5.' (Eds L Mandelker, P Vajdovich.) pp. 191-231. (Humana Press, Totowa, NJ)

Celi P, Merlo M, Barbato O, Gabai G (2012) Relationship between oxidative stress and the success of artificial insemination in dairy cows in a pasture-based system. *The Veterinary Journal* **193,** 498-502.

Celi P, Merlo M, Da Dalt L, Stefani A, Barbato O, Gabai G (2011) Relationship between late embryonic mortality and the increase in plasma advanced oxidised protein products (AOPP) in dairy cows. *Reproduction, Fertility and Development* **23,** 527-533.

Ehrenwald E, Fox PL (1996) Role of endogenous ceruloplasmin in low density lipoprotein oxidation by human U937 monocytic cells. *The Journal of Clinical Investigation* **97,** 884-890.

Gabai G, Testoni S, Piccinini R, Marinelli L, Stradaioli G (2004) Oxidative stress in primiparous cows in relation to dietary starch and the progress of lactation. *Animal Science* **79,** 99-108.

Golder HM, Celi P, Rabiee AR, Lean IJ (2014) Effects of feed additives on rumen and blood profiles during a starch and fructose challenge. *Journal of Dairy Science* **97,** 985-1004.

Hussein AH, Staufenbiel R, Müller AE, A. E-S, Abd-El-Salam M (2012) Ceruloplasmin activity in Holstein dairy cows: Effects of lactation stages and anticoagulants. *Comparative Clinical Pathology* **21,** 705-710.

Leslie K, Bosu W (1983) Plasma progesterone concentrations in dairy cows with cystic ovaries and clinical responses following treatment with fenprostalene. *The Canadian Veterinary Journal* **24,** 352.

Mwaanga E, Janowski T (2000) Anoestrus in dairy cows: Causes, prevalence and clinical forms. *Reproduction in Domestic Animals* **35,** 193-200.

Peter AT, Vos PLM, Ambrose DJ (2009) Postpartum anestrus in dairy cattle. *Theriogenology* **71,** 1333-1342.

Riley JC, Behrman HR (1991) Oxygen radicals and reactive oxygen species in reproduction. *Experimental Biology and Medicine* **198,** 781-791.

Rizzo A, Minoia G, Trisolini C, Mutinati M, Spedicato M, Jirillo F, Sciorsci RL (2009) Reactive oxygen species (ROS): Involvement in bovine follicular cysts etiopathogenesis. *Immunopharmacology and Immunotoxicology* **31,** 631-635.

Rizzo A, Roscino MT, Binetti F, Sciorsci RL (2012) Roles of reactive oxygen species in female reproduction. *Reproduction in Domestic Animals* **47,** 344-352.

Santulli P, Borghese B, Lemaréchal H, Leconte M, Millischer A-E, Batteux F, Chapron C, Borderie D (2013) Increased serum oxidative stress markers in women with uterine leiomyoma. *PloS One* **8,** e72069.

Silvia WJ, Hatler TB, Nugent AM, Laranja da Fonseca LF (2002) Ovarian follicular cysts in dairy cows: An abnormality in folliculogenesis. *Domestic Animal Endocrinology* **23,** 167-177.

Sunderman FWJ, Nomoto S (1970) Measurement of human serum ceruloplasmin by its *p*-phenylenediamine oxidase activity. *Clinical Chemistry* **16,** 903-910.

Talukder S, Kerrisk KL, Ingenhoff L, Gabai G, Garcia SC, Celi P (2014*a*) Changes in plasma oxidative stress biomarkers in dairy cows after oestrus synchronization with controlled internal drug release (CIDRs) and prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$). *Animal Production Science.* **54,** 1490–1496.

Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P (2014*b*) Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture based system. *Theriogenology* **81,** 925-935.

Witko-Sarsat V, Friedlander M, Khoa TN, Capeillère-Blandin C, Nguyen AT, Canteloup S, Dayer J-M, Jungers P, Drüeke T, Descamps-Latscha B (1998) Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure 1, 2. *The Journal of Immunology* **161,** 2524-2532.

