When to inseminate the cow?

Insemination, ovulation and fertilization in dairy cattle

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When to inseminate the cow?

Insemination, ovulation and fertilization in dairy cattle

Judith Bernardine Roelofs

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Abstract

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In dairy practice, calving rates after first insemination are often less than 50%. Part of this low percentage might be explained by wrongly timed inseminations. The aim was to establish the relationship between various oestrus characteristics and ovulation time in order to investigate whether these oestrus characteristics could predict ovulation time and to study the consequences of variation in the interval between insemination and ovulation on the success of fertilization and embryonic characteristics. The ultimate goal of the project was to come to an optimal insemination strategy that can be used in practice. The relationship between behavioural oestrous signs, changes in activity (measured by pedometers) and progesterone profiles and time of ovulation were. It was shown that monitoring progesterone profiles was not suitable to predict time of ovulation because of the large variation found in the decrease of progesterone relative to ovulation. Although the prediction of ovation time using behavioural oestrus signs was quite accurate, it seems not suitable for practice because of the high labour requirements. The increase in the number of steps measured by pedometers seems useful to predict time of ovulation accurately and could be easily implemented in practice. Effects of the interval between insemination and ovulation on success of fertilization and embryonic characteristics were also studied. It was shown that the insemination-ovulation interval in which high fertilization rates were observed was quite long (insemination from 36 to 12h before ovulation), while the interval in which the majority of fertilized ovum developed into a good quality embryo was considerably shorter (insemination from 24 to 12h before ovulation); the interval between insemination and ovulation did not affect the sex ratio of the embryos.
In conclusion, the best chance to increase calving rates seems to be to use the insemination strategy, in which cows are inseminated between 5 to 17h after the first increase in the number of steps.

Keywords: dairy cattle; oestrus; behaviour; pedometer; reproductive hormones; ovulation time; insemination strategy

Voorwoord

Na eerst de vakgroep te hebben leren kennen als afstudeervakker, toegevoegd onderzoeker en secretaresse ben ik vier jaar geleden begonnen aan mijn promotie-onderzoek. Deze vier jaar kan ik achteraf misschien het best beschrijven als een achtbaan, zowel van emoties als van gebeurtenissen, die elkaar in een snel tempo hebben afgewisseld. Vele mensen hebben vol enthousiasme op hun eigen manier een bijdrage geleverd aan deze achtbaanrit, waarvoor ik ze hartelijk wil bedanken.

Op het moment dat je voor het eerst in een achtbaan stapt, zijn er spanning en onzekerheid; 'wat gaat er komen?', 'zal ik het wel aan kunnen en leuk vinden?'. Gelukkig zijn er dan mensen langs de kant die controleren of je riemen wel goed vast zitten, je geruststellen en je de rest van de rit in de gaten houden om zonodig het karretje bij te sturen. Nicoline en Bas, mijn dank voor jullie enthousiasme, positief-kritische houding en betrokkenheid (zelfs tijdens zwangerschap- en ouderschapsverloven). Naar mijn mening kan een AIO zich geen betere begeleiders wensen.

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Dan zijn er de mensen die ergens in het karretje gaan zitten, vaak niet wetende hoe de rit zal verlopen. Zonder deze mensen zou de rit niet zo gezellig zijn geweest en niet tot een goed einde zijn gekomen. Graag wil ik de mensen van de Ossekampen, Frans, Arie, Ilona en Leen, bedanken voor hun hulp en in het bijzonder Ronald die alle inseminaties op de vreemdste tijdstippen heeft uitgevoerd. Govert, 'mijn embryospoeler', bedankt voor al het werk dat je verzet hebt en de gezelligheid. Emmy en Frits, reproductie-onderzoek betekent veel nacht- en weekendwerk, dankzij jullie hulp en meedenk-werk is de kar op de rails gebleven. Een afstudeervak bij mij betekende een aanslag op je nachtrust, daarom wil ik de studenten, Bram, Carlijn, Carolijn, Erwin, Frank, Gert-Jan, Hanneke, Janmar, Jasper, Joost, Josina, Karin, Klaas-Jan, Kristel, Laura, Lia, Linda, Luis en Willemiek bedanken voor hun inzet en het harde werken.

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Bij een achtbaan zijn er ook mensen die langs de kant blijven staan en de rit van een afstandje volgen. Al kunnen zij niet precies bevatten wat de reis in de achtbaan inhoudt, ze zijn altijd bereid om naar de verhalen te luisteren en om je soms even de hectische rit te laten vergeten. Graag wil ik dan ook mijn vrienden en familie bedanken voor hun interesse en begrip wanneer mijn standaard antwoord werd 'ik kan vanavond wel komen, mits mijn koeien niet tochtig worden…'.

Op het eind van de rit heb je mensen nodig die, wanneer de kar tot stilstand is gekomen, je begeleiden naar de uitgang, omdat je benen nog wat slapjes aanvoelen van de heftige rit. Pap en Maartje, bedankt dat jullie de taak als paranimfen op jullie nemen.

Ik wil graag eindigen met een citaat van Midas Dekkers; *'Koeien, dat zijn pas beesten! En zo worden ze nog steeds gemaakt'*.

De rit was fantastisch en leerzaam, ik kan niet wachten om in de volgende achtbaan te stappen…

Judith
Wageningen, september 2005

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1

General Introduction

Introduction

For conception to occur, insemination must take place at the correct stage of the cow's oestrus cycle. Successful fertilization highly depends on the interval from insemination to ovulation. When cows are inseminated too early, sperm are aged by the time ovulation occurs and they cannot successfully fertilize an ovum anymore (Hawk 1987). When insemination takes place too late, fertilization and formation of a viable embryo may not be possible anymore because of ageing of the egg (Hunter & Greve 1997). Not only the chance of fertilization can be affected by the timing of insemination, also early embryonic development can be altered when inseminations are performed too early or too late (Dalton et al. 2001). Indications exist that, in practice, the timing of insemination relative to ovulation is highly variable with subsequent negative consequences for pregnancy- and calving rate. The causes of the variation in the timing of insemination relative to ovulation include variation in oestrus detection strategy and -accuracy, in moment of ovulation after onset or end of oestrus and in the timing of insemination after detection of oestrus. In a recent study, it was found that true calving rates after first insemination are only 42% in The Netherlands (17 representative farms, Poelarends & Smolders 2004). The question arises how much of these low calving rates can be attributed to incorrect timing of insemination. To determine the best time for insemination relative to the time of ovulation there is a need for practical predictors of ovulation time and information on fertilization rates and embryo survival rates at various insemination-ovulation intervals.

Possibilities to predict time of ovulation

For a parameter to be useful as a predictor of ovulation time, several prerequisites need to be met. It should have a small variation in time to ovulation, and the measurements should be easy to carry out, repeatable and preferably be automated and it should be present in a high proportion of the animals. In this paragraph various parameters will be discussed in light of their usefulness as ovulation predictors.

Oestrus behaviour

The behaviour of a cow changes during oestrus. This change in behaviour is mediated by the oestrogens produced by the preovulatory follicle. The clearest sign of oestrus behaviour is the standing heat, i.e. a cow is standing immobile when she is mounted by another cow. However, this behaviour is expressed by less than 50% of the cows (Heres et al. 2000, Lyimo et al. 2000, Van Eerdenburg et al. 2002). (Attempts to) mount other cows are seen in 80% of the cows in oestrus and 54% of the cows in oestrus are mounted but will not stand (Van Vliet & Van Eerdenburg 1996). Other behavioural signs that are observed frequently during oestrus are

sniffing the vulva of another cow and resting with the chin on another cow (Van Eerdenburg et al. 1996). A large variation exists in expression of oestrous behaviour between individuals.

Several experiments have studied the interval between onset or end of the behavioural oestrus, defined by first or last display of standing heat, and the time of ovulation (Table 1.1). Intervals between the onset of standing heat and time of ovulation ranged from 16 to 61h, with an average of 24.0 to 38.5h. Intervals between end of standing heat and time of ovulation ranged from 3 to 18h with an average of 9.2 to 12.4h. Thus, the end of showing standing heat is a rather accurate predictor of ovulation time. However, as already mentioned, standing heat is displayed in a low proportion of the oestrous periods.

Table 1.1. Overview of intervals between onset or end of oestrus, based on standing heat, and time of ovulation found in different studies

interval to ovulation (h) (range)	n	assessment of ovulation time (frequency)	assessment of oestrus (frequency)	Reference
onset of oestrus				
27.8 (16-38)	125	?	?	Gerasimova 1940
(20.5-27.0)	3	?	?	Fietta et al. 1968
32.8	13	palpation (2h)	visual (continuously)	Randel et al. 1973
31.3 (26->36)	21	laparoscopy (once)	visual (12h)	Christenson et al. 1975
26.0 (21-29)	4	laparoscopy (continuously)	visual (4h)	Bernard et al. 1983
24.0/30.0 (pluri-/biparous)	8	ultrasound (2h)	visual (?)	Rajamahendran et al. 1989
27.6 (16-40)	93	ultrasound (2h)	mounting detector (continuously)	Walker et al. 1996
37.7 (18-60)	30	ultrasound (12h)	visual (?)	Augusto et al. 1997
26.6 (18-35)	42	ultrasound (2h)	visual (continuously)	Pinheiro et al. 1998
24.9	23	ultrasound (24h)	mounting detector (continuously)	Lopez et al. 2002
38.5 (28-61)	12	ultrasound (4h)	visual (4h)	Saumande & Humblot 2005
end of oestrus				
10.5 (3-18)	132	palpation (2h)	visual (2h)	Trimberger 1948
11.1	50	palpation (4h)	visual (8h)	Aschbacher et al. 1956
12.4	51	palpation (6h)	visual (6h)	Hall et al. 1959
9.2 (6-14)	6	palpation (2h)	visual (2h)	Wishart 1972

A cow displays a number of other behavioural signs during oestrus (Van Eerdenburg et al. 1996). In a study of van Eerdenburg et al. (2002) it was shown that the intensity of oestrus behaviour was related to the time of ovulation. Cows that

showed more oestrus behaviour (based on a combination of standing heat, being mounted but not standing, mounting other cows, sniffing, flehmen and chin resting) ovulated earlier than cows that showed less oestrus behaviour (r=0.31, P=0.01). In that study, ovulation was assessed at 24h intervals by ultrasound examinations. Based on those results, it seems that a more accurate monitoring of oestrus expression could lead to a more accurate prediction of ovulation time. However, up to now the relationship between behavioural signs other than display of standing heat and time of ovulation has not been studied. Therefore, in Chapter 3 of this thesis, an experiment is described in which the relationship between various behavioural oestrous signs and time of ovulation was established, to study whether a (combination of) behavioural oestrous signs could be used to predict time of ovulation.

LH, oestrogens and progesterone

LH

Bernard et al. (1983) found an interval between onset of LH-surge and ovulation of 27.3±1.6h and between end of LH-surge and ovulation of 17.5±1.5h. Other studies indicate that the peak in LH-surge occurs approximately 25h before ovulation (Schams et al. 1977, Rajamahendran et al. 1989, Saumande & Humblot 2005). The LH-surge, therefore, is a reliable predictor of ovulation time. In practice, however, it is not feasible to determine the LH-peak to predict time of ovulation, because blood samples need to be taken at short intervals. Unfortunately, LH profiles cannot be established in milk (Johnson & Reeves 1988).

Oestrogens

Few reports exist regarding the relationship between levels of oestradiol and timing of ovulation. In heifers, Mosher et al. (1990) found an interval between oestradiol-peak (in plasma) and time of ovulation of 22.3±3.9h. Lopez et al. (2002) found larger intervals between oestradiol-peaks and ovulation. They measured oestradiol in plasma and milk samples and found mean intervals from highest measured plasma and milk oestradiol until ovulation of 30.7±6.3h and 46.7±5.3h, respectively. A large variation was found in the time that the highest oestradiol concentration was detected relative to the onset of oestrus. Monitoring of oestrogen-levels is unsuitable as predictor of time of ovulation on the farm, because of the large variation between animals to time of ovulation and because of the fact that, currently, measurements of oestrogen levels cannot be easily automated in practice.

Progesterone

Another hormone that might predict time of ovulation is progesterone. Progesterone concentrations start to decrease two to three days before ovulation and remain low up to six days after ovulation (Dieleman et al. 1986). Progesterone concentrations can be measured in blood as well as in milk (Dobson et al. 1975). In numerous studies the possibility of using progesterone concentrations to detect oestrus has been investigated (Phillips & Schofield 1988, Sawyer et al. 1990, Delwiche et al. 2001, Friggens & Chagunda 2005). It was found that low progesterone concentrations not necessarily indicate oestrus, because progesterone levels are also low during the post partum period when cyclicity has not yet been resumed. High progesterone concentrations, however, do indicate that ovulation will not occur, even though the cow may show oestrous behaviour (Nebel et al. 1987). To our knowledge, no information is available about the relationship between progesterone concentrations and the actual time of ovulation. Therefore, we designed an experiment to study the feasibility of predicting time of ovulation by monitoring progesterone levels, which is described in Chapter 5 in this thesis.

Vaginal mucus conductivity/resistance

Several studies have shown that vaginal mucus conductivity/resistance changes during oestrus (reviewed by Rorie et al. 2002). Electrical resistance is lowest during oestrus because of changes in cell density, fluid volume and electrolyte content of the bovine vulva and vagina (Ezov et al. 1990). Leidl & Stolla (1976) found evidence that the resistance of vaginal mucus is affected by oestrogen. Several other studies have shown that the lowest resistance coincides with the LH-surge; the interval between lowest resistance and time of ovulation was between 32 and 24h (Schams & Butz 1972, Leidl & Stolla 1976, Schams et al. 1977, Aboul Ela et al. 1983). The resistance dropped from an average of 48 Ohm between oestrous periods to an average of 30 Ohm during oestrus (Schams & Butz 1972). Various studies reported variation, both among and within cows, in conductivity measurements, resulting in undesirably high rates of false positives and false negatives (Elving et al. 1983, Lehrer et al. 1995). Also the high labour requirement and hygiene risks greatly limit the practicality of this approach. So, measurements of vaginal mucus conductivity/resistance do not seem useful for the prediction of time of ovulation. We have conducted a pilot-experiment to establish the relationship between changes in vaginal mucus resistance and time of ovulation. Because of the high variation in conductivity values found during and in between oestrus, between animals as well as within animals, this parameter seemed not a reliable predictor of ovulation time and, therefore, the results of that study are not published in this thesis.

Body temperature

During the oestrous cycle significant changes in body temperature occur (reviewed by Firk et al. 2002). During oestrus the temperature rises about 0.3°C (Nieuwenhuizen et al. 1979, Mosher et al. 1990). This may be caused by the higher activity during oestrus, but the mechanism behind the rise is not clear. Although this temperature rise has been related to oestrus-characteristics, only little is known about the relationship of this parameter with time of ovulation. In heifers a rather variable interval between the peak in vaginal temperature and ovulation of 21.1±6.1h has been found (range: 16-33h; Mosher et al., 1990). From this experiment, Mosher et al. (1990) concluded that since ovulation occurs within a consistent interval from the onset of a temperature spike, the onset of a temperature spike might be as good a predictor of ovulation as the LH peak. Rajamahendran et al. (1989) found that the peak in vaginal temperature occurred in heifers 22.0±3.5h and in cows 27.0±3.5h before ovulation. They found a high correlation between vaginal and rectal temperature, but the rise in temperature before ovulation was only significant in the vaginal temperature measurements. Their study demonstrated that the rise in vaginal temperature was a reliable measure of the time of ovulation and the time of the LH-surge. Several authors dispute, however, the usefulness of body temperature measurements as an oestrus detection method and therefore as predictor of time of ovulation (Boyd et al. 1969, Lewis & Newman 1984, Firk et al. 2002). The measurements could eventually be automated if one would measure the milk temperature as a derivative of the body temperature. However, as already mentioned, the temperature rise during oestrus is small, only 0.3°C. Therefore, body temperature is not a specific indicator of the incidence of oestrus, because a rise in temperature can also be caused by inflammatory reactions and variation in environmental temperature. In conclusion, temperature measurements do not seem a practical tool to predict time of ovulation. We have conducted a pilot-experiment to establish the relationship between changes in rectal temperature and time of ovulation. Because of the high variation in rectal temperature, found during and in between oestrus, between animals as well as within animals, this parameter seemed not a reliable predictor of ovulation time and, therefore, the results of that study are not published in this thesis.