CHAPTER 8

Rumination Patterns, Locomotion Activity and Milk Yield for a Dairy Cow Diagnosed With a Left Displaced Abomasum (LDA)

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OVERVIEW OF CHAPTER 8

During the investigation reported in Chapter 5, a cow was diagnosed with left displaced abomasum. Her activity, rumination patterns and milk yields were compared to cows in the same cohort and are compiled and presented here as a case study.

ABSTRACT

A cow (body weight 600 kg; age 3 years; 76 days in milk) was identified by herdspersons due to decreased milk yield and inappetence and drafted for veterinarian examination. The veterinary practitioner diagnosed her for left displaced abomasums (LDA). Neck collars (SCR HR LD) were fitted 7 days before the start of the study that continuously monitored the individual cow activity level (AL) and rumination level (RL) for 2-hour time blocks. A decrease in RL and AL was observed as early as 6 days and 3 days, respectively before the LDA clinical diagnosis followed by a rapid increase in RL and AL during the postoperative period. The LDA cow also displayed decreased milk yield for the 5 days before the day of clinical diagnosis which remained lowered after treatment. Changes in rumination along with changes in activity and milk yield may enable the early prediction of a LDA, which may result in improvements in animal well-being and recovery speed.

Keywords: left displaced abomasums, rumination, activity, milk yield, dairy cow.

INTRODUCTION

Left displaced abomasum (LDA) is an important metabolic disease in dairy cattle, with diagnosis based on signs such as anorexia, decreased milk production and clinical examination by simultaneous auscultation and percussion (van Winden *et al.* 2003). One possible method to identify potential health problems in dairy cows before they show clinical signs is to use an automated system that records the activity profiles of an individual cow (Edwards and Toze 2004). Dramatic changes in rumination patterns can be attributed to sudden changes in intake and ill health (Bar and Solomon 2010), so a decrease in daily rumination along with a decrease in daily activity may provide an early warning to detect LDA in dairy cows. Here we report changes in the levels of rumination, activity and milk yield associated with an LDA before clinical diagnosis and after surgical correction.

CASE DESCRIPTION

Thirty Holstein cows were enrolled in a field study for evaluating the potential of SCR heat and rumination long distance (HR LD) tags (SCR Engineers Ltd., Netanya, Israel) for oestrus detection. All experimental procedures were approved by the University of Sydney Animal Ethics Committee. All cows had access to pasture as well as concentrates and maize silage. The SCR HR LD tags continuously monitored individual cow activity level (AL) and rumination level (RL) for 2 hour time blocks (Bar and Solomon 2010). Using a mathematical algorithm, a weighted activity and rumination index was calculated that expressed the momentary deviation of activity and rumination, respectively, from the average activity and rumination in the same period during the previous 7 days (Kamphuis *et al.* 2012). Daily milk production was also recorded for each animal.

One 3 year old cow (cow 1471), that was 76 days in milk, was identified by herdspersons due to decreased milk yield and inappetence, and drafted for veterinary examination. The veterinary practitioner diagnosed a left displaced abomasum (LDA) based on auscultation of tympanic, resonant, high-toned ping between approximately the ninth and thirteenth ribs. Surgical correction by right flank omentopexy was carried out on the same day (Day 0). Data were collated of 24-hour average weighted AL, RL, and

milk yield during the period 10 days either side of Day 0. All data were compared to those of the 29 healthy cows.

During the entire period, milk yield was lower in cow 1471 than in healthy cows, with a marked reduction from Day −6 to −5 that continued until Day 1 (Figure 1). On Days −9 and −7, RL was higher, and from Day −6 to 0, was lower compared to healthy cows. Subsequently, an increase in RL was observed that was similar to that observed for healthy cows (Figure 2). Activity level was lower in cow 1471 from Days −3 to Day 0 compared to that of the healthy cows. The day after the LDA was surgically corrected activity level for Cow 1471 resumed to similar levels of the healthy cows (Figure 3).