Activity measurements

Activity of a cow can be measured automatically by pedometers that record the number of steps during a period of time (Farris 1954). Many studies have shown that there is an increase in the number of steps taken by a cow during oestrus (Farris 1954, Kiddy 1977, Lewis & Newman 1984, Arney et al. 1994, López-Gatius et al. 2005). In most studies the number of steps was measured in 12h periods; for the use as predictor of ovulation time, smaller periods might be more accurate. Maatje

et al. (1997) used pedometers that recorded the number of steps in 2h periods and studied the effect of different intervals between the onset of oestrus and time of insemination on conception rates. The conception rate was 84.2% when insemination was performed 6 to 17h after the increase in activity and declined rapidly when insemination took place outside this interval. This strong relationship between time of insemination and conception rate assumes that the increase in activity has a good relationship with time of ovulation, but up to now this relationship has not been studied. Therefore, in Chapter 4 of this thesis, an experiment is reported in which the relationship between an increase in the number of steps (measured with a pedometer that stored the number of steps in 2h periods) and time of ovulation is established and the applicability of pedometer readings to predict time of ovulation, in practice, is discussed.

Conclusions

Several parameters (standing heat, hormone-profiles, body temperature, vaginal conductivity and walking activity) have been studied for their use in oestrus detection or prediction of ovulation time. The best predictor of ovulation time seems to be the LH-surge. However, LH-concentrations cannot easily be assessed in practice; therefore the use of monitoring LH-concentrations to predict to time of ovulation is limited to experimental studies. Apart from a low feasibility, monitoring oestradiol-concentrations, body temperature and conductivity of vaginal mucus do not seem to be closely enough related to ovulation time to predict time of ovulation. Onset of showing standing heat might be useful to predict time of ovulation. However, standing heat is displayed in only 50% of all oestrous periods, which limits its use as a predictor of ovulation time. Therefore, next to standing heat, the relationships between other behavioural oestrous signs, progesterone concentrations and the increase in activity and ovulation time are established in this thesis to study their usefulness as predictor of ovulation time (Chapter 3, 4 and 5). Further, in the general discussion (Chapter 8) also the combination of parameters to predict ovulation time is discussed.

Insemination-ovulation interval

Influence on fertilization/pregnancy

As mentioned before, the optimal time for artificial insemination depends on the one hand on the lifespan of spermatozoa to ensure fertilization and on the other hand on the viable lifespan of the ovum after ovulation. The lifespan of spermatozoa in the cow's oviduct is reported to be 24 to 48h for fresh and 12 to 24h for frozen-thawed semen, the viable lifespan of the ovum is suggested to be around 6 to 12h (Gordon 2003).

In pigs, the optimal time for insemination lies between 0 and 24h before ovulation (reviewed by Kemp & Soede 1997). Not much is known concerning fertilization rates of cows at various insemination-ovulation intervals. Studies from the 1940's showed that conception rates in cattle differed when cows were bred during different stages of oestrus (Trimberger & Davis 1943, Barrett & Casida 1946). The best conception rates (varying from 73% to 86%) were obtained when inseminations were performed 24 to 6h before ovulation (Trimberger 1948). As conception rate, in his study, was assessed by pregnancy diagnoses, it is not known whether the low conception rates were a result of fertilization failure, (early) embryonic death or both. Surprisingly, since then not much new information has appeared concerning the optimal time of insemination relative to ovulation in dairy cattle (reviewed by Hunter 1994).
Based on research that was performed in the 1970's, it was assumed that in cattle, insemination should take place between 12 and 18h before ovulation to get good fertilization results (Hunter 1994). However, he assumed that ovulation takes place at a rather stable 12h after the end of oestrus. From recent studies, it is known that the moment of ovulation relative to oestrus is far more variable. In recent years, research has been done to study effects of different insemination times relative to oestrus characteristics on conception rate (Maatje et al. 1997, Dransfield et al. 1998) but time of ovulation was not assessed in these studies. These studies show variable results. Maatje et al. (1997) found conception rates (at 42 to 49 days after insemination) of around 80% for inseminations 0 to 24h after the onset of increase in walking activity and of 17.6% for inseminations more than 24h after the onset of increase in walking activity. Dransfield et al. (1998) found highest conception rates of 51% when inseminations were performed 4 to 12h after the first display of standing heat (as detected by mount detectors). In their studies, it is not known whether suboptimal insemination times resulted in higher fertilization failure or higher (early) embryonic death. This distinction could be made in a study of Dalton et al. (2001). They assessed fertilization rate and embryo quality seven days after insemination when cows were inseminated 0, 12 or 24h after the onset of standing heat (using mount detectors). Inseminations performed 24h after the onset of standing heat resulted in the highest fertilization rate, whereas inseminations performed at the onset of standing heat resulted in the highest percentage of good quality embryos. They concluded that inseminations performed 12h after the onset of oestrus provided a compromise between a potentially lower fertilization rate (AI at the onset of standing heat) and lowered embryo quality (AI at 24h after the onset of standing heat). Therefore, in Chapters 6 and 7 of this thesis, an experiment is described in which effects of different insemination-ovulation intervals on fertilization and characteristics of seven-day-old embryos are studied.

Influence on the sex ratio

It has been suggested that early inseminations (i.e. long before ovulation) would result in more female calves whereas late inseminations (i.e. close to ovulation) would result in more male calves, due to different timing of capacitation and survival time of the X- and Y-chromosome bearing spermatozoa in the female reproductive tract (Martinez et al. 2004). Some studies indeed found an influence on sex ratio by different insemination times (Wehner et al. 1997, Pursley et al. 1998), although others did not (Yadav et al. 1990, Rorie et al. 1999). In order to determine if the sex ratio can be influenced by the insemination-ovulation interval, this parameter is included in the experiment described in Chapters 6 and 7.

Conclusions

In conclusion, no clear picture has been found concerning conception rates with varying oestrus to insemination intervals. Since 1948, no studies have been conducted evaluating the effects of different insemination to ovulation intervals. The studies that have been conducted in this area can not distinguish between effects on success of fertilization and effects on embryonic survival rates. In this thesis the results of experiments with varying insemination-ovulation intervals are reported and discussed in Chapters 6 and 7.

Aim and outline of the thesis

The aim of the project was two-fold. Firstly, to establish the relationship between various oestrus characteristics and ovulation time in order to investigate whether these oestrus characteristics could predict ovulation time. Secondly, to study the consequences of variation in the interval between insemination and ovulation on the success of fertilization and embryonic characteristics. The ultimate goal of the project was to come to an optimal insemination strategy that can be used in practice.

In studies in which the interval between onset or end of standing heat and the ovulation time was assessed, ovulation was often assessed using rectal palpation (Table 1.1). This is not the ideal method to assess time of ovulation because the preovulatory follicle can rupture during palpation. In the study of Rajamahendran et al. (1989), frequent rectal ultrasound examinations appeared not to affect ovulation. However, as no control animals were included in their study, it could not be concluded that frequent ultrasound examinations had no effect. Therefore, the experiment described in Chapter 2 aimed at investigating whether repeated rectal ultrasound examinations around ovulation influenced oestrous behaviour and hormone-profiles, thereby assessing whether repeated rectal ultrasound could be used to determine the time of ovulation.

In the experiments described in Chapters 3-5, the relationships between various oestrus characteristics and ovulation time were established and the possibilities to predict time of ovulation was studied. The oestrus characteristics studied were:

- Behavioural oestrous signs, since in dairy practice primarily the behaviour of the cows is observed to determine the insemination strategy (Chapter 3)
- The increase in activity, since the literature indicates a close relationship between this parameter and ovulation time and it can be easily automated (Chapter 4)
- The decrease in progesterone, since it occurs in all cycling animals before ovulation. When the measurements can be done on-line during milking the measurements would be ease to carry out in practice (Chapter 5)

The experiments studying the second aim of the project are described in Chapters 6 and 7. In Chapter 6 effects of variable insemination to ovulation intervals are described on fertilization and embryonic characteristics seven days after ovulation. The following embryonic characteristics were assessed: embryo quality and –morphology, number of cell cycles and number of accessory sperm cells. In Chapter 7, the effect on the sex of the embryo is described.

Finally, in Chapter 8, the results of the experiments described in Chapter 3-7 are combined to theorize on an optimal insemination strategy based on ovulation prediction.

2

Influence of repeated rectal ultrasound examinations on hormone profiles and behaviour around oestrus and ovulation in dairy cattle

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Abstract

Frequent rectal ultrasound is often used to assess time of ovulation. This study investigated whether frequent rectal ultrasound examination, affects behavioural oestrus and peri-ovulatory hormone profiles (LH, oestradiol and progesterone). Additionally, the relationship between peri-ovulatory hormone profiles, oestrous behaviour and time of ovulation was studied. Oestrus was synchronised in two consecutive cycles of Holstein Friesian cattle (parity from 1 to 6; n=24 cycles). In 12 of these cycles, time of ovulation was assessed by three-hourly rectal ultrasound (assessment of ovulation time=UG) the other half served as controls (n=12; no assessment of ovulation time=CG). There were no significant differences between the onset of oestrus (33.8±1.6h), duration of oestrus (13.4±0.9h) or intensity of oestrous behaviour (1047±180 points) between UG and CG treated animals. Furthermore, LH, oestradiol and progesterone profiles were similar between UG and CG. For UG, ovulation took place 30.2±1.9h after onset of oestrus. This interval had the largest variation (21h) of all parameters studied, ranging between 19 and 40h after onset of oestrus. The smallest variation (6h) was found in the timing of ovulation in relation to the LH-peak; ovulation took place 25.3±0.6h (range 21.5 to 27.5h) after the peak in LH. This study demonstrated that repeated rectal ultrasound does not alter behavioural oestrus or peri-ovulatory hormone and is therefore a useful tool for assessing time of ovulation. Further research, using ultrasound, can now be carried out to find predictors for time of ovulation.

Introduction

For conception to occur, insemination must take place at the correct stage of the cow's oestrous cycle. Fertilization is highly dependent on the interval from insemination to ovulation. When cows are inseminated early, the aged sperm may not be capable of fertilizing the ovum (Hawk 1987). When insemination is late, fertilization and formation of a viable embryo may not be possible because of an ageing egg (Hunter & Greve 1997). In practice, enormous variability exists in the timing of insemination relative to ovulation, with subsequent negative consequences for pregnancy rate. The causes of variability include variation in oestrus detection strategy and -accuracy, variation in the moment of ovulation after detection of oestrus and in the timing of insemination after detection of oestrus. It is best to inseminate according to characteristics that have a clear relationship with time of ovulation. To find such 'characteristics', time of ovulation needs to be assessed accurately, without interference with the characteristics studied. Ultrasound is often used to assess ovulation (Pierson & Ginther 1984, Quirk et al. 1986, Pierson & Ginther 1987, Rajamahendran et al. 1989). Using once daily ultrasound, Sirois and Fortune (1988) observed normal oestrous cycle length, normal progesterone levels,

and typical preovulatory LH surges; there was no evidence that repeated ultrasound modified the course of a normal oestrous cycle. However, for more precise detection of ovulation time more frequent ultrasound examinations are desired.
The present study investigated whether frequent rectal ultrasound examinations, during and after oestrus, affects behavioural oestrus characteristics and peri-ovulatory hormone profiles. Ultrasound was performed every 3h from the onset of oestrus until ovulation had occurred. Additionally, the relationship between peri-ovulatory hormone profiles, oestrous behaviour and time of ovulation was studied.

Materials and Methods

Experimental design

The experiment was conducted at the experimental dairy farm “de Ossekampen” at Wageningen University and Research Centre, the Netherlands. The Ethical Committee for Experimentation with Animals, Wageningen, the Netherlands, approved the experimental protocol in advance of the study. Two batches of animals were used. Animals in both batches were followed during two synchronised oestrous periods (first synchronisation=FS, second synchronisation=SS). Frequent rectal ultrasound was performed to assess time of ovulation. For each animal, time of ovulation was assessed in one of the two synchronised oestrous periods (assessment of ovulation time with ultrasound group=UG, no assessment of ovulation time group=CG). A jugular vein catheter was placed for blood sampling for hormone-profiles and visual observation of oestrous behaviour took place.

Animals, feed and housing

Data were collected from eight primiparous and six multiparous cycling lactating Holstein-Friesian cows. The parity of the animals varied between 1 and 6. The animals were 65±7.7 (SD) days in lactation (range: 39 to 96 days) when the ear-implant was inserted. The animals were fed an ad lib mixture of grass silage, corn silage and mineral supplements, and concentrates according to production level (CVB, 2000). The animals were housed in a free stall with slatted floor and cubicles. Milking was twice a day at 6.30h until 7.00h and 16.00h until 16.30h.

Synchronisation

Oestrus was synchronised with an ear-implant containing 3 mg norgestomet and an im injection of 3 mg norgestomet and 5 mg oestradiol valerate in oil (the Crestar method, Intervet, Boxmeer, The Netherlands). Implants were removed after 10 days. Four days before the implant was removed, an im injection of 15 mg of Prostaglandin F2-α analogue (Prosolvin, Intervet, Boxmeer, The Netherlands) was

given. The second ear-implant was inserted eleven days after removal of the first implant.
Before synchronisation, ovulation was induced with an im injection of 0.021 mg busereline-acetate (Receptal, Hoechst Roussel Vet, Brussel, Belgium) in four cases. In three cases no expression of oestrous signs was seen after subsequent synchronisation; these cases were excluded from the analyses.

Visual observation of oestrous behaviour
Behavioural observations commenced 30h after the removal of the ear implant after the first synchronisation in the first batch and 12h after removal of the implant after all other synchronisations. Two observers monitored behaviour simultaneously every 3h for 30 min (at 8.00h, 11.00h, 14.00h, 17.00h, 20.00h, 23.00h, 2.00h and 5.00h) from a platform at 2.5m, which did not disturb the animals. Oestrous behaviours were monitored according to the methods of Van Eerdenburg et al. (1996). Each time an animal showed an oestrous sign, the assigned number of points was recorded (Table 2.1). If the sum of points during consecutive observation periods exceeded 95, the animal was considered to be in oestrus. Onset of oestrus was defined as the first observation period the animal showed oestrous behaviour minus 1.5h. End of oestrus was defined as the last observation period the animal showed oestrous behaviour plus 1.5h.

Table 2.1. Scoring scale for observed oestrous signs[1]

Oestrous signs	Points
Flehmen	3
Restlessness[2]	5
Sniffing the vulva of another cow	10
Mounted but not standing	10
Resting with chin on the back of another cow	15
Mounting other cows (attempt)	35
Mounting head side of other cows (attempt)	45
Standing heat	100

[1] Each time an oestrous sign is observed, the assigned number of points is recorded (modified after Van Eerdenburg, et al., 1996)
[2] Can be recorded only once during an observation period

Blood sampling
Immediately after removal of the ear-implant, a blood sample was taken by venipuncture of the coccygeal vein. Thereafter, animals were fitted with semi-permanent jugular vein catheters (Braun Cavafix Certo 255, 40 cm, 18G, Instruvet, Amerongen, The Netherlands) for 10 days. Every 1.5h, 10 ml of blood was taken until the end of oestrus. After oestrus, blood samples were taken every 3h following the behavioural observations until ovulation had taken place. After ovulation, blood sampling continued for 4 days every 6h (at 12.00h, 18.00h, 0.00h

and 6.00h). Blood samples were immediately put on ice in vacuettes (sterile, K3E EDTA, Greiner) and centrifuged at 3000 rpm for 10 min at 4°C. Plasma was decanted and stored at -20°C until analysed.

Hormone assays

Ten to 18 blood samples (at 1.5h intervals) around visual oestrus were analysed per cow to determine the LH-surge. Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al. (1983, 1986). The intra- and interassay coefficients of variation were <9%. The sensitivity was 0.4µg/l NIH-LH-B4. The basal concentration of LH for every cow was calculated based on four values well before or after the LH-surge. An increase in LH concentration was defined as 2*SD above the basal concentration. The start of the LH-surge was defined as the time calculated by interpolation between the last sample with basal concentration and the first sample with an increase in concentration. The end of the LH-surge was defined as the time calculated by interpolation between the last increased sample and the first basal sample.

Five to 10 blood samples (at 3h intervals) around visual oestrus were analysed per cow to determine the decline in oestradiol-17ß (E_2) before ovulation. Concentrations of E_2 in the peripheral blood were estimated by validated solid-phase ^{125}I RIA method (TKE: Diagnostic Products Corporation, Los Angeles, CA, USA) as described by Dieleman and Bevers (1987). The sensitivity was 7.5pmol/l, and the interassay coefficient of variation was 8.9%. The maximum E_2 concentration was defined as the sample after which the concentration did not increase anymore and the minimum difference with the next sample was 0.4pg/ml. The decline of E_2 was defined as 1.5h after the sample of maximum E_2 concentration.

Sixteen blood samples (at 6 and 12h intervals) around and after visual oestrus were analysed per cow to determine the increase in progesterone (P_4) after ovulation. The concentration of progesterone in plasma of peripheral blood was estimated in duplicate by a validated solid-phase ^{125}I RIA method (Coat-A-Count TKPG, Diagnostic Products Corporation, Los Angeles, CA) as described before by Dieleman and Bevers (1987). The sensitivity was 0.15nmol/l, and the interassay coefficient of variation was 11%. The increase in P_4 was defined as 3h before the first sample with a concentration >0.1ng/ml after which a consistent rise of P_4 levels occurred.

Ultrasound

The ovaries of the cows were examined rectally using an ultrasound scanner (Scanner 200, Pie Medical, Maastricht, The Netherlands). The scanner was equipped with a 7.5 MHz sector transducer. The transducer was inserted into the rectum to examine the ovaries. The reproductive tract was not manipulated or palpated before or during the ultrasound examination. Immediately after removal of the ear-implant, ultrasound was performed to determine the number and size of

the follicles >5mm. Subsequently, ultrasound was performed every 3h in all UG cows from the moment the first cow in the group expressed behavioural oestrous signs. During the first three-hourly ultrasound session, each ovary was scanned to determine on which ovary the preovulatory follicle was located. Thereafter, scanning the ovary containing this follicle was continued every 3h until the disappearance of the follicle, which marked ovulation (Rajamahendran et al. 1989). Each time, the diameter of the follicle was measured. Time of ovulation was defined as the first ultrasound the preovulatory follicle had disappeared minus 1.5h. For animals in the control group, one day after oestrus, ultrasound was performed to determine if ovulation had taken place. Six days after oestrus, ultrasound was performed in all cows to determine if a corpus luteum had appeared which confirmed ovulation.

Statistics

Data were analysed with a multivariate Analyses of Variance, using the GLM-procedure of the Statistical Analysis System (The SAS system for windows V8, 1999). The following parameters were analysed: duration of oestrus (h), onset and end of oestrus (h after removal ear-implant), total behaviour points during oestrus, maximum behaviour points during oestrus, time of maximum behaviour points during oestrus (h after removal ear-implant), basal LH concentration (ng/ml), onset and end of LH-surge (h after removal ear-implant), duration of LH-surge (h), height of the peak of LH-surge (ng/ml), maximum E_2 concentration (pg/ml), start of declining E_2 (h after removal ear-implant), start of increasing P_4 (h after removal ear-implant) and the intervals between the different events.

Preliminary analyses with the Analyses of Variance revealed no differences between batches for any of these parameters; therefore data of the two batches were combined. Preliminary analyses also revealed no differences for any of the parameters between primiparous and multiparous cows, so parity was not included in the final model.

The initial experimental set-up was a Latin square, where animals served as their own control. However, because of the high number of missing values, a model was used in which the effect of Animal was not included, so more observations could be used. In these final models, the factors Use of ultrasound (UG vs. CG) and Synchronised oestrous period (FS vs. SS) were taken into account. Analyses were also performed including the factor Animal; in these analyses only animals were included with two observations per parameter. The results are presented in a separate paragraph.

Also with SAS, Pearson correlations where computed between the different timing events, between the maximum E_2 concentration and the total behaviour points during oestrus and maximum behaviour points during oestrus, between the maximum E_2 concentration and the number of the behavioural oestrous signs expressed and between the maximum E_2 concentration and the follicle size after

removal of the ear-implant and at the time of ovulation. When time of ovulation was assessed, also Pearson correlations were computed for the different events relative to time of ovulation. All means are presented as least square means (lsmeans)±SEM, unless otherwise stated. P-values <0.05 mean a significant difference and P-values between 0.05 and 0.1 mean there is a tendency for difference.

Results

Initially, 16 cows were selected for the experiment to be followed for two synchronised oestrous periods. One cow was replaced before the second synchronisation because she became ill after the first synchronisation (Control). Six oestrous periods were excluded from the analyses for the following reasons; observed oestrus when the ear-implant was still inserted (1* Control (SS)), silent oestrus after inducing ovulation before synchronisation (2* Ultrasound (both FS), 1* Control (SS)), developing a cyst (1* Control (FS)) and illness (1* Control (SS)). In total, 24 oestrous cycles in 14 cows were observed. Ultrasound examinations were performed in five animals after the first synchronisation and in seven animals after the second synchronization. Ultrasound was performed on average 15.7±0.7 (range: 10 to 20) times per cow consecutively in the Ultrasound Group (n=12).

Behaviour

The various expressions of oestrous signs during oestrus are presented in Figure 2.1. One animal did not show any oestrous signs after both synchronisations. Expression of oestrous signs did not differ between the Ultrasound and Control Group (Figure 2.1).

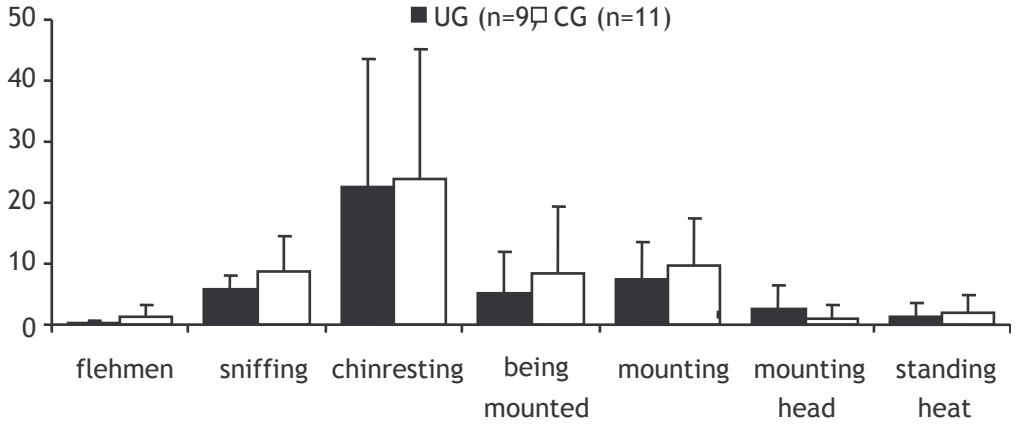

Figure 2.1. Number of different oestrous signs (mean+SD) occurring during the entire oestrus in the Ultrasound and Control Group. Additionally, one animal had a silent oestrus in both periods and in two animals the onset of oestrus was missed.

Chin resting on another cow occurred with the greatest frequency; on average 23.3±4.6 times per oestrus. Flehmen occurred with the lowest frequency with an average of 0.9±0.4 times during oestrus. Only signs of sniffing and chin resting during oestrus were demonstrated by all cows. Other behavioural signs were not shown by all cows. Ninety-five percent of cows mounted other cows during oestrus, while only 45% of cows showed standing heat. Seventy percent of cows were mounted during oestrus with 64% of those demonstrating a standing heat. Fourty percent of the animals mounted the head-side of another cow and flehmen occurred in 25% of the cows during oestrus.

Table 2.2. Parameters of behavioural oestrus, hormone profiles and intervals between parameters (lsmeans[1]±se) in ultrasound- and control group and overall means

Parameter	UG (n)	CG (n)	Overall (range)
Oestrus			
Onset of oestrus[2]	34.4±1.8 (9)	32.4±1.7 (11)	33.8±1.6 (19.5;46.5)
End of oestrus[2]	46.0±1.3 (11)	46.3±1.3 (11)	46.5±1.2 (31.5;58.5)
Duration of oestrus (h)	12.9±1.5 (9)	13.8±1.3 (11)	13.4±0.9 (6;21)
Total points oestrus	957±281 (9)	1135±259 (11)	1047±180 (95;2692)
Max. points oestrus	386±85 (9)	456±78 (11)	418±56 (85;843)
Average points/observation	211±48(9)	236±44 (11)	223±31 (45;423)
LH			
Basal concentration LH (ng/ml)	0.83±0.1 (11)	0.87±0.1 (11)	0.85±0.07 (0.32;1.46)
Duration LH-surge (h)	9.6±0.5 (10)	9.2±0.5 (10)	9.5±0.4 (5.1;11.9)
Timing of peak LH-surge[2]	40.7±2.2 (10)	38.7±2.0 (11)	39.4±1.6 (15.5;51.5)
Height LH-peak (ng/ml)	9.4±0.9 (10)	9.3±0.9 (11)	9.3±0.6 (4.7;14.5)
E_2			
Timing of decline of E_2[2]	40.1±2.3 (12)	41.0±2.4 (12)	40.9±1.9 (15.5;56.0)
Max. E_2-concentration[3] (pg/ml)	11.2±0.5 (12)	10.6±0.5 (12)	11.0±0.4 (7.3;14.4)
P_4			
Time of increase P_4[2]	97.5±2.0 (12)	98.0±3.9 (11)	97.8±2.1 (66.5;115)
P_4 concentration[4]	1.55±0.14 (10)	1.69±0.14 (11)	1.62±0.10 (0.98;3.05)
Intervals			
Onset oestrus-LH peak (h)	5.2±1.9 (8)	6.1±1.7 (10)	5.7±1.2 (-4;14)
End oestrus-LH peak (h)	-7.6±1.4 (11)	-8.1±1.3 (11)	-7.9±0.9 (-16;2)
Onset oestrus-decline E_2 (h)	6.5±2.2 (9)	7.9±2.0 (11)	7.4±1.4 (-4;18.5)
End of oestrus-decline E_2 (h)	-7.0±1.8 (12)	-5.8±1.8 (11)	-6.3±1.2 (-16;9.5)
Time max. score-LH-peak (h)	-0.8±1.5 (8)	0.4±1.6 (10)	-0.2±1.1 (-8.5;8)
Time max. score-decline E_2 (h)	0.8±2.2 (9)	2.4±2.1 (11)	1.7±1.5 (-11.5;12.5)
LH-peak–decline E_2 (h)	0.9±1.1 (10)	1.2±1.1 (11)	1.0±0.7 (-6;9)
Onset oestrus- increase P_4	64.5±2.2 (9)	63.1±2.6 (10)	63.7±1.7 (47;77.5)
End oestrus-increase P_4	50.8±1.5 (11)	49.0±2.2 (10)	49.9±1.3 (35;59.5)
LH-peak-increase P_4	59.5±1.2 (10)	58.0±1.9 (11)	58.7±1.1 (50;69.5)
Decline E2-increase P_4	58.3±1.1 (12)	56.6±1.6 (11)	57.5±0.9 (50;65)

[1] corrected for oestrous synchronisation period (FS, SS); [2] h after removal of ear-implant; [3]corrected for E_2-concentration immediately after removal of Crestar; [4] P_4 concentration 120h after LH-peak
In total 12 oestrous periods in each group are observed. One animal had a silent oestrus in both periods.

Repeated ultrasound started on average 9.6±1.6h before the onset of oestrus. Onset of oestrus, duration of oestrus and intensity of oestrous behaviour did not differ between the Ultrasound and Control Group (Table 2.2). Oestrus started 33.8h after removal of the ear-implant with a range of 27h. The duration of oestrus ranged from 6h to 21h. A large variation was seen in expression of oestrous signs between cows. The number of points recorded per oestrus ranged between 95 and 2692 points. During oestrus the average profile of the number of points received during an observation period did not differ between the Ultrasound and Control Group (Figure 2.2A).

After the first synchronisation, the animals showed oestrus later (FS: 38.0±1.7h) compared to the second synchronisation (SS: 28.7±1.8h; $P<0.05$, Figure 2.3). Duration and intensity of oestrus did not differ after the first and second synchronisation.

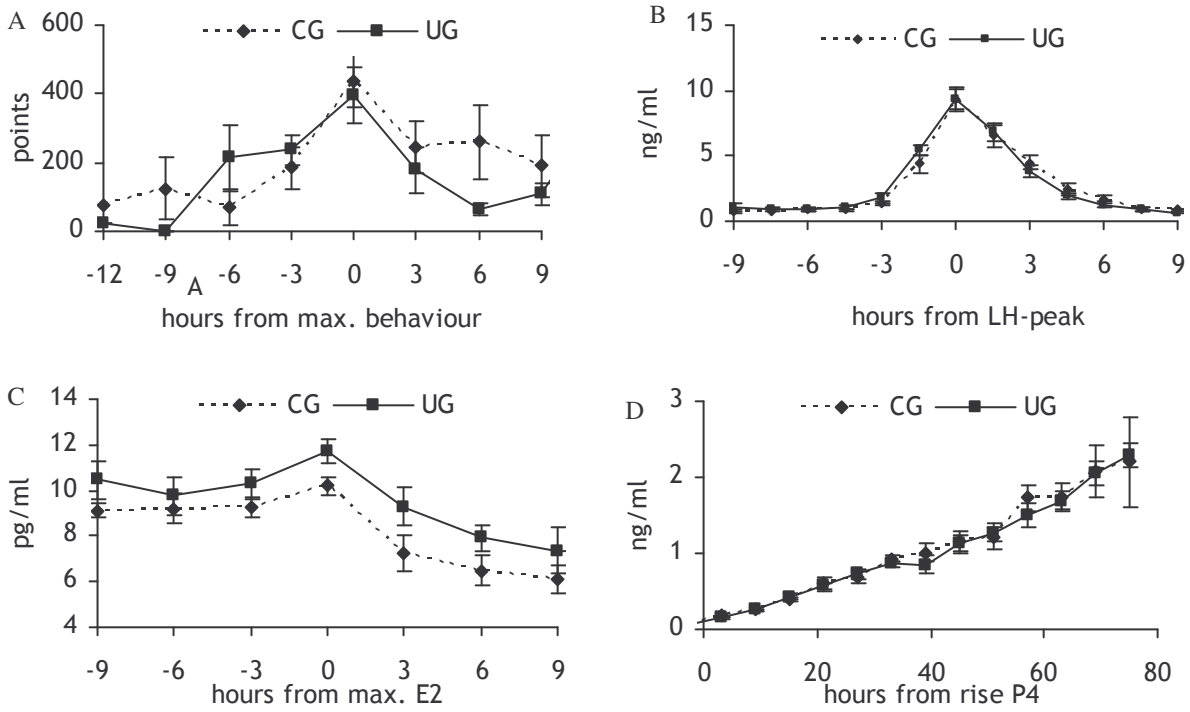

Figure 2.2.

A. Mean number of points during entire oestrus for UG and CG (mean±se), for cows that were in oestrus at that time. The first ultrasound in the UG was performed 9.6±1.6h before the onset of oestrus
B. LH profiles for UG and CG (mean±se). The first ultrasound in the UG was performed 11.8±1.7h before the onset of the LH-surge
C. E_2 profiles for UG and CG (mean±se). The first ultrasound in the UG was performed 15.6±1.9h before the decline of E_2. After correction for E_2 levels immediately after removal of ear-implant, no differences were found between UG and CG
D. P_4 profiles for UG and CG (mean±se). The first ultrasound in the UG was performed 11.8±1.7h before the 73.1±1.7h before the increase in P_4

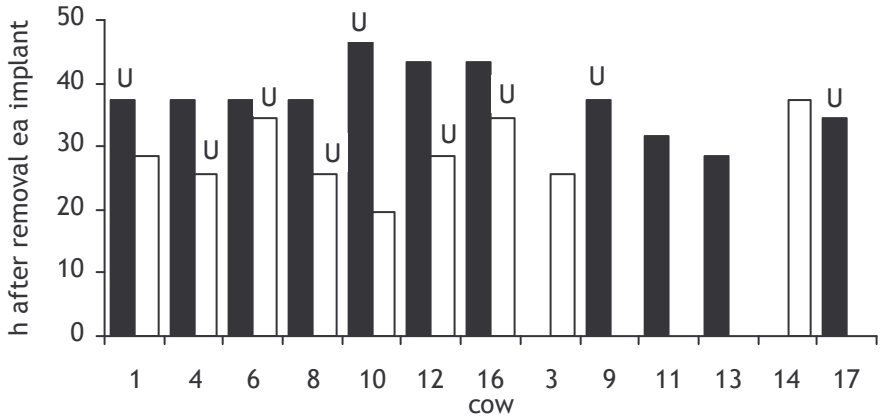

Figure 2.3. Onset of oestrus after removal of ear implant. After the first synchronisation (black bars) the animals showed oestrus later (38.0±1.7h) compared to the second synchronisation (light bars, 28.7±1.8h; P<0.05). 'U' above the bars means the animals were in the UG.