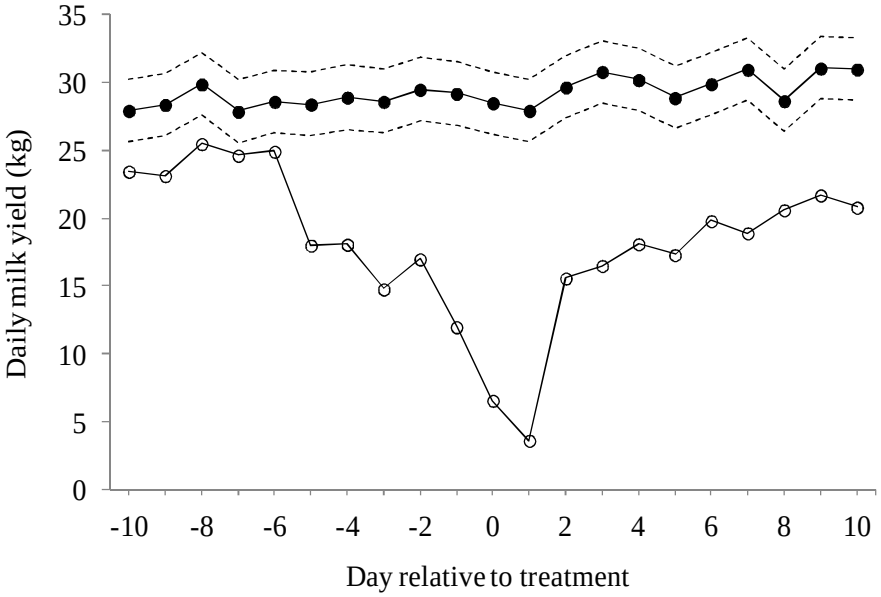

Figure 1. Average daily milk yield of healthy cows (●) and LDA cow (○). The day relative to treatment in X-axis relates to the relative day of diagnosis and surgical correction of the LDA (cow 1471). The dashed lines indicate the 95% confidence interval (CI) limits and the vertical dotted line (at Day 0) indicates the day of clinical diagnosis of the LDA for cow 1471. Milk yields are referred to as higher or lower if they are outside the CI of healthy cows.

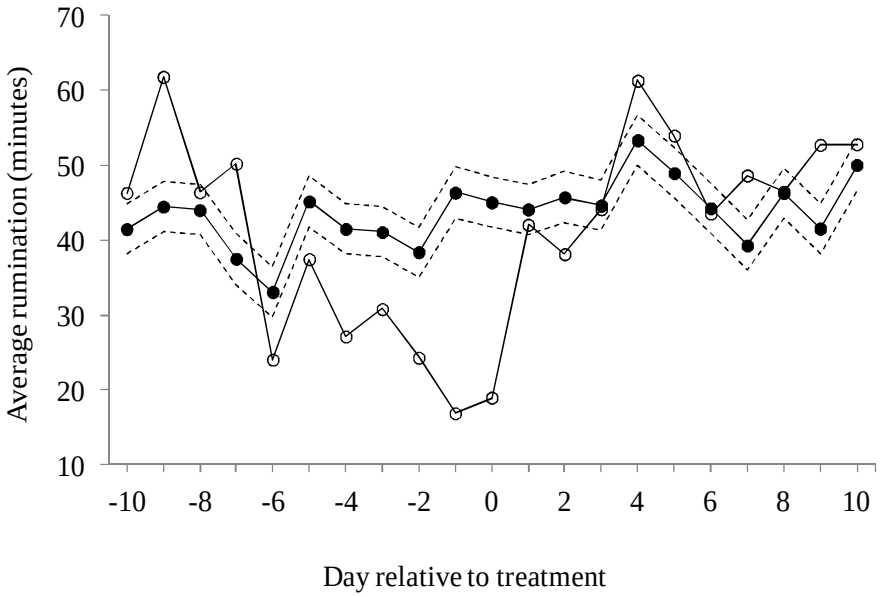

Figure 2. Average rumination level of healthy cows (●) and LDA cow (○). The day relative to treatment in X-axis relates to the relative day of diagnosis and surgical correction of the LDA (cow 1471). The dashed lines indicate the 95% confidence interval (CI) limits and the vertical dotted line (at Day 0) indicates the day of clinical diagnosis of the LDA for cow 1471. The levels are referred to as higher or lower if they are outside the CI of healthy cows.