Hormone profiles

The results of the hormone assays from the UG and CG and the overall means are presented in Table 2.2. Timing of LH-surge, decline in E_2 and increase in P_4 did not differ between the UG and CG (see also Figure 2.2). Concentrations of LH and P_4 did not differ between the UG and CG (Figure 2.2). Peak LH-concentrations were 9.3±0.6ng/ml and P_4 concentrations 120h after the LH-peak were 1.62±0.10ng/ml (Table 2.2). The maximum E_2 concentration differed between the UG and CG (11.7±0.48 vs. 10.2±0.48pg/ml, P<0.05, Figure 2.2C). However, when the concentrations were corrected for the level of E_2 at removal of the ear-implant, E_2-profiles did not differ between the UG and CG (Table 2.2).

The onset of the LH-surge after removal of the ear-implant was later after the first synchronisation compared to the second synchronisation (FS vs. SS: 40.8±2.1 vs. 31.0±2.0h, P<0.05). Duration of the LH-surge and maximal concentration of LH did not differ between the first and second synchronisation. The time of decline of E_2-concentration also occurred later after the first synchronisation compared to the second synchronisation (FS vs. SS: 45.5±2.3 vs. 35.5±2.5h, P<0.05). Maximum E_2 concentration did not differ between the first and second synchronisation. The increase in P_4 after removal of the ear-implant tended to be later after the first synchronisation compared to the second synchronisation (FS vs. SS: 101.6±2.8 vs. 93.5±3.0h, P<0.1).

Follicle size

The size of the dominant follicle at removal of the ear-implant was 15.6±0.7mm, ranging from 8mm to 21mm. The size of the dominant follicle tended to be smaller after the first synchronisation (14.5±1.1mm) compared to the second synchronisation (17.2±0.8mm, P<0.1). No significant correlations were found

between the maximum E_2 concentration and the size of the dominant follicle at removal of the ear-implant (r=-0.04; P>0.1) or the size of the follicle at time of ovulation (r=-0.04; P>0.1). Also no significant correlation was found between the size of the dominant follicle at removal of the ear-implant and the timing of ovulation (h after removal of the ear-implant, r=0.46; P>0.1) or the size of the follicle at time of ovulation (r=0.38; P>0.1). There was no difference in size of the follicle just before ovulation between the first and second synchronisation (average 21.1±0.6, n=12).

Correlations

In Table 2.3 the timing of ovulation (h after removal of ear-implant for the Ultrasound Group) relative to oestrus and the hormone profiles is shown. Ovulation took place 30.2±1.9h after onset of oestrus and this interval had the largest variation (21h) of all parameters studied. The smallest variation (6h) was found in the timing of ovulation in relation to the LH-peak; ovulation took place 25.3±0.6h after the peak in LH. Timing of ovulation (h after removal of ear-implant) had a significant correlation (r>0.7; P<0.05) with end of oestrus, time of decline of E_2, time of maximum LH concentration after removal of the ear-implant, and time of increase in P_4 but not with onset of oestrus (r=0.56, P>0.1).

Table 2.3. Timing of ovulation after removal of ear-implant for the Ultrasound Group, onset- and end of oestrus, time maximal behavioural score, decrease of E_2, peak LH and increase in P_4

	Mean±se (n)	(range)
Timing of ovulation (h after removal ear-implant)	63.2±1.5 (12)	(53.5;71.5)
Onset oestrus-ovulation (h)	30.2±1.9 (9)	(19;40)
End oestrus-ovulation (h)	16.7±1.1 (11)	(10;22)
Time max. score-ovulation (h)	24.7±1.8 (9)	(17.5;35.5)
Decline E_2-ovulation (h)	23.9±1.1 (12)	(17.5;32)
Peak LH-ovulation (h)	25.3±0.6 (10)	(21.5;27.5)
Ovulation-increase P_4 (h)	33.8±1.1 (12)	(28.5;38.5)

Figure 2.4 represents the mean E_2 concentration and behaviour score relative to timing of peak LH for individual animals (+SEM). On average the LH-peak occurred at the same time as the beginning of the decline in E_2 (ranging from 6h before until 9h after the decline in E_2, see also Table 2.2). On average when E_2 started to decline, the oestrous behaviour score started to decline (1.7±1.5h). However, profiles of individual animals were far more variable as is clear from the variation that ranged from 11.5h before until 12.5h after the decline in E_2. Significant correlations were observed between timing of the onset of oestrus, the end of oestrus, the time of the maximum number of points during oestrus, the time of the LH-peak, the time of the decline of E_2 and the time of increase in P_4 after removal of the ear-implant (r>0.6; P>0.05).

The maximum E_2 concentration was positively correlated with the maximum number of behaviour points acquired during one observation period (r=0.46, P<0.05), but not with total oestrous behaviour score or duration of oestrus. Animals that showed standing heat had a higher maximum E_2 concentration compared with animals that did not show standing heat (12.1±0.52 (n=9) vs. 10.0±0.48ng/ml (n=11), P<0.05), but maximal E_2 was not correlated with the number of any of the other oestrous signs.

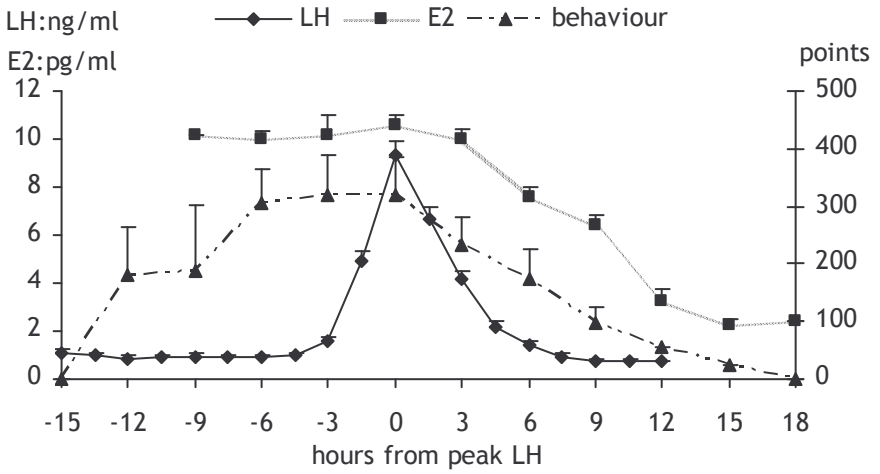

Figure 2.4. Mean E_2 concentration and behaviour points relative to timing of peak LH for individual animals (+se). For each individual animal, the sample with the highest concentration of LH is appointed as time 0.

Animals with two observations per parameter

Analysis on only animals for which data were available for both the UG and the CG, revealed significant Animal effects in about half of the parameters. Similar differences in the first and second synchronization were found as described in the previous paragraphs. However, some additional effects of first and second synchronisation were found; the duration of oestrus was shorter after the first synchronization (11.7±0.6h) compared to the second synchronization (15.5±0.6h, P<0.05; n=7 animals). The number of maximum and total oestrous points received tended to be less after the first synchronisation (245±58 and 580±140 points) compared to the second synchronisation (431±58 and 1005±140 points, P<0.1; n=7 animals). The time of increase in P_4 was significantly longer after the first synchronisation (101.9±2.6h) compared to the second synchronisation (91.3±2.6h, P<0.05; n=9 animals).

No differences were found between the ultrasound group compared to the control group for any of the parameters, except for the interval between beginning of oestrus until the time of increase in P_4 (UG vs. CG: 62.9±0.4 vs. 59.0±0.4h, P<0.05, n=6 animals).

Discussion

The results from this study demonstrate that frequent rectal ultrasound examinations during and after oestrus do not affect behavioural oestrous characteristics. Additionally, LH-, E_2- and P_4-profiles are comparable between ultrasound and control treated animals. In a study by Rajamahendran et al. (1989) the relationship between onset of oestrus, endocrine changes and time of ovulation was examined, time of ovulation was assessed by 2-hourly rectal ultrasound. Frequent ultrasound of the reproductive tract and the ovary containing the largest follicle appeared not to affect the time of ovulation. However, as no control animals were included in the study, it could not be concluded that frequent ultrasound examinations had no effect. In the current study the investigated behavioural and endocrine parameters and the intervals between different events are similar for the Ultrasound and Control Group. The only interval affected was the onset of oestrus and the time of increase in P_4 (using the model including Animal). However, the number of animals is very low (n=6) and the difference found in this interval (4h) lies within the interval blood sampling (6h). The intervals between the different events and time of ovulation in the Ultrasound Group are in agreement with other studies (Schams et al. 1977, Bernard et al. 1983, Rajamahendran et al. 1989, Mosher et al. 1990, Walker et al. 1996). Therefore, it can be concluded that repeated ultrasound examination does not affect these events and is a reliable method to assess ovulation time.

The timing of onset of oestrus, end of oestrus, LH-surge, decline in E_2 and increase in P_4 after removal of the ear-implant were highly correlated and occurred on average 8 to 10h earlier after the second synchronisation compared to the first synchronisation. The onset of oestrus after removal of the ear-implant in the first period (38.0±1.7h) is comparable with the findings of other studies that used that same method to induce oestrus (Salaheddine et al. 1992, Tregaskes et al. 1994, Cavalieri et al. 1997, Diop et al. 1998, Singh et al. 1998). The timing of the LH-surge after removal of the ear-implant in the first period agrees with the findings of Cavalieri et al. (1997). Therefore, the timing of events after the first synchronisation appeared ‘normal’ whereas they seemed more ‘advanced’ after the second synchronisation. However, Walker et al. (1996) found no difference in onset of oestrus (defined by standing heat) after a first or second induced cycle (75.8±4.0h vs. 70.7±4.4h), which was induced the first time with an injection of PGF2α in the presence of a corpus luteum and the second time with an injection of PGF2α 8 to 13 days later. It is not clear why the described events (behaviour, hormone profiles) were advanced after the second synchronisation compared to the first synchronisation in the current study. After the first synchronisation the follicle size at removal of the ear-implant was smaller compared to the second synchronisation (on average 2.7mm smaller). At ovulation, follicle sizes were

similar for both groups. When assuming similar growth rate of the dominant follicle up to ovulation one would expect a later ovulation after the first synchronisation. This could be an explanation why after the second synchronisation the described events were advanced. Another explanation for the differences found between the first and second synchronisation could be that animals were not habituated to the frequent blood sampling and therefore were more stressed after the first synchronisation compared to the second synchronisation. In pigs, an impairment of oestrus, a delayed LH-surge and delayed ovulation was seen after stress during the follicular phase (Hennessy & Williamson 1983). However, Turner et al. (1999) showed that cortisol needed to be elevated for more than four days to impair the secretion of LH in pigs. Not much research is done on the effects of acute stress during the follicular phase on reproduction in dairy cattle. Stoebel and Moberg (1982) found a disruption in the LH-surge, but no effects on oestrous behaviour and timing of the LH-surge when animals were stressed for 3.5 days during the follicular phase. So, if animals in the current experiment were (more) stressed after the first synchronisation, it would have been for such a short period of time that it is unlikely that this is the cause of the difference in oestrous expression and timings of several reproductive events.

The duration of oestrus, on average 13.4±0.9h, corresponds with the findings of van Vliet et al. (1996), who used the same method for defining oestrus. With this definition of oestrus all behavioural signs related with oestrus are taken into account (Cavalieri et al. 1997). When only standing behaviour is taken into account, oestrous periods could be missed, because not all cows in oestrus show standing heat (Van Eerdenburg et al. 1996, Heres et al. 2000, Lyimo et al. 2000, Van Eerdenburg et al. 2002). In the current study only 45% of the animals showed standing heat. The quantity and duration of observing the behaviour of the animals are important factors in comparing results of different studies. In the current study 25% of the animals showed behavioural oestrous signs less than 12h. So when the observations are less frequent then in the current study, oestrous periods will be missed (Holtz & Meinhardt 1993, Van Eerdenburg et al. 1996) and comparisons with intervals concerning e.g. onset and end of oestrus will be difficult. In the current experiment, onset of standing heat to ovulation was 27.4±1.5h (n=5), which is comparable with other studies that define oestrus by standing heat (Gerasimova 1940, Christenson et al. 1975, Walker et al. 1996, Cavalieri et al. 1997, Pinheiro et al. 1998, Lopez et al. 2002).

In the current study, a large variation between onset of oestrus and time of ovulation was found (19-40h). Although a correlation would be expected, no significant correlation between onset of oestrus and time of ovulation was found, probably due to the large variation mentioned above. The question arises if a relationship between individual oestrous signs and time of ovulation exists. The amount of observations in this study is not sufficient to confirm this. More research

needs to be done to study this relation. The duration of the LH-peak in the current study (5.1 to 11.9h) is the same range as found by other authors (Lemon et al. 1975, Schams et al. 1977, Bernard et al. 1983, Dieleman et al. 1986). The interval between peak LH and time of ovulation was 25.3±0.6h and had the smallest variation between animals (range 21.5-27.5h, Table 2.3) compared to intervals between behavioural oestrus, E_2 or P_4 and time of ovulation. This observation is in agreement with other studies (Schams et al. 1977, Bernard et al. 1983, Rajamahendran et al. 1989, Mosher et al. 1990, Cavalieri et al. 1997, Bage et al. 2002).

The interval between decline of E_2 and ovulation ranged from 17.5 to 32h (Table 2.3), which is comparable with the data of Mosher et al. (1990), who assessed time of ovulation every 4h by laparoscopy and took blood samples every 4h. The intervals are shorter than those reported by Lopez et al. (2002), who assessed time of ovulation once a day, by ultrasound and took blood samples every 12h. The larger interval found in that study may be due to the longer intervals between blood sampling and assessment of time of ovulation compared to our study.

In the current study, P_4 began to increase 63.7±1.7h after the onset of oestrus and 33.8±1.1h after ovulation. Dieleman et al. (1986) found a comparable average interval between oestrus and increase in P_4 of approximately 72h. By our knowledge, no data is available about the interval between time of ovulation and increase in P_4.

In the current study, a positive correlation between intensity of oestrous behaviour and the maximum E_2 concentration was found, but this correlation existed only with the maximum behaviour score. In a study of Lyimo et al. (2000) the same oestrus detection method was used. They also found a positive correlation between intensity of oestrous behaviour and the maximum E_2 concentration, but they found this correlation with the total behaviour score and not with the maximum behaviour score. In both studies no significant correlation was found between the maximum E_2 concentrations and any of the individual behavioural signs of oestrus. Several studies did not find a relation between the E_2 concentration and intensity of oestrous behaviour (Glencross et al. 1981, Coe & Allrich 1989). Coe et al. (1989) hypothesised that after a threshold concentration of E_2 is reached, induction of oestrous behaviour is initiated and the expression of oestrus is relatively independent of serum concentrations of E_2. Figure 2.5 shows that in our study the correlation mainly exists when the E_2-concentration is relatively low. Contradictory with the findings of Coe et al. (1989), it seems from the current experiment that oestrous behaviour is initiated when E_2-concentrations are relatively low and the intensity of oestrous behaviour is dependent of E_2 concentrations up to a certain concentration (threshold) of E_2. When this threshold is reached, the expression of oestrus is relatively independent of concentrations of E_2 as found by Coe et al. (1989). The difference in the findings of Coe et al. (1989) and the current study could be because in their study only mounting behaviour was taken into account.

Consequently animals with very low oestrous expression, as seen in the current experiment, were not taken into account. Glencross et al. (1981) did look at other behavioural signs besides mounting behaviour, but only took blood samples once a day, which possibly explains the lack of correlation between E_2 and intensity of oestrus.

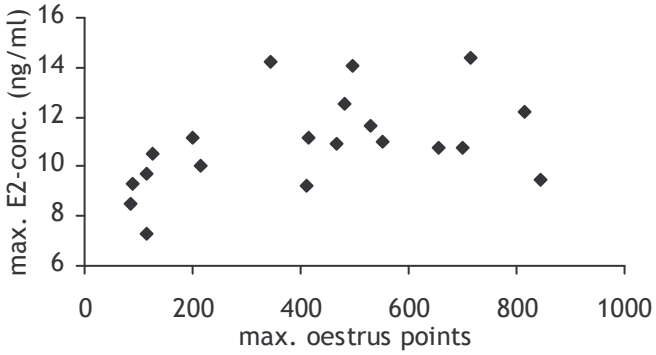

Figure 2.5. Maximum E_2-concentration relative to maximum points acquired during oestrus ($r=0.46$, $P<0.05$, $n=20$)

E_2 levels decreased 1.7h after the behaviour score was at its maximum (Table 2.2), which is in agreement with the study of Lyimo et al. (2000). They found that E_2 levels dropped immediately after the behaviour score was at its maximum.
The results of the present study show that repeated rectal ultrasound examination is a useful tool to assess the time of ovulation, because oestrous behaviour and peri-ovulatory hormone profiles are not influenced by repeated rectal ultrasound examination. The relationship between oestrous behaviour and time of ovulation is very variable between animals. The LH-peak has the best relationship with time of ovulation but is in practice hard to assess. Further research, using ultrasound, can now be carried out to find predictors for time of ovulation in practice.

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3

Various behavioural signs of oestrus and their relationship with time of ovulation

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Abstract

The objective of this study was to investigate the relationship between various behavioural signs of oestrus and time of ovulation and, determine which behavioural oestrous sign(s) best predicted time of ovulation. In total 94 ovulations were observed in 67 Holstein-Friesian dairy cows. Different behavioural oestrous signs were observed at three-hourly intervals and their relationship with time of ovulation (ultrasound examinations at three-hourly intervals) was investigated. In all oestrous periods, sniffing and chin resting was displayed, while mounting was displayed in 90% and standing heat in 58% of oestrous periods. Oestrus was more intense in primiparous cows compared to multiparous cows and when more animals were in oestrus at the same time. Although these factors influenced intensity of behavioural oestrous signs, they did not influence time of ovulation. Ovulation occurred 30.0±5.1h after onset of oestrus (ranging between 18.5 and 48.5h) and 18.8±4.4h after end of oestrus (ranging between 9.5 and 33.5h). Although informative, these predictors are highly variable between individuals and the method used to determine the onset and end of oestrus is time consuming, this therefore limits in their use as a practical predictor of ovulation time. Sniffing and chin resting were displayed during the non-oestrous period and are therefore not useful predictors of ovulation time. For animals that displayed standing heat, onset of standing heat was a good predictor for ovulation time (occurring 26.4±5.2h before ovulation). However, standing heat was only displayed in a limited number of cows, especially when only one cow was in oestrus at a time. Onset of mounting was the best predictor for time of ovulation (occurring 28.7±5.3h before ovulation), and it was displayed in 90% of the oestrous periods. However, mounting cannot yet be assessed automatically, which limits its practical use as ovulation predictor.

Introduction

For conception to occur, insemination must take place at the correct stage of the cow's oestrous cycle. Successful fertilization highly depends on the time interval from insemination to ovulation. When insemination takes place too early, the sperm is aged and by the time ovulation occurs it cannot fertilize the ovum (Hawk 1987). When insemination takes place too late, the egg is aged and fertilization and formation of a viable embryo is not likely (Hunter & Greve 1997). Indications exist that, in practice, an enormous variability exists in the timing of insemination relative to ovulation. The causes of variation in the timing of insemination relative to ovulation include variation in oestrus detection strategy and accuracy, variation in moment of ovulation after detection of oestrus and in the timing of insemination after detection of oestrus.

Next to standing heat, which is commonly used in practice, different behavioural signs can be used for oestrus detection, e.g. sniffing the vagina of another cow, resting chin on another cow, or mounting behaviour (Van Eerdenburg et al. 1996). The relationship between the expression of these different behavioural oestrous signs and the time of ovulation is not yet established. It is known however, that the moment of ovulation in relation to behavioural oestrous signs is far more variable than was assumed in the past (Walker et al. 1996, Kaim et al. 2003). In recent years, research has been done to study effects of different insemination times relative to oestrus characteristics on conception rate (Maatje et al. 1997, Dransfield et al. 1998, Xu et al. 1998, Dalton et al. 2001), unfortunately time of ovulation was not assessed in these studies. To determine the best time for insemination relative to the time of ovulation there is a need for predictors of ovulation time.
The present study investigated the relationship between different behavioural oestrous signs and time of ovulation to see if behavioural oestrous signs can be used as a predictor of time of ovulation.

Materials and Methods

Experimental design

The experiment was conducted at the experimental dairy farm “de Ossekampen” at Wageningen University and Research Centre, the Netherlands. The Ethical Committee for Experimentation with Animals (Wageningen, the Netherlands) approved the experimental protocol. Every 3h visual observation of oestrous behaviour took place. Rectal ultrasound was performed to assess time of ovulation.

Animals, feed and housing

Data were collected from an existing herd of approximately 70 lactating Holstein-Friesian cows. Parity of the animals varied between one and six and the animals were 105.7±72.6 (mean±SD) days in milk, with a range of 14 to 369 days. Animals were fed an ad lib mixture of grass silage, corn silage and mineral supplements, and concentrates according to production level (CVB (Central Animal Feed Bureau)-norms, 2000). The animals were housed in a free stall with slatted floor and cubicles. The animals were milked by an automated milking system (Liberty, Prolion, Vijfhuizen, The Netherlands). Estimated 305-day milk production was 8274±1412 (mean±SD) kg.

Visual observation of oestrous behaviour

Two observers monitored behaviour simultaneously every 3h for 30 min (at 8.00h, 11.00h, 14.00h, 17.00h, 20.00h, 23.00h, 2.00h and 5.00h) from an elevated chair, which did not disturb the animals. Oestrus was defined according to Van

Eerdenburg et al. (1996). Each time an animal displayed a behavioural oestrous sign, the assigned number of points was recorded by hand (Table 3.1). If the sum of points during consecutive observation periods exceeded 100, the animal was considered to be in oestrus. Onset of oestrus was defined as the first observation period the animal showed oestrous behaviour minus 1.5h. End of oestrus was defined as the last observation period the animal showed oestrous behaviour plus 1.5h. A regular oestrus was defined as an oestrus in which in every observation period (during oestrus) at least one of the defined oestrous signs was displayed. An irregular oestrus was defined as an oestrus in which there was at least one observation period (during oestrus) in which the animal did not express any of the oestrous signs. The number of animals in oestrus during an observation period was recorded. Because of another experiment, oestrus was induced in 23 cases with an i.m. injection of 15 mg of prostaglandin F2-α analogue (Prosolvin, Intervet, Boxmeer, The Netherlands).

Table 3.1. Scoring scale for observed oestrous signs[1]

Oestrous signs	Points
Flehmen	3
Restlessness[2]	5
Sniffing the vulva of another cow	10
Mounted but not standing	10
Resting with chin on the back of another cow	15
Mounting other cows (attempt)	35
Mounting head side of other cows (attempt)	45
Standing heat	100

[1]Each time an oestrous sign is observed, the assigned number of points is recorded (modified after Van Eerdenburg, et al., 1996); [2]Can be recorded only once during an observation period

Ultrasound

The ovaries of the cows were examined rectally using an ultrasound scanner (Scanner 200, Pie Medical, Maastricht, The Netherlands). The scanner was equipped with a 7.5 MHz sector transducer. The transducer was inserted into the rectum to examine the ovaries. The reproductive tract was not manipulated or palpated before or during the ultrasound examination. Ultrasound examinations started between 8 and 11h after the end of an observed oestrus. During the first three-hourly ultrasound examination, each ovary was scanned to determine on which ovary the preovulatory follicle was located. Thereafter, scanning the ovary containing this follicle was continued every 3h until the disappearance of the follicle, which marked ovulation time (Rajamahendran et al. 1989). Each time, the diameter of the follicle was measured. Time of ovulation was defined as the first ultrasound the preovulatory follicle had disappeared minus 1.5h. Six days after oestrus, ultrasound examination was performed to determine if a corpus luteum had appeared which confirmed ovulation.

Statistics

Data on intensity of behavioural oestrous signs and intervals between behavioural oestrous signs and time of ovulation were analyzed by means of a multivariate analysis of variance (adjusted for multiple comparisons by Bonferroni), using the Statistical Analysis System (The SAS system for windows V8, 1999). Preliminary analyses revealed no differences between natural and induced oestrous periods for any of the parameters; therefore data of induced (n=23) and natural (n=72) oestrous periods were combined. The factors Parity, Regular Oestrus and Animals in Oestrus were used as class variables, in which Parity consisted of two classes (primiparous (n=32) versus multiparous (n=57) cows), Regular Oestrus consisted of two classes (regular (n=72) versus irregular (n=17) oestrus) and Animals in Oestrus consisted of three classes (1, 2 and 3). Class 1 oestrous periods occurred when no other animal in the herd was in oestrus (n=25). Class 2 oestrous periods occurred (partly) simultaneously with at most one other oestrus (n=36). Class 3 oestrous periods occurred (partly) simultaneously with at least two other oestrous periods (n=28). The chi-square analysis was used to investigate differences between the percentages of oestrous periods in which a behavioural oestrous sign was displayed at least once. Sniffing, chin resting and mounting were analyzed as individual behavioural oestrous signs, because these signs were displayed in at least 90% of the oestrus cases. Standing heat was analyzed as individual behaviour, because this behavioural oestrous sign has always been the most discriminative sign of oestrus (Van Eerdenburg et al. 1996). Student's t-tests were performed to investigate differences between the start (or end) of expressing the behavioural oestrous signs mentioned above (h before ovulation) at an animal level.

All means are presented as mean±SD, unless otherwise stated. P-values <0.05 are defined as a significant difference and P-values between 0.05 and 0.10 are defined as a tendency for difference.

Results

In total 94 ovulations were observed in 67 animals. In five cases the onset of oestrus was missed, so 89 entire oestrous periods were observed. In 25 cases only one animal in the herd was in oestrus, in 36 cases a maximum of two animals were in oestrus at the same time during at least one observation period and in 28 cases more than two animals were in oestrus at the same time during at least one observation period. In 82% of the oestrous periods, oestrus was regular; the number of animals in oestrus at the same time or parity did not influence this percentage.

Intensity of oestrus

Table 3.2 shows the average duration of oestrus and total-, maximum- and average number of points acquired during oestrus. The number of animals in oestrus at the

same time did not influence the duration of oestrus, but when more animals were in oestrus at the same time, total-, maximum- and average number of behavioural points acquired during oestrus increased (Table 3.2). Primiparous cows had a longer duration of oestrus and higher maximum number of behavioural points compared to multiparous cows. They tended (P=0.064) to acquire more behavioural points in total during oestrus compared to multiparous cows (Table 3.2). Duration of oestrus was shorter when oestrus was regular (11.0±4.2h) compared to irregular oestrus (15.4±3.3h; P<0.05).

Table 3.2. Intensity of oestrous behaviour when one, two or more than two cows were in oestrus at the same time during at least one observation period and intensity of oestrous behaviour for primiparous and multiparous cows (mean±SD (range))

	Animals in oestrus			Parity		Average
	1	2	More than 2	Primiparous	Multiparous	
Duration of oestrus (h)	11.3±4.2 (6-21)	11.4±5.3 (3-24)	12.8±3.2 (6-18)	13.6±4.8[a] (3-24)	10.8±3.8[b] (3-21)	11.8±4.4 (3-24)
Tot points[1]	782±526[a] (114-2035)	1067±605[a] (180-3303)	1529±851[b] (332-3857)	1381±709[c] (165-3150)	993±706[d] (114-3857)	1132±728 (114-3857)
Max. points[2]	367±201[a] (85-880)	540±269[a] (170-1243)	734±379[b] (203-1635)	679±377[a] (85-1635)	482±265[b] (101-1268)	553±323 (85-1635)
Avg points[3]	207±122[a] (33-458)	314±200[ab] (90-910)	360±193[b] (139-788)	342±217 (33-910)	273±165 (57-860)	298±187 (33-910)

[1]Number of points acquired during the entire oestrus; [2]The maximum number of points acquired during an observation period during oestrus; [3]The average number of points acquired during an observation period during oestrus

[a,b]Different superscripts within a row mean a significant difference for Animal in oestrus or Parity (P<0.05); [c,d]Different superscripts within a row mean a tendency for a difference for Animal in oestrus or Parity (P<0.1)

Individual behavioural signs

Expression of behavioural oestrous signs is presented in Figure 3.1. Not all signs were displayed during every oestrus. Sniffing and chin resting were displayed in all oestrous periods. In 90% of the oestrous periods mounting occurred and in 56% of the oestrous periods an animal was mounted but did not stand. Standing heat was seen in 58% of the oestrous periods. Sniffing (25.7±17.7 times) and chin resting (24.5±18.5 times) was displayed most frequent during oestrus. A large variation was seen in the expression of behavioural oestrous signs between animals, e.g. sniffing ranged between one and 82 times during oestrus and mounting was displayed between zero and 25 times during oestrus. The frequency of expressing a behavioural oestrous sign was lower when only one cow was in oestrus, compared to when multiple animals were in oestrus at the same time (Table 3.1). The frequency of chin resting (18.8±14.2 versus 30.2±18.0 times, P=0.076) and mounting (4.1±3.7 versus 7.8±6.2 times, P=0.057) tended to be less when one animal was in oestrus compared to when more than two animals were in oestrus at

the same time. An increase in the number of animals in oestrus at the same time, resulted in an increase in percentages of oestrous periods in which being mounted, mounting or standing heat was displayed at least once.

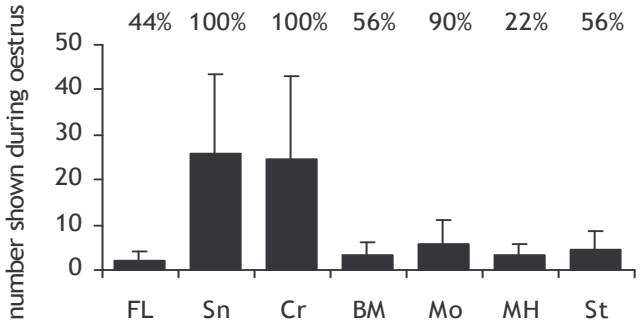

Figure 3.1. Mean number of different signs (Fl=flehmen, Sn=sniffing the vulva of another cow, CR=resting with the chin on another cow, BM=being mounted, Mo=mounting, MH=mounting the headside, St=standing heat) shown during oestrus (mean+SD) if the sign was displayed at least once during oestrus. Above the bars, the percentage of oestrous periods in which the sign was displayed at least once is shown.

Primiparous cows displayed sniffing more frequent during oestrus than multiparous cows (31.6±18.7 vs 22.4±16.3 times; P<0.05). Also mounting was displayed more frequent by primiparous than by multiparous cows (8.0±6.1 vs 4.7±4.3 times; P<0.05). Display of the other behavioural oestrous signs did not differ between primiparous and multiparous cows. Percentages of oestrous periods in which a certain behavioural oestrous sign was shown did not differ between primiparous and multiparous cows.

Table 3.3 The number of times (mean±SD) an oestrous sign was displayed during oestrus (when the sign was displayed at least once), and the percentage of oestrous periods in which the sign was displayed at least once, related to number of animals in oestrus at the same time

	One cow in oestrus (n=25)		Two cows in oestrus (n=36)		More than two cows in oestrus (n=28)	
	Times	%	Times	%	Times	%
Sniffing	28.9±19.3	100	23.3±16.9	100	26.0±17.3	100
Flehmen	2.4±2.8	40	2.0±1.3	42	2.1±1.5	50
Chin resting	18.8±14.2^{c}	100	24.0±20.1^{c,d}	100	30.2±18.0^{d}	100
Mounted	2.4±2.2	20$^{\#}$	3.1±3.3	66$^{\#}$	3.4±2.5	75$^{\#}$
Mounting	4.1±3.7^{c}	76*	5.5±4.9^{c,d}	92*	7.8±6.2^{d}	100*
Mounting head	2.3±1.5	16	3.9±3.1	19	3.0±2.1	32
Standing heat	3.6±3.1	20$^{\#}$	3.3±3.1	69$^{\#}$	5.9±5.2	79$^{\#}$

^{c,d}Different superscripts within a row mean a tendency for a difference (P<0.1)

*Percentages within rows differ (chi-square analysis, P<0.05); $^{\#}$Percentages within rows differ (chi-square analysis, P<0.01)

Table 3.4 shows the periods of time in which individual behavioural oestrous signs were displayed for primiparous and multiparous cows. Display of the behavioural signs sniffing and chin resting was longer in primiparous cows compared to multiparous cows (Table 3.4). The number of animals in oestrus did not influence the time displaying sniffing, chin resting, mounting or standing heat.

Table 3.4 Duration (h) of different behavioural oestrous signs for primiparous and multiparous cows (mean±SD, range)

Behavioural oestrous sign	Primiparous	Multiparous	Average
Sniffing (n=89)	12.8±4.5[a] (3-24)	10.2±4.1[b] (3-21)	11.1±4.4 (3-24)
Chin resting (n=89)	12.3±5.0[a] (3-24)	9.8±3.9[b] (3-18)	10.7±4.5 (3-24)
Mounting (n=80)	8.8±4.8 (3-21)	7.3±4.4 (3-21)	7.9±4.6 (3-21)
Standing heat (n=52)	5.2±2.8 (3-12)	4.9±3.2 (3-15)	5.0±3.0 (3-15)

[a,b]Different superscripts within a row mean a significant difference (P<0.05)

Some of the individual behavioural oestrous signs were also displayed while animals were not in oestrus. During approximately 20 days of non-oestrus, 87% of the animals that were not in oestrus displayed sniffing 5.5±5.2 times (ranging between one and 29 times). In that period, chin resting was displayed 1.9±1.3 times (ranging between one and seven times) in 46% of the animals that were not in oestrus. Seven percent of the animals mounted another cow one time during that period and standing heat was not displayed at all by animals that were not in oestrus.