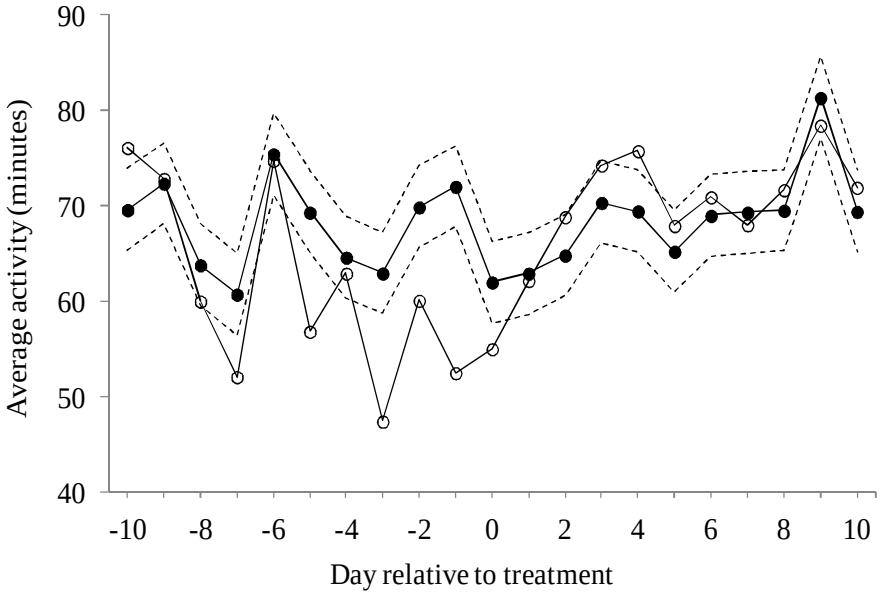

Figure 3. Average activity level of healthy cows (●) and LDA cow (○). The day relative to treatment in X-axis relates to the relative day of diagnosis and surgical correction of the LDA (cow 1471). The dashed lines indicate the 95% confidence interval (CI) limits and the vertical dotted line (at Day 0) indicates the day of clinical diagnosis of the LDA for cow 1471. The levels are referred to as higher or lower if they are outside the CI of healthy cows.

Based on the changes in RL profiles and milk yield, it may be possible to detect LDA at least 5 days earlier than the actual date of the clinical diagnosis. The decreased rumination observed in Cow 1471 was likely due to abomasal enlargement and reduced rumen fill, and the decreased activity may have been attributed to a loss of appetite and reduced grazing time, restricted movement, discomfort and spending more time lying down (Schultz 1988; Edwards and Toze 2004). This was associated with decreasing milk yield from Day −5 to 1. The AL and RL in Cow 1471 returned to similar levels of the healthy cows after treatment; however, milk yield was lower during the entire period after treatment, which agrees with the findings of Edwards and Toze (2004). One possible reason for this is that the most common treatment method for LDA is surgery and surgery itself has been shown to have long term negative effects on milk production (Edwards and Toze 2004).

A decline in activity for cows clinically diagnosed with ketosis and digestive disorders 8 days earlier than clinical diagnoses of those disorders has also been reported (Edwards and Toze 2004). However, rumination changes across the different metabolic conditions were not evaluated in that study. Further studies would be required to understand the specific pattern of activity and rumination in relation to different types of metabolic conditions. The early postpartum period is considered to be the major risk period for developing LDA (van Winden *et al.* 2003), however, in the present study, Cow 1471 was diagnosed as having an LDA on Day 76 of lactation, which may render her case less representative of the typical LDA population.

The development of detection models/algorithms based on both rumination level and activity level might provide a real-time indication of the health status of cows, providing cattle veterinarians and herdspersons with an accurate early diagnosis that may result in improvements in animal well-being and speed of recovery. Considering the increase in herd size and production per cow, the use of automated technology and sensors is becoming more prevalent in livestock farming (Maltz 2010). Further studies regarding the sensitivity and specificity of this technology for early prediction of LDA and other digestive disorders during the transition period are warranted.

REFERENCES

Bar D, Solomon R (2010) Rumination collars: What can they tell us. In 'Proceedings of the First North American Conference'. pp. 214–215. (Precision Dairy Management, Toronto, Canada) Available at http://www.precisiondairy2010.com/proceedings/s11bar.pdf [verified 2 October 2014]

Edwards JL, Tozer PR (2004) Using activity and milk yield as predictors of fresh cow disorders. *Journal of Dairy Science* **87,** 524-531.

Kamphuis C, DelaRue B, Burke CR, Jago J (2012) Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. *Journal of Dairy Science* **95,** 3045-3056.

Maltz E (2010) Novel technologies: Sensors, data and precision dairy farming. In 'The First North American Conference on Precision Dairy Management 2010.'

Schultz LH (1988) Milk fever, ketosis, and the fat cow syndrome. In 'The Ruminant Animal: Digestive Physiology and Nutrition'. (Ed. DC Church) pp. 493–509. (Prentice-Hall, Inc: Englewood Cliffs, NJ)

Van Winden SCL, Jorritsma R, Müller KE, Noordhuizen JPTM (2003) Feed intake, milk yield, and metabolic parameters prior to left displaced abomasum in dairy cows. *Journal of Dairy Science* **86,** 1465-1471.

CHAPTER 9

General Discussion and Conclusions

GENERAL DISCUSSION

One of the objectives of this research was to evaluate the reproductive performance of dairy cows in automatic milking systems (AMS) and to identify the possible factors associated with reproduction. This was achieved by conducting a retrospective cohort study (Chapter 2). The initial extensive literature review (Chapter 1) indicated that in intensive housing systems, a number of studies have been conducted to evaluate reproductive performance in AMS. Whilst some of these had relatively short data collection periods and others reported on a very limited number of parameters to quantify reproductive performance, there was no strong or consistent indications that the incorporation of AMS in an indoor system impacted negatively (or positively) on reproductive performance. Those that did show an impact on reproduction were not able to conclusively show that the cause could be attributed to changes in ovulation detection or fertility per se due to the confounding effect of milking frequency and milk production. Regardless, there is no literature reporting on the impact of pasture-based AMS on reproductive performance. The review of literature (Chapter 1) identified several factors associated with poor reproduction in dairy cows, which indicates the value of planning prospective studies and reproductive interventions to increase the reproductive performance in the Australian dairy industry.

Even though numerous factors are known to impact on reproductive performance, detection of ovulation is frequently reported as one the most limiting factors to achieve high in-calf rates and short intervals between calving and conception. In pasture-based dairying systems, the process of herding cows for set milking sessions creates an opportunity for personnel to detect oestrus expression in dairy cows. The incorporation of AMS into the pasture-based farming system impacts on these opportunities as the farming operation is reliant on voluntary cow traffic with very few cows (only those that are overdue for milking) being herded/encouraged to the milk harvesting facility. In Chapter 2, it was observed that at the reported production levels and milking frequencies for the AMS research herd, milk yield and milking frequency during 100 days in milk had no effect on reproductive measures. However, the probability of first oestrus being recorded by 49 days in milk and conception by 100 days in milk tended to be higher for cows milked at least 2.3 times/day, on average, compared with cows milked <1.5 times/day. Only 1% of cows averaged three or more milkings per day during the first

100 days in milk (Chapter 2). It may be possible that these cows moved around the system voluntarily more frequently and may have gained access to more fresh allocations of feed, thereby reducing the duration of early-lactation, negative energy balance. It is also possible that cows with higher milking frequencies were inadvertently observed by farm staff more frequently, which could have increased the chance of an oestrus event being detected. Conversely, it was possible that lower milking frequency cows (in early lactation) were suffering a subclinical (or clinical) disease, which reduced their willingness to move around the farm system thereby resulting in reduced intakes, milking frequency and milk production.