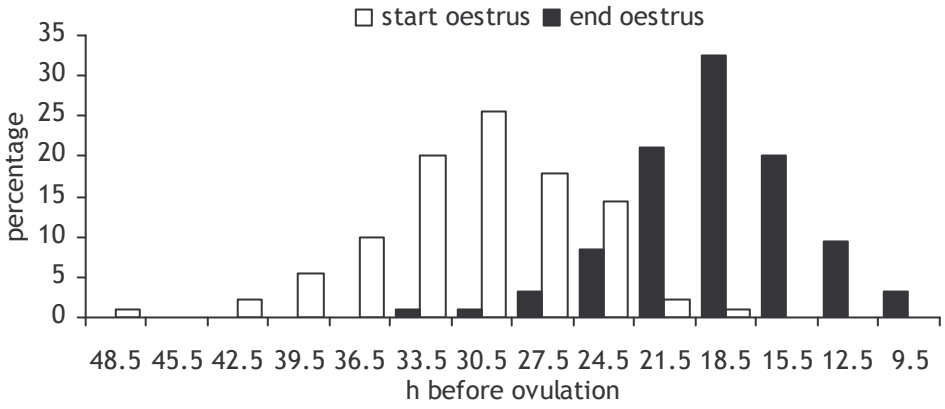

Figure 3.2. Distribution of interval between start (n=89) and end (n=94) of oestrus and time of ovulation.

Table 3.5. Interval between onset and end of oestrus until time of ovulation (h) for different behavioural oestrous signs (mean±S.D.) and distribution (%)

	Mean±SD	Distribution													
		48.5	45.5	42.5	39.5	36.5	33.5	30.5	27.5	24.5	21.5	18.5	15.5	12.5	9.5
All signs															
Start	30.6±5.1	1	0	2	6	10	20	26	18	14	2	1	0	0	0
End	18.8±4.4	0	0	0	0	0	1	1	3	8	21	33	20	9	3
Sniffing															
Start	30.3±5.0[a]	0	1	0	3	11	22	25	15	17	3	2	0	0	0
End	19.2±4.1[d]	0	0	0	0	0	1	1	1	10	26	35	17	7	2
Chin resting															
Start	30.2±5.1[a]	1	0	1	2	10	21	24	20	13	4	2	0	0	0
End	19.5±4.0[e]	0	0	0	0	0	1	2	0	11	28	34	17	6	1
Mounting															
Start	28.7±5.3[b]	0	1	0	3	5	17	21	23	19	6	4	1	0	0
End	20.8±4.1[f]	0	0	0	0	0	1	3	6	12	36	27	12	4	0
Standing heat															
Start	26.4±5.2[c]	0	0	2	0	2	10	14	20	30	12	8	2	0	0
End	21.4±5.4[f]	0	0	0	2	0	2	4	10	10	32	20	14	6	0

Different letters mean that the difference in onset (a, b and c) or end (d, e and f) of expressing the behavioural oestrous signs (h before ovulation) was significant ($P<0.05$) at an animal level.

Relationships between behavioural estrous signs and time of ovulation
Figure 3.2 shows the distribution of the interval between onset- and end of oestrus and time of ovulation. The average interval between onset of oestrus and ovulation was 30.6±5.1h. Ovulation took place 18.8±4.4h after the end of oestrus (Table 3.5). When intervals between onset of displaying individual behavioural oestrous signs and time of ovulation were analyzed, no significant difference was seen between these intervals (Table 3.5). Also intervals between end of displaying individual behavioural oestrous signs and time of ovulation did not differ between signs (Table 3.5). Intervals did not differ between primiparous and multiparous cows and were not influenced by the number of cows in oestrus at the same time.
In 86% of the animals that displayed mounting, ovulation occurred between 21.5 and 33.5h after onset of mounting. In 93% of the animals, ovulation occurred between 15.5 and 27.5h after end of mounting behaviour. Onset of standing heat occurred between 21.5 and 33.5h before ovulation and end of standing heat occurred between 15.5 and 27.5h before ovulation in 86% of the animals (Table 3.5).
The time interval between maximum behavioural points recorded during oestrus and ovulation was 24.7±5.0h (ranging from 14 to 41h). This interval was not influenced by parity or number of animals in oestrus.

Discussion

For a parameter to be a useful predictor for time of ovulation it should be highly accurate in identifying the appropriate behavioural event(s) which correlate with time of ovulation in the majority of individuals and must occur before time of ovulation. To our knowledge, no recent data are available on fertilization rates of cows at various insemination-to-ovulation intervals. In the fifties, Trimberger (1948) and Hall (1959) found that insemination should take place between 7 and 18h (12h window) before ovulation to achieve high fertilization rates. Based on these intervals, a predictor for time of ovulation should preferably determine time of ovulation with an accuracy of 12h and should be able to predict time of ovulation at least 18h before ovulation. Otherwise, inseminations based on the predictor will be too late to achieve good fertilization rates.
For a predictor to be useful in practice, the measurements should be easy to carry out, repeatable and preferably be automated. In the current study, the interval between onset of oestrus (ranging between 18.5 and 48.5h) or end of oestrus (ranging between 9.5 and 33.5h) and time of ovulation is quite variable when all the behavioural oestrous signs are taken into account. This interval between onset of oestrus and time of ovulation corresponds with an earlier study done in the same herd (19 to 40h, Roelofs et al. 2004) using synchronized oestrous periods. Because of the large variation between animals, onset of oestrus is not useful as predictor

for time of ovulation. Similarly, end of oestrus is not useful either. Moreover, assessing onset or end of oestrus the way it was done in this experiment requires a high input of labour from the farmer and to date is not yet automated.
An animal in oestrus starts with sniffing and chin resting, then starts to mount other animals and lastly displays standing heat. Sniffing and chin resting are not useful as predictors for time of ovulation; these signs were displayed by all animals in every oestrus, but were not exclusive for oestrus, as also found in other studies (Phillips & Schofield 1990, Van Eerdenburg et al. 1996). In the present study, 87% of the animals occasionally displayed sniffing when they were not in oestrus, and chin resting was occasionally displayed by 46% of the animals outside oestrus. Mounting behaviour might be interesting as predictor for ovulation time; it is displayed in 90% of oestrous periods, and although two animals displayed mounting behaviour when they were not in oestrus, they displayed it only once during the whole non-oestrous period. Similar to other studies, mounting seems to be an accurate sign of oestrus (Holtz & Meinhardt 1993, Van Eerdenburg et al. 1996). The variation in interval between the first mount and time of ovulation was 30h (ranging between 15.5 and 45.5h). In 86% of the oestrous periods mounting was displayed within a 12h interval (21.5 and 33.5h before ovulation). This means that in 77% of the cases (86% of the 90%), onset of mounting behaviour could predict ovulation time with an accuracy of 12h. However, to our knowledge no devices to automatically register mounting behaviour are available. Standing heat was displayed in 58% of the oestrous periods. Other studies found that less than 50% of the animals displayed standing heat during oestrus (Van Eerdenburg et al. 1996, Heres et al. 2000, Lyimo et al. 2000). In the present study standing heat was displayed in 20% of the oestrous periods when only one cow was in oestrus. The percentage of oestrous periods in which standing heat was displayed increased when more animals were in oestrus at the same time. Ovulation occurred on average 26.4±5.2h after the first display of standing heat. This interval is in agreement with other studies that investigated interval between onset of standing heat and time of ovulation (Bernard et al. 1983, Walker et al. 1996, Lopez et al. 2002, Kaim et al. 2003). Variation in the interval of first standing heat and time of ovulation was 27h (ranging between 15.5 and 42.5h). In 86% of the oestrous periods standing heat was displayed within a 12h interval (first standing heat between 21.5 and 33.5h before ovulation). This means that in 50% of all oestrous periods (86% of the 58%) onset of standing heat could predict ovulation time with an accuracy of 12h. Standing heat can be assessed automatically by means of mounting detectors (Walker et al. 1996, Xu et al. 1998, Shipka 2000, Rorie et al. 2002).

Accuracy and success of prediction of time of ovulation mainly depends on two things; how often the behavioural sign is displayed in how many animals and on the relationship with time of ovulation. When a behavioural oestrous sign is displayed intensively (i.e. often during oestrus) the chance of detection is higher. Therefore,

it would be important to search for factors that intensify oestrous behaviour. In the current experiment it was shown that the number of animals in oestrus at the same time affects intensity. Animals acquired twice the number of behavioural oestrous points when more than two cows were in oestrus for at least one observation period compared to when only one cow was in oestrus. Van Vliet et al. (1996) also found almost twice the number of behavioural oestrous points using the scoring system of Van Eerdenburg (1996) when two or more cows were in oestrus at the same time compared to one cow in oestrus. Mounting behaviour and standing heat seem to be responsible for the higher number of points acquired when more cows are in oestrus in the present study. With an increasing number of animals in oestrus, the percentages of oestrous periods in which animals were mounted, and the percentages of oestrous periods in which standing heat was displayed increased significantly. Also the number of times mounting was displayed tended to be higher when more animals were in oestrus at the same time. When more than one cow is in oestrus at the same time in the herd, a better oestrous expression is realized (i.e. behavioural oestrous signs are expressed more often). On one hand, this better oestrous expression may result from the fact that standing heat, and to a lesser extent mounting behaviour, are behavioural oestrous signs in which the animal displaying the behavioural sign needs at least one other animal (in oestrus) to be able to express it. On the other hand, this better oestrous expression may result from the sexual stimulation provided by other animals in oestrus (Orihuela 2000).

In the present study, oestrous duration for primiparous cows was approximately 3h longer compared to multiparous cows. This is in contrast with studies that found either no difference in oestrous duration between primiparous and multiparous animals (Lyimo et al. 2000) or found a shorter oestrous duration in primiparous cows (Van Vliet & Van Eerdenburg 1996, Walker et al. 1996). When individual oestrous behavioural signs are analyzed it appears that oestrous duration for primiparous cows is longer because of the longer period of time primiparous cows displayed sniffing and chin resting. Most studies that investigated oestrus duration, define oestrus as ‘standing when being mounted’ (Esslemont & Bryant 1976, Walker et al. 1996). Duration of mounting behaviour and standing heat did not differ between primiparous and multiparous cows in the present study.

It is also apparent, that intensity of displaying behavioural oestrous signs can differ between farms. In a study that used the same scoring system (Van Vliet & Van Eerdenburg 1996), total points acquired during oestrus with one or multiple cows in oestrus was twice as low as found in the current experiment. Although the number of animals in oestrus at the same time and also parity influenced intensity of behavioural oestrous signs, they did not influence relationships with time of ovulation. Further investigation of factors influencing intensity of oestrus could be worthwhile.

In conclusion, onset of mounting behaviour is the best predictor for time of ovulation, however this behavioural sign cannot yet be assessed automatically, thus limiting it practical use on the farm. Standing heat can be a good predictor for time of ovulation and can be assessed automatically (i.e. mounting detectors). Unfortunately, the disadvantage of using standing heat as predictor for time of ovulation is that only a limited number of cows display standing heat, especially when few animals are in oestrus at the same time. Although, the behavioural oestrous sign of 'mounting' is promising as predictor for time of ovulation, it is not useful as of yet in practice. Therefore, it is worthwhile to investigate other parameters to predict time of ovulation in practice (e.g. increase in walking activity by means of pedometers readings). When the time of ovulation can be predicted accurately, the next step is to investigate effects of the timing of insemination relative to ovulation on fertilization rates.

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4

Pedometer readings for oestrus detection and as predictor for time of ovulation in dairy cattle

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Theriogenology (in press)

Abstract

The objective of this study was to study the relationship between increase in number of steps measured by pedometers, behavioural oestrous parameters and time of ovulation, in order to investigate whether the number of steps can be used as a tool for oestrus detection and as a predictor for time of ovulation. In total, 63 ovulations were observed in 49 Holstein-Friesian cows. Different behavioural signs of oestrus were observed at three-hourly intervals. Cows were equipped with pedometers, which stored number of steps in two-hourly time periods and pedometer oestrus alerts were defined using different algorithms and thresholds. The percentage of behavioural oestrous periods also detected by pedometers measurements, ranged between 51% and 87% for all oestrous periods. When only oestrous periods where taken into account in which more than one animal was in oestrus, detection percentages increased up to 95%. Number of steps taken during the oestrous period was higher when more animals were in behavioural oestrus at the same time, and number of steps taken during the oestrous period was also higher for primiparous cows compared to multiparous cows. Ovulation occurred 29.3±3.9h after onset of increased number of steps (ranging between 39 and 22h) and 19.4±4.4h after the end of increased number of steps (ranging between 35 and 12h). The intervals were not influenced by the number of animals that were in oestrus at the same time or by parity. In conclusion, pedometers can detect oestrus accurately and appear to be a promising tool for prediction of ovulation time and hence could be a tool for improving fertilization rates.

Introduction

Methods to detect oestrus include e.g. visual observation, changes in body temperature, changes in vaginal mucus resistance, recording of mounting activity and also increase in number of steps around oestrus (Firk et al. 2002). Activity (measured by pedometers) of dairy cows during oestrus was first studied in the early fifties of the last century (Farris 1954). That study showed that the oestrous period in dairy cows is characterized by an increased number of steps. Later research showed that the increase in number of steps is a promising tool for accurate detection of oestrus (Lehrer et al. 1992, Firk et al. 2002), a prerequisite for good insemination results. Most studies utilizing pedometers, however, have focused on improving efficiency of oestrous detection (Rorie et al. 2002) and not on improving fertilization rates.

The chance of fertilization highly depends on the interval from insemination to ovulation. When cows are inseminated too early, chances are that sperm is aged by the time ovulation occurs and can not fertilize the ovum anymore (Hawk 1987). And when insemination takes place too late, chances are that because of the

ageing of the egg, fertilization and formation of a viable embryo is not possible anymore (Hunter & Greve 1997). Therefore, insemination time should be based on time of ovulation rather than on detection of oestrus.
If time of ovulation can be predicted by an increase in number of steps, pedometer readings could be a tool for improving fertilization rates. The present study investigated the relationship between increase in number of steps measured by pedometers during behavioural oestrus and time of ovulation to see if increase in number of steps can be used as a tool for oestrous detection and as predictor of time of ovulation.

Materials and Methods

The experiment was conducted at the experimental dairy farm "de Ossekampen" at Wageningen University and Research Centre, the Netherlands. The Ethical Committee for Experimentation with Animals (Wageningen, the Netherlands) approved the experimental protocol.

Animals, feed and housing
Data were collected from a herd of approximately 70 lactating Holstein-Friesian cows. Forty-nine of these animals showed oestrus and a subsequent ovulation at least once during the experimental period. Parity of these 49 animals varied between one and six and the animals were 100.0±65.8 (mean±SD) days in milk, with a range of 17 to 370 days. Animals were fed an ad lib mixture of grass silage, corn silage and mineral supplements, and concentrates according to production level (CVB (Central Animal Feed Bureau)-norms, 2000). The animals were housed in a free stall with slatted floor and cubicles. The animals were milked by an automated milking system (Liberty, Prolion, Vijfhuizen, The Netherlands). Estimated 305-day milk production was 8297±1334 (mean±SD) kg.

Measurement of number of steps
For activity measurements, animals were equipped with pedometers (Nedap Agri B.V, Groenlo, The Netherlands) on a front leg. The pedometer recorded the number of steps a cow made in two-hourly time periods (0-2 am, 2-4 am, ..., 10-12 pm). Receivers were placed by the entrance and exit of the automatic milking system and the data were transferred to a computer. Approximately 18 days of pedometer measurements around behavioural oestrus were analyzed for animals that showed visual signs of oestrus; onset and end of increased number of steps around visual oestrus were evaluated using different methods and thresholds

Calculation of an increase in number of steps

Increase in number of steps (measured by a pedometer) was calculated using two different methods, one based on the median number of steps and the other based on the standard deviation of the average number of steps. In both methods, the number of steps taken in a 2h time period was compared with the number of steps taken in the same time period during the 10 preceding days. Comparisons were done per 2h time period, because of the presence of a diurnal pattern in number of steps during dioestrus (Figure 4.1).