With AMS, the practice and routines to detect ovulation must be modified to ensure that cows are detected, drafted, and inseminated in a timely manner to ensure that reproductive performance of the herd is not negatively impacted. Some brands of AMS incorporate ovulation detection aids including activity meters, rumination monitoring devices, and inline milk progesterone monitoring. However, not all aids are available with any one chosen brand or the farmer may chose not to incorporate these into the farming system due to perceived value/reliability or cost. In addition some may not be available to some markets e.g. the inline milk progesterone monitoring (Herd Navigator) is not currently available to Australian farmers.

An advantage of most AMS is that in many ways cows are managed as individuals rather than as a herd. This aspect of AMS lends itself to automation of many management practices since cows' traffic around the farm and through the dairy as individuals or small groups. The use of infrared thermography (IRT) as an ovulation detection aid is one such technology that might suit automation and incorporation into an AMS facility since cows are generally directed into and out of the milk harvesting facility in single file through automatic drafting gates. If data capture with IRT requires the cows to be held in one location for more than a few moments it might be better suited to the actual milking event. Even in this case, the number of IRT devices would be limited to the number of milking stations which might be considerably less than the number of bails in a conventional milking parlour. Field studies were conducted to evaluate the potential of IRT for predicting the time of ovulation in synchronized (Chapter 3) and 21-day natural cycles (Chapter 5) under pasture-based grazing conditions. Of the alert thresholds and algorithms evaluated in Chapter 3, the 1.0

standard deviation threshold of adjustment 2 resulted in the greatest sensitivity (Se); however, the specificity (Sp) and positive predictive values (PPV) were lower than desirable.

The sharp increase in muzzle and vulval IRT temperature 48 h to 24 h before ovulation observed in Chapter 3 was deemed likely to coincide with the timing of oestrus. However, it has frequently been assumed that under pasture-based system the influence of ambient temperature variation on cow body temperature may obscure the physiological changes in skin temperature during the oestrus cycle.

In Chapter 3, cows oestrus cycles were synchronized and IRT data capture was conducted at four hour intervals while in Chapter 5 IRT was performed twice daily in unsynchronized cows. Whilst it is possible that the synchrony of the oestrus cycle might have impacted on the ovulation detection accuracy achieved in chapter 3, performing IRT twice in a day in the investigation reported in Chapter 5 may have resulted in ovulation cases going undetected. The number of false negatives can potentially be reduced if IRT could be performed continuously rather than twice in a day although such a practice would have limited application potential (particularly in pasture based dairy systems). Whilst there is not reported literature regarding the ideal frequency of data capture for IRT or even if continuous data capture is necessary – it is challenging to imagine the application feasibility of a higher data capture frequency which will impact on the value of further evaluations.

Accelerometers are another technology that deserve investigation regarding their value and in accuracy as ovulation detection aids in pasture-based AMS. Whilst some work has been reported with the use of accelerometers in indoor AMS facilities, the results cannot be extrapolated with confidence to grazing based farms. This is primarily because the level of variability in activity and grazing behaviour is likely to be considerably higher in pastured cows than it might be in indoor facilities. This factor alone may impact significantly on the value and accuracy of such technology in pasture-based AMS farms particularly since AMS cows traffic around the farming system voluntarily and can have considerably high levels of variation in trafficking frequency both within cows and between cows in the same herd. As observed in Chapter 5, when the SCR tags activity threshold was set < 20, it yielded the highest detection performance (80.0% Se, 94.3% Sp and 66.7% PPV). When the SCR tags' activity

threshold was increased to 50, it resulted in the greatest Se but poorest Sp. Visual detection of mounting indicators resulted in higher Sp and PPV but lower Se than those measured by SCR HR LD tags. For automated detection systems to be of practical use in pasture based herds, they should perform at least at the level achieved by the average farmer using manual detection methods (Kamphuis *et al.* 2012). Considering that when using visual observation aided by tail paint it is possible to achieve a performance level of 80% Se with 80% PPV, these values should could be considered as the minimum performance targets for automated oestrus detection systems (Xu *et al.* 1997; Verkerk *et al.* 2001; McGowan Je 2007; Kamphuis *et al.* 2012). The SCR HR LD tags used in Chapter 5 were able to achieve 75% Se when used as an independent system. This performance level may be of benefit to dairy herds with lower level of manual detection (<80%) and larger herds relying more on inexperienced staff and handling a higher number of cows per person (Newman 2011; Kamphuis *et al.* 2012).

Besides using SCR HR LD tags for automatic ovulation detection, the development of detection models/algorithms based on both rumination level and activity level might provide a real-time indication of the health status of cows, providing cattle veterinarians and herdspersons with an accurate early diagnosis, which may result in improvements in animal well-being and speed of recovery. The rumination and activity level recorded by SCR HR LD tags was able to make an early prediction of left displaced abomasum in a cow (Chapter 8). A decrease in rumination level and activity level was observed as early as 5 days and 3 days, respectively before its clinical diagnosis followed by a rapid increase in rumination level and activity level during the postoperative period. The occurrence of metabolic and infectious diseases may decrease the productive and reproductive performance in dairy cow and therefore establishment of methods to monitor health and reproductive status simultaneously might be beneficial to maintain the farm profitability.

Ovulation, one of the most important physiological events in female reproduction has been compared to inflammation (Espey 1980). The mechanism of inflammation is associated with an increase of blood circulation resulting to an increase of temperature in the respective inflamed tissue. In Chapters 3 and 5 IRT has been used to evaluate its potential in monitoring the increase in skin temperature and hence detecting the ovulation event in dairy cows. The inflammatory process is often associated with

oxidative stress which has been reported to be involved in follicle rupture and ovulation as mentioned in the review (Chapter 1). Assessing the biomarkers of oxidative stress in biological samples (plasma and milk) can be a novel approach for the prediction of ovulation in dairy cows. These hypotheses were tested in Chapters 4 and 6. Lower plasma biological antioxidant potential (BAP) and glutathione (GSH) levels were observed in ovulated cows compared to those of an-ovulated cows (Chapter 4). Milk superoxide dismutase level was higher in ovulated cows while glutathione peroxidase and GSH concentrations were lower in ovulated cows compared to those in an-ovulated cows (Chapter 6). These findings supported that the decrease in antioxidant status during the peri-ovulatoty stage may be an essential event preceding the ovulatory response and further studies might lead to cow side tests to monitor ovulation.

Plasma concentration of oxidative stress biomarkers were measured in a cow diagnosed with an ovarian follicular cyst (Chapter 7) and were compared to those of the 12 ovulated cows reported in Chapter 3. The cystic cow had lower reactive oxygen metabolites (ROMs) and oxidative stress index (OSI) but higher antioxidant levels (BAP and GSH) compared to her ovulated herd mates. These findings suggested that insufficient concentrations of ROMs might have resulted in the observed higher levels of BAP and GSH that may have inhibited the oxidative reactions involved in the breakdown of the follicular wall and the resultant transformation of the preovulatory follicle into a follicular cyst. Further research needs to be conducted to assess whether or not the milk OS biomarkers reflect similar changes to those observed with plasma OS biomarker monitoring. If so, measuring the milk OS biomarkers in dairy species may enable the automation of monitoring concentrations (of biomarkers) allowing the identification of reproductive disorders prior to clinical manifestation.

Unfortunately, there are no specific reference values for free radicals and antioxidants status in dairy cows. Moreover, using different types of assay kits for measuring oxidative stress biomarkers may influence the values reported in the literature. There would be considerable value in the development of the specific referral ranges and cow-side test to allow implementation of adequate interventions aimed at improving on farm reproductive management.

As a result of technical progress in real-time livestock/cow monitoring systems, automatic oestrus detection systems have become a practical reality (Nebel 2014). An

added advantage is that it is becoming apparent that the data generated by some of these systems can also be used to generate real-time alerts regarding other physiological and health conditions. Such additional values enhance the value proposition of these technologies and also allow for improved productivity through the early detection (and intervention) of some health conditions and improved animal wellbeing through the improved timing of treatment and potentially improved cure-rates and reduced production losses.