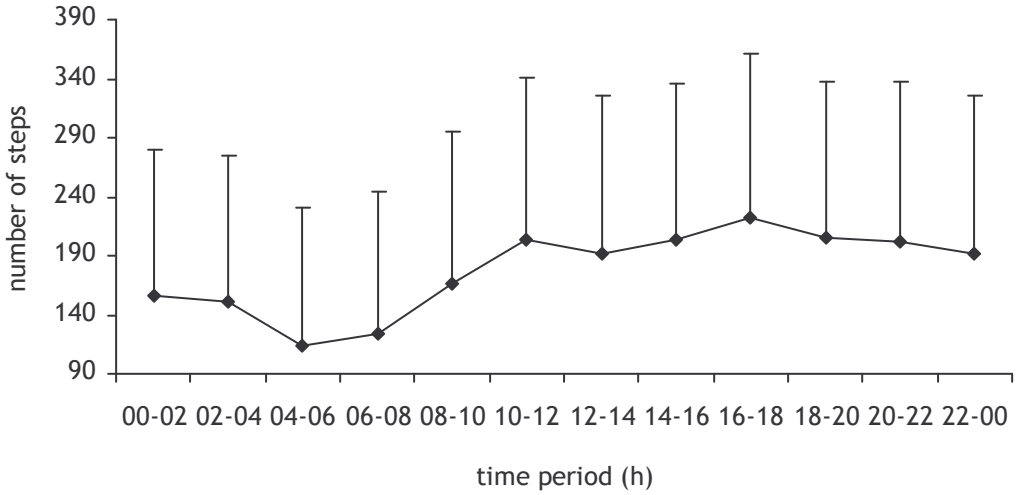

Figure 4.1. Number of steps taken in 2h time periods (mean+SD) for all animals and days, periods of oestrus excluded (n=1419-1455). The model also included the class-variable Cow ($P<0.05$) and the interaction between Period and Cow ($P<0.05$).The bars show the 95% confidence limits.

In the median method, the number of steps taken in a particular 2h time period was divided by the median number of steps of the 10 preceding days. Using this approach, a ratio was calculated for every 2h time period. If this ratio exceeded a threshold, either 10.0 for one period (MED10) or 5.0 for two consecutive periods (MED5), this was defined as an actual increase in number of steps and designated as an oestrus alert based on pedometer readings (pedometer oestrus alert).

In the second method based on the standard deviation, the mean and standard deviation of the number of steps was calculated for the 10 preceding days. If the number of steps for a particular 2h time period exceeded a threshold, i.e. the mean plus 2 (2SD), 2.5 (2.5SD), 3 (3SD) or 3.5 (3.5SD) times the standard deviation of the preceding 10 days, this was defined as an actual increase in number of steps. If this increase occurred for two consecutive periods, this was defined as a pedometer oestrus alert. A pedometer oestrus alert was defined as correct, when it was accompanied by behavioural oestrus and ovulation was confirmed. A pedometer oestrus alert was defined as false, when it was not accompanied by a behavioural oestrus. A pedometer oestrus alert was defined as missed, when behavioural oestrus (followed by ovulation) was not accompanied by a pedometer

oestrus alert. In other words, during one oestrous cycle, there was either a correct or a missed pedometer oestrus alert, and there could be zero, one or several false pedometer oestrus alerts. Figure 4.2 shows the activity pattern of an individual animal; Day 0 is the day of behavioural oestrus followed by ovulation. The dotted line indicates the calculated threshold for increase in number of steps (using 3SD). This animal had a false pedometer oestrus alert on day -7 (the continuous line exceeds the dotted line two consecutive times) and a correct pedometer oestrus alert on day 0.

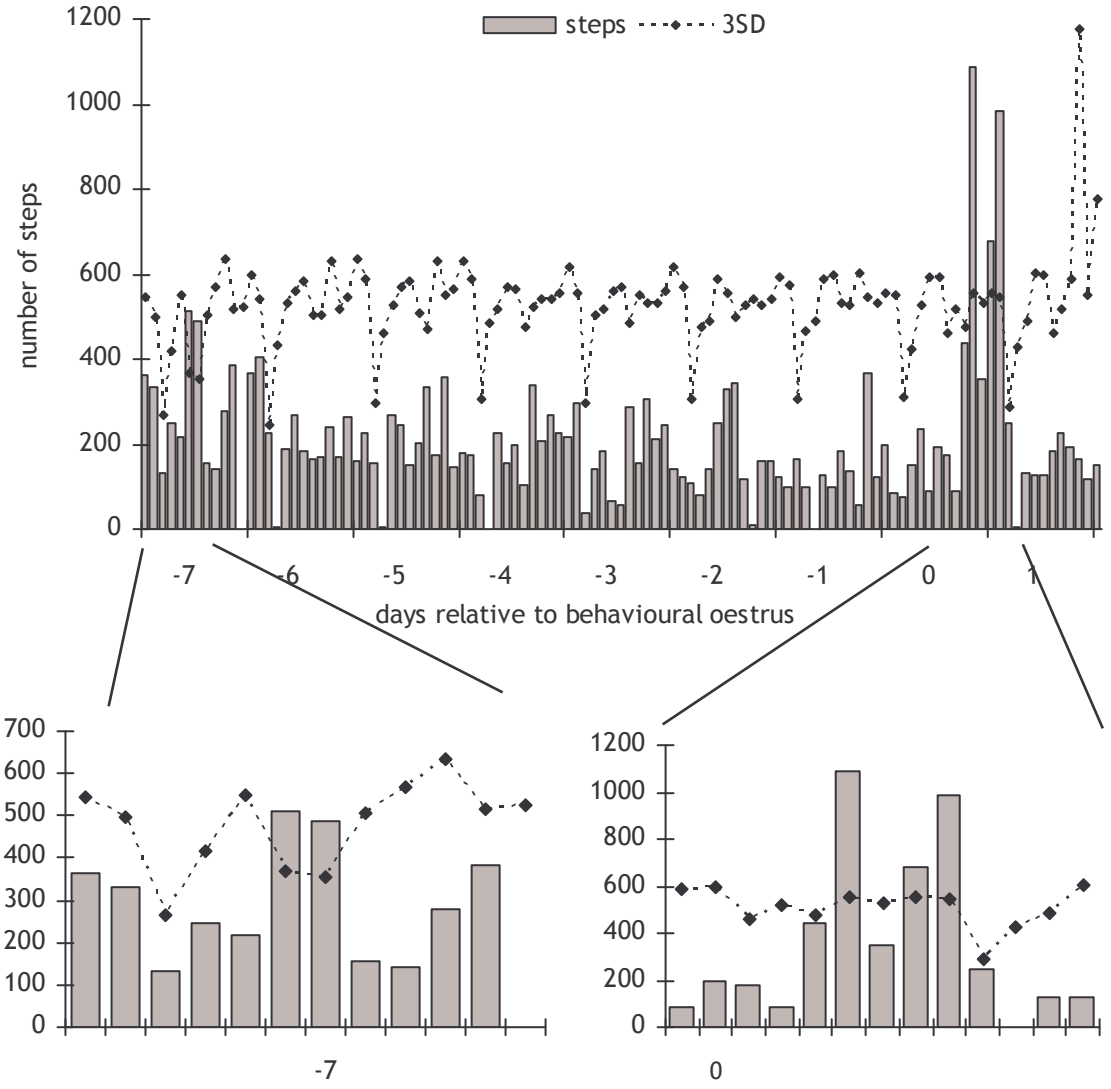

Figure 4.2. The activity pattern of an individual animal. The grey bars are the actual number of steps taking in 2h periods. The dotted line shows the threshold for 3SD, which is the mean of the preceding 10 days for that 2h period plus three times the standard deviation of that mean. When the grey bars exceed the dotted line two consecutive times, this is considered a pedometer oestrus alert. This animal had a false pedometer oestrus alert on day -7 (enhanced) and a correct pedometer oestrus alert on day 0 (enhanced, day 0=day of behavioural oestrus).

Onset of oestrus based on pedometer readings (pedometer oestrus) was defined as the first time at which an actual increase in number of steps was found (beginning of the 2h time period). End of pedometer oestrus was defined when at least two consecutive time periods no increase was found. It was defined as the last time at which an actual increase in number of steps was found (end of the 2h time period).

Visual observation of oestrous behaviour

Two observers monitored behaviour simultaneously every 3h for 30 min (at 8.00h, 11.00h, 14.00h, 17.00h, 20.00h, 23.00h, 2.00h and 5.00h) from an elevated chair, which did not disturb the animals. Each observer observed half of the stable and after an observation period the results of the observers were summed. Oestrus was defined according to Van Eerdenburg et al. (1996). Each time an animal displayed a behavioural oestrous sign, the assigned number of points was recorded (Table 4.1). If the sum of points during consecutive observation periods exceeded 100, the animal was considered to be in behavioural oestrus.

Table 4.1. Scoring scale for observed oestrous signs[1]

Oestrous signs	Points
Flehmen	3
Restlessness[2]	5
Sniffing the vulva of another cow	10
Mounted but not standing	10
Resting with chin on the back of another cow	15
Mounting other cows (attempt)	35
Mounting head side of other cows (attempt)	45
Standing heat	100

[1]Each time an oestrous sign is observed, the assigned number of points is recorded (modified after Van Eerdenburg, et al., 1996); [2]Can be recorded only once during an observation period

Onset of behavioural oestrus was defined as the first observation period the animal showed oestrous behaviour minus 1.5h. End of behavioural oestrus was defined as the last observation period the animal showed oestrous behaviour plus 1.5h. The number of animals in behavioural oestrus during an observation period was recorded. Data were collected in 23 cases from animals with an oestrus, induced by i.m. injection of 15 mg of prostaglandin F2-α analogue (Prosolvin, Intervet, Boxmeer, The Netherlands) when a corpus luteum was present. In 40 cases data were collected from naturally cycling cows.

Ultrasound detection of ovulation

The ovaries of the cows were examined rectally using an ultrasound scanner (Scanner 200, Pie Medical, Maastricht, The Netherlands). The scanner was equipped with a 7.5 MHz sector transducer. The transducer was inserted into the rectum to examine the ovaries. The reproductive tract was not manipulated or

palpated before or during ultrasound examination. Ultrasound examinations started between 8 and 11h after the end of an observed behavioural oestrus. During the first ultrasound examination, each ovary was scanned to determine on which ovary the preovulatory follicle was located. Thereafter, scanning the ovary containing this follicle was continued every 3h until the disappearance of the follicle, which marked ovulation time (Rajamahendran et al. 1989). Each time, the diameter of the follicle was measured. Time of ovulation was defined as the first ultrasound the preovulatory follicle had disappeared minus 1.5h. Six days after oestrus, ultrasound examination was performed to determine if a corpus luteum had appeared which confirmed ovulation.

Statistics

Because ovulation was assessed only in cows that showed behavioural oestrus, relationships between pedometer readings and ovulation can only be calculated for cows that showed behavioural oestrus and not for cows with silent oestrus. Preliminary analyses revealed no differences between natural and induced oestrous periods for any of the parameters; therefore data of induced (n=23) and natural (n=40) oestrous periods were combined. Influences of 2h time periods on number of steps outside oestrous periods were calculated by means of a multivariate analysis of variance (adjusted for multiple comparisons by Bonferroni), using the Statistical Analysis System (The SAS system for windows V8, 1999). In the model were included Time Period and Cow as class-variables and the interaction between these class-variables. Data on intensity of behavioural and pedometer oestrus and intervals between pedometer oestrus and time of ovulation were also analyzed by means of a multivariate analysis of variance (adjusted for multiple comparisons by Bonferroni). The factors Parity and Animals in Oestrus were used as class variables, in which Parity consisted of two classes (primiparous (n=20) and multiparous (n=43) cows) and Animals in Oestrus consisted of three classes (1= no other animal in behavioural oestrus (n=15), 2= (partly) simultaneously with at most one other animal in behavioural oestrus (n=26) and 3= (partly) simultaneously with at least two other animals in behavioural oestrus (n=19)). Chi-square analysis was used to investigate differences between percentages of correct pedometer oestrus alerts for the different classes of Animal in Oestrus. All means are presented as mean±SD, unless otherwise stated. P-values <0.05 are defined as a significant difference and P-values between 0.05 and 0.1 are defined as a tendency for difference.

Results

In total, 63 ovulations were observed in 49 animals. For each ovulation, pedometer readings from 18 to 0 days before behavioural oestrus and 1 to 17 days after behavioural oestrus (on average 18.3±2.9 days) were analyzed. Basal number of

steps per 2h time period was on average 177±134, but differed between time periods. The lowest number of steps was found in the early morning (4-8 am, see Figure 4.1).

Efficiency of different methods of defining pedometer oestrus

Table 4.2 shows number and percentage of correct, false and missed pedometer oestrus alerts for the different methods calculating increase in number of steps. The threshold of 2SD and 2.5SD resulted in the highest percentage of correct pedometer oestrus alerts (87%). The methods that use the median (MED10 and MED5) resulted in poor percentages of correct pedometer oestrus alerts (51% and 52%, respectively). MED10 also resulted in a high number of false pedometer oestrus alerts (80 in 63 oestrous cycles). The thresholds of 2.5SD, 3SD and 3.5SD resulted in high percentages of correct pedometer oestrus alerts (87%, 83% and 79% respectively) and relatively low percentages of false pedometer oestrus alerts (17%, 8% and 5% respectively).

Table 4.2. Percentage and number of correct and missed pedometer oestrus alerts and average number of false pedometer oestrus alerts for each threshold of increase of activity (n=63).

Pedometer oestrus alert	Correct		Missed		False		
	Number	%	Number	%	% oestrus[1]	Average[2]	Range[2]
MED10	32	51	31	49	40	3.2	1-17
MED5	33	52	30	48	14	2.1	1-6
2SD	55	87	8	13	37	1.3	1-3
2.5SD	55	87	8	13	16	1.3	1-2
3SD	52	83	11	17	6	1.1	1-2
3.5SD	50	79	13	21	5	1.0	1

[1]Percentage of all behavioural oestrus periods in which at least one false pedometer oestrus alert was present; [2]Number of false pedometer oestrus alerts in oestrous cycles with at least one false pedometer oestrus alert

Of the 63 behavioural oestrous periods with ovulation, 11 were not detected by pedometer readings using 3SD (missed pedometer oestrus alerts). The mean and standard deviation of number of steps taken in the 2h time periods during 10 days prior to behavioural oestrus did not differ between the 52 correct pedometer oestrus alerts (mean: 179±50 steps; SD: 121±31 steps) and the 11 missed pedometer oestrus alerts (mean: 184±40 steps; SD: 131±29 steps, $P>0.1$). In other words, the reason why oestrus was not detected by pedometers readings was not because those animals had a different activity pattern 10 days before behavioural oestrus. Nine of the 11 missed pedometer oestrus alerts did have one 2h time period with increased number of steps. In other words, there was an increase in activity, but of too short a duration to be considered as pedometer oestrus alert. Using 3SD, five times a pedometer oestrus alert was given when no behavioural oestrus with ovulation occurred (false pedometer oestrus alert). Of these five false pedometer

oestrus alerts, two can be explained by the fact that, the animals were forced to go through a footbath, which increased the number of steps in that particular 2h period. The remaining three false pedometer oestrus alerts cannot be explained by clear events. Mean and standard deviation of number of steps taken in the 2h period during 10 days prior, did not differ between correct (mean: 179±50 steps; SD: 121±31 steps) and false (mean: 168±33 steps; SD: 127±41 steps, $P>0.1$) pedometer oestrus alerts. However, the actual number of steps in the 2h period in which the activity was increased, was significantly lower for false pedometer oestrus alerts (477±322 steps) compared to correct pedometer oestrus alerts (841±259 steps; $P<0.05$).

Relationship between behavioural oestrus and increase in number of steps

Table 4.3 shows characteristics of behavioural and pedometer oestrus (using 3SD) for primiparous and multiparous animals and for different numbers of animals in behavioural oestrus. Duration of behavioural oestrus was on average 2h longer (11.8h) compared to pedometer oestrus (10.0h; Table 4.3). Primiparous cows had a longer duration of pedometer oestrus and were more active during pedometer oestrus compared to multiparous cows; the same was true for behavioural oestrus. The number of animals in behavioural oestrus did not influence any of the parameters of pedometer oestrus, but animals displayed more behavioural oestrous signs when more than two cows were in oestrus. Although the number of animals in behavioural oestrus at the same time did not significantly influence the number of steps during pedometer oestrus, more animals were detected in oestrus by the pedometers when more than two animals were in behavioural oestrus at the same time (95%) compared to when two animals (85%) or only one animal (67%) was in behavioural oestrus at the same time. Total, maximum and average number of steps were positively correlated with total, maximum and average number of points acquired during behavioural oestrus ($r:0.32$-0.56; $P<0.05$). Total, maximum and average number of steps were also positively correlated with most of the individual behavioural signs (sniffing, chin resting, mounting and standing heat) displayed during behavioural oestrus ($r:0.28$-0.46; $P<0.05$). Only total and maximum number of steps was not correlated with chin resting and average number of steps was not correlated with sniffing. Duration of pedometer oestrus was positively correlated with duration of behavioural oestrus ($r=0.53$) and with the number of times sniffing ($r=0.30$) and standing heat ($r=0.38$; $P<0.05$) were displayed.