In this thesis, multidisciplinary approaches (technologies and biomarkers of oxidative stress) were employed as indicators of imminent ovulation in dairy cows. Accelerometer data proved to be a more accurate indicator of ovulation than IRT. Ovulated cows presented higher oxidative stress index and lower antioxidant status compared to those in an-ovulated cows whilst changes in oxidative stress biomarkers were observed in cystic cows compared to those of ovulated cows. These findings may provide a foundation for future development of an in-line automated biosensor suitable to identify the reproductive events in dairy species.

CONCLUSIONS

The first aim of this research thesis was to examine the risk factors associated with reproductive performance in pasture-based AMS. Although the likelihood of first oestrus being detected decreased steadily in relation to year of AMS commissioning, the general lack of reported long-term reproductive performance data in AMS makes it challenging to ascertain the true cause/effect relationship in this findings. Certainly, without the inclusion of data from additional AMS farms, we cannot conclude that AMS directly (or even indirectly) caused the trend.

The second aim was to evaluate technologies (IRT and accelerometer) for ovulation detection in dairy cows and to assess whether these technologies can replace current methods (visual detection and Estrotect) in pasture-based farming systems. Performing IRT at 4-hour intervals may have an acceptable level of oestrus detection accuracy; however, it is challenging to imagine a feasible and practical application on farm. Accelerometer technology provided a good indication of when ovulation is likely to occur. Although the Sp estimated in this study were deemed acceptable, the Se and PPV using different thresholds were considerably lower than the desirable 100% detection

rate. Prospective studies regarding the pattern and the cause of false negatives may help to identify the possible ways to improve the sensitivity of the system-generated activity alerts.

The third aim was to examine the changes of milk and plasma OS biomarkers between ovulatory and an-ovulatory oestrus cycles in dairy cows. The observed decrease in antioxidants (BAP and GSH) in ovulated cows suggested that the antioxidant status present during the peri-ovulatoty stage may be an essential event preceding the ovulatory response. It would be worthwhile to conduct further studies comparing the oxidant and antioxidant status in ovarian fluids and utero-ovarian circulation to allow a full understanding of the physiological role of OS biomarkers on the ovulatory process.

The findings contained within this thesis will provide valuable information for veterinarians and farmers who are interested in technologies and biosensors for reproductive management in pasture-based system however, it is accepted that at least some of the technologies are still a way off adoption for on-farm application. In addition, the findings will provide a foundation for research scientists in the field of oxidative stress regarding the possible involvement of oxidants and antioxidants in reproductive events in dairy cows; however, further research is required to justify the development of cow-side tests that would allow implementation of adequate interventions aimed at improving on farm reproductive management.

REFERENCES

Espey Ll (1980) Ovulation as an inflammatory reaction—a hypothesis. *Biology of Reproduction* **22,** 73-106.

Kamphuis C, DelaRue B, Burke CR, Jago J (2012) Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. *Journal of Dairy Science* **95,** 3045-3056.

McGowan JE, Burke CR, Jago JG (2007) Validation of a technology for objectively measuring behaviour in dairy cows and its application for oestrous detection. In 'Proceedings of the New Zealand Society of Animal Production.' pp. 136-142. (New Zealand Society of Animal Production, New Zealand)

Nebel RL (2014) Attaining reproductive solutions through activity and health monitoring. In Dairy Cattle/Technical articles/Genetics – Reproduction Available at http://en.engormix.com/MA-dairy-cattle/genetic/articles/attaining-reproductive-solutions-through-t3081/103-p0.htm [verified 22 June 2015]

Newman M (2011) DairyNZ economic survey 2009–10. (DairyNZ Ltd., Newstead, New Zealand)

Verkerk G, Claycomb RW, Taufa VK, Copeman P, Napper A, Kolver E (2001) CowTrakker technology for improved heat detection. In 'Proceedings of the New Zealand Society of Animal Production.' pp. 172-175. (New Zealand Society of Animal Production, New Zealand)

Xu ZZ, McKnight DJ, Vishwanath R, Pitt CJ, Burton LJ (1997) Estrus detection using radiotelemetry or visual observation and tail painting for dairy cows on pasture. *Journal of Dairy Science* **81,** 2890-2896.