Table 4.3. Characteristics of behavioural oestrus and pedometer oestrus for one, two or more than two animals in behavioural oestrus at the same time and primiparous and multiparous animals (mean±SD (range))

	Animals in oestrus			Parity		Average
Pedometer oestrus	1 (n=10)	2 (n=22)	More than 2 (n=18)	Primiparous (n=16)	Multiparous (n=34)	
Duration (h)	10.0±4.4	9.0±3.8	11.3±4.5	12.4±4.1[a]	8.9±3.7[b]	10.0±4.2
	(6-18)	(4-18)	(4-18)	(6-18)	(4-18)	(4-18)
Total steps[1]	3313±1577	3904±2116	5415±27479	6090±2331[a]	3455±1792[b]	4298±2317
	(832-5520)	(1320-8416)	(820-168)	(2640-9168)	(820-8416)	(820-9168)
Maximum steps[2]	910±329	1114±351	1235±436	1397±383[a]	980±308[b]	1113±384
	(312-1344)	(544-1760)	(480-2080)	(784-2080)	(312-1648)	(312-2080)
Average steps[3]	662±218	852±210	929±290	984±234[a]	773±236[b]	840±254
	(277-932)	(539-1213)	(410-1524)	(660-1524)	(277-1308)	(277-1524)
Behavioural oestrus	n=15	n=26	n=19	n=20	n=40	
Duration (h)	11.0±4.0	11.1±5.0	13.3±3.4	13.5±5.2[y]	10.9±3.6[z]	11.8±4.4
	(6-21)	(3-24)	(9-18)	(3-24)	(3-21)	(3-24)
Total points[1]	810±543[a]	1097±659[a]	1751±890[b]	1621±718[y]	1038±771[z]	1232±797
	(114-2035)	(235-3303)	(441-3857)	(185-3150)	(114-3857)	(114-3857)
Maximum points[2]	379±188[a]	551±283[ab]	806±394[b]	791±389[a]	488±268[b]	589±342
	(100-690)	(170-1243)	(203-1635)	(100-1635)	(101-1268)	(100-1635)
Average points[3]	217±131[y]	324±197[y]	405±211[z]	414±236[y]	277±160[z]	323±198
	(57-458)	(117-910	(139-788)	(62-910)	(57-771)	(57-910)

Different letters (a, b) within a row mean a significant difference for the variable 'animal in oestrus' or 'parity' ($P<0.05$). Different letters within a row mean a tendency for a difference for the variable 'animal in oestrus' or 'parity' ($P<0.1$)

[1]Number of points/steps acquired during the entire oestrus; [2]The maximum number of points/steps acquired during a period during oestrus; [3]The average number of points/steps acquired during a period during oestrus

When behavioural oestrus was more intense, the chance that this oestrus was detected with the pedometer was higher; animals with a pedometer oestrus had a longer duration of behavioural oestrus (12.3±4.1h) compared to the duration of behavioural oestrus for animals with a missed pedometer oestrus alert (9.0±4.7h, $P<0.05$). Also, animals that had a pedometer oestrus acquired more total (1366±787 points) and maximum (639±334 points) number of points for behavioural oestrus compared to the total (565±444 points, $P<0.05$) and maximum (337±271 points, $P<0.05$) number of points for animals that had a missed pedometer oestrus alert.

Relationship between increase in steps and time of ovulation

Figure 4.3 shows the distribution of the interval between onset- and end of pedometer oestrus and time of ovulation. The average interval between onset of pedometer oestrus and ovulation was 29.3±3.9h (range: 39-22h), and ovulation took place 19.4±4.4h (range: 35-12h) after the end of pedometer oestrus. Interval between onset of pedometer oestrus and time of ovulation did not differ between primiparous and multiparous cows and was not influenced by the number of cows in behavioural oestrus at the same time. The interval between end of pedometer oestrus and time of ovulation was shorter for primiparous cows (16.9±3.0h) compared to multiparous cows (20.6±4.5h), which was probably due to the longer duration of oestrus for primiparous cows (Table 4.3). The interval between end of pedometer oestrus and time of ovulation was not influenced by the number of animals in behavioural oestrus at the same time.

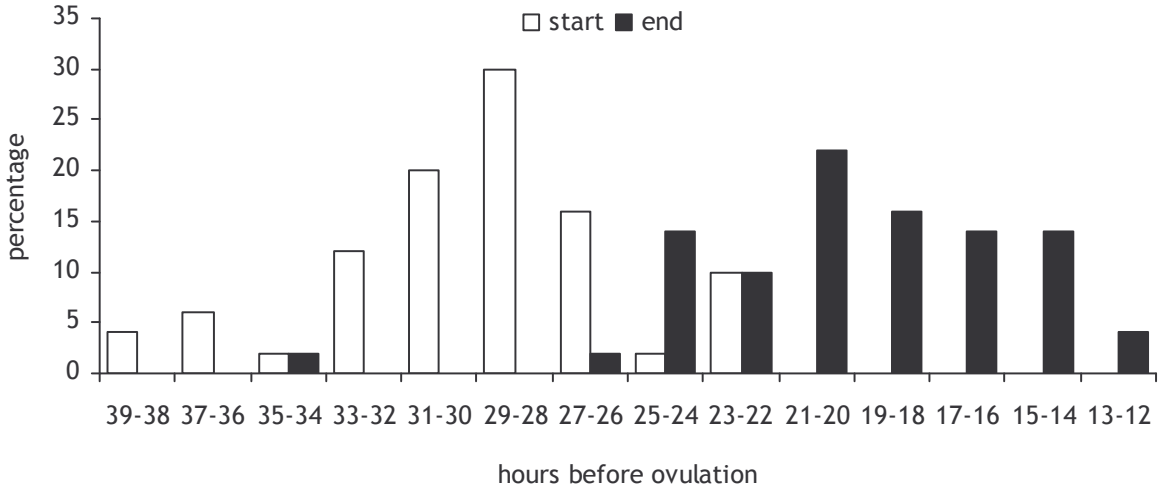

Figure 4.3. Distribution of interval between start (n=51) and end (n=49) of pedometer oestrus and time of ovulation.

Discussion

The results of this study show that the increase in the number of steps preceding ovulation can be used to detect oestrus and to predict time of ovulation fairly accurate. Ovulation occurred approximately 29h (ranging from 22 to 39h) after onset of pedometer oestrus (using 3SD). If time of ovulation can be predicted, insemination can be timed to get good fertilization results. Not many studies have looked at the effects of different intervals of insemination in relation to ovulation time on conception rates in natural cycling dairy cattle. More then 50 years ago, Trimberger et al. (1948) found highest conception rates when cows were inseminated between 7 and 24h before ovulation. The best conception rate (85.7%) was found when insemination took place between 13 and 18h before ovulation. If these results still hold, it would mean that insemination should take place between 11 and 16h after onset of pedometer oestrus in our study to get the best fertilization results. Maatje et al. (1997) designed an experiment to estimate the optimal time interval from the onset of oestrus, determined by pedometers, to artificial insemination. In that study the chance of conception was highest (84.2%) when cows were inseminated between 6 and 17h after increased pedometer activity; the estimated optimum was at 11.8h. Unfortunately, time of ovulation was not assessed in that experiment, but when ovulation time would be calculated by the means of the results of our experiment, the results of Maatje et al. (1997) would be in agreement with the results of Trimberger et al. (1948).

For pedometer readings to be useful as oestrous detection as well as predictor of ovulation time, it is important to have a high accuracy and a high detection percentage. The number of steps during 10 days prior to a pedometer oestrus gives no indication if a pedometer oestrus alert is false or correct. However, looking at the actual number of steps during the pedometer oestrus, a low number of steps might indicate that the pedometer oestrus alert is false. The number of correct, missed and false pedometer oestrus alerts depends on the definition of the threshold for ‘increased number of steps’. Different thresholds have been used in experiments to study the increase in number of steps around oestrus (Kiddy 1977, Williams et al. 1981, Moore & Spahr 1991, Maatje et al. 1997, López-Gatius et al. 2005). In our experiment, using a threshold based on the mean of the 2h time periods during 10 days prior plus several times the standard deviation seemed to give the best results in terms of accuracy and detection. Because the number of steps was not normally distributed, another threshold was calculated using a ratio based on the median of the 2h time periods during 10 days prior, to see if results would improve. This was not the case; the number of missed and false pedometer oestrus alerts was much higher using the method with the median compared to the other method. Using 2SD, 2.5SD, 3SD and 3.5SD, the percentages of correct pedometer oestrus alerts ranged between 79 and 87%, which is a similar range as

found in other experiments (Kiddy 1977, Moore & Spahr 1991, Schofield et al. 1991). Using SD as a threshold, the average number of false pedometer oestrus alerts ranged from 1.0 to 1.3 in 5% to 37% of the 63 behavioural oestrous periods. The literature differs considerably in the percentage of false pedometer oestrus alerts measured, ranging from 0% (Peter & Bosu 1986) to 205% (Moore & Spahr 1991) or more (Holdsworth & Markillie 1982). These large differences may be explained by different threshold definitions, different periods (e.g. 12h instead of 2h periods) or different management (e.g. different floor surface, no automatic milking system) used in the different experiments.

In our experiment, the method using 3SD (so, comparison of the number of steps in a 2h period with the mean plus 3 times the standard deviation of the same 2h periods for the 10 days prior) showed a good result with 83% correct pedometer oestrus alerts and only five false pedometer oestrus alerts in only four of the 63 oestrous cycles. Two of the five false pedometer oestrus alerts can be explained by management factors; at the time the false pedometer oestrus alert was given, the cows were forced through a footbath, which increased the number of steps. Koelsch et al. (1994) also found that altered routines were a likely source of false oestrous indications by pedometer readings. Thus, occurrence of pedometer oestrus alerts after alteration of the daily routine should be interpreted with caution. In nine of the 11 missed pedometer oestrus alerts, one 2h time period with increased number of steps was detected. In other words, there was an increase in activity, but of too short a duration to be considered a pedometer oestrus (which requires at least two consecutive 2h time periods with increased activity). If one period with increased activity would be considered a pedometer oestrus, the percentage of correct pedometer oestrus alerts would increase to 97%. However, the number of false pedometer oestrus alerts would increase tremendously; 79% of the oestrous cycles would have one to eight false pedometer oestrus alerts with an average of 3.4 per cycle. This shows that the definition used to define increased number of steps (and as a consequence, number of correct, false and missed pedometer oestrus alerts) is very important and dependant on several factors, e.g. herd, management, etc. Because of the presence of a diurnal rhythm in the number of steps (Liu & Spahr 1993, Arney et al. 1994, Koelsch et al. 1994) it should be more precise to store the number of steps in small time periods, and use this in the definition for increased number of steps, as done in our experiment. Past experiments used pedometers that stored the number of steps in time periods of 12h (Kiddy 1977, Holdsworth & Markillie 1982, Pennington et al. 1986). Several experiment have shown that smaller time periods (up to 2h) improved efficiency (number of correct pedometer oestrus alerts) but decreased accuracy (higher number of false pedometer oestrus alerts (Moore & Spahr 1991, Liu & Spahr 1993). The conclusion of those studies was that number of steps measured for time periods of 12h was potentially satisfactory for recording cow activities to detect oestrus. However, our data show that for 15 of the pedometer oestrous periods, an

increase in activity (using 3SD as a threshold) was present for only 6h or less. These short pedometer oestrous periods would probably have been missed when data storage took place in 12h time periods. Furthermore, if the increase in number of steps is used to predict time of ovulation, timing of insemination would be based on onset of pedometer oestrus. Therefore, it is important to have data storage for small time periods, so timing of insemination in relation to ovulation can be accurate.

Visual observations for behavioural oestrous signs were carried out frequent in this experiment (for 30 min every 3h), which ensured detection of short behavioural oestrous periods. In practice some behavioural oestrous periods will be missed when visual observations are carried out less frequent (Moore & Spahr 1991, Liu & Spahr 1993, Van Vliet & Van Eerdenburg 1996). Also behavioural oestrous periods that occur during the evening and night could be missed by visual observations in practice. Standing heat is the most discriminative sign of oestrus (Van Eerdenburg et al. 1996) but not all cows express this behavioural sign (Van Eerdenburg et al. 1996, Heres et al. 2000, Lyimo et al. 2000, Roelofs et al. 2005c). When, in practice, oestrous periods are detected only using this behavioural sign, many oestrous periods will be missed. When the three mentioned causes for possibly missed behavioural oestrous periods (short oestrus, oestrus during night and no standing heat) are taken into account, 28 behavioural oestrous periods (44%) would have been missed in our experiment, 68% of these 28 oestrous periods were detected by pedometers (using 3SD) which shows the importance of pedometers for oestrus detection. Oestrous periods can also be 'silent', i.e. no expression of oestrous behaviour at all preceding ovulation. Because correct and missed pedometer oestrus alerts are based on expression of behavioural oestrus in this experiment, it is not known whether animals with silent oestrus show an increase in the number of steps and would have been detected in oestrus using pedometer readings. However, in previous experiments with the same visual observation method, all oestrous periods were detected (Van Vliet & Van Eerdenburg 1996, Lyimo et al. 2000).

Several studies show that oestrus is more intense when more animals are in oestrus at the same time: animals received more points (Van Vliet & Van Eerdenburg 1996), displayed more mounts and stands (Hurnik et al. 1975, Helmer & Britt 1985, Van Vliet & Van Eerdenburg 1996) and took more steps (Varner et al. 1994). In our study, intensity of behavioural oestrus was increased when more animals were in oestrus at the same time and the number of steps increased when more animals were in oestrus at the same time, although the latter increase was not significant ($P=0.14$ for average number of steps during oestrus). When a behavioural oestrous sign is displayed intensively (i.e. often during oestrus) or the increase in number of steps is more obvious (higher), the chance of detection may also be higher. In our study, overall detection percentage of pedometer oestrus was 83%, but when only one animal was in behavioural oestrus, significantly less pedometer oestrous

periods were detected compared to when two or more animals were in behavioural oestrus. These results indicate that more animals being in oestrus at the same time, not only stimulates behavioural signs of oestrus, but also increases walking activity during oestrus, because of which oestrous detection percentages by pedometers increased up to 95%.
Another factor that influenced intensity of oestrus was parity. Behavioural- and pedometer oestrous durations for primiparous cows were 3h longer compared to multiparous cows. This is in contrast with studies that found either no difference in behavioural oestrous duration between primiparous and multiparous animals (Lyimo et al. 2000) or that found a shorter behavioural oestrous duration in primiparous cows (Van Vliet & Van Eerdenburg 1996, Walker et al. 1996). Also oestrus was more intense (more number of points received and number of steps taken during oestrus) in primiparous cows compared to multiparous cows. A study that used the same system for behavioural observations found the opposite; multiparous cows acquired on average more points during oestrus compared to primiparous cows (Van Vliet & Van Eerdenburg 1996). Why these differences exist is unclear. To our knowledge, no information is available on the influence of parity on activity patterns.
In conclusion, pedometer measurements can detect oestrus accurately and appear to be a promising tool for prediction of ovulation time and hence could be a tool for improving fertilization rates. In practice, a daily routine is important for pedometer readings to have a high detection percentage and a high accuracy. In the end it is up to the farmer to choose a threshold that best fits his wishes. If he wants to know for sure that a pedometer oestrus alert is correct, there will be more missed pedometer oestrus alerts. Vice versa, if a farmer wants to detect as many oestrous periods as possible, the consequence is that there will be more false pedometer oestrus alerts. The percentages of false, correct and missed pedometer oestrus alerts for the different thresholds in this study only apply to one experimental farm. Further research on factors that can influence activity patterns (e.g. number of animals in oestrus, parity etc.) and validation of the results for different farms (to investigate whether the same thresholds apply to different herds) could be worthwhile to optimize the use of pedometers as a tool for oestrous detection and prediction of ovulation time.

Acknowledgments

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5

Relationship between progesterone concentrations in milk and blood and time of ovulation in dairy cattle

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Animal Reproduction Science (in press)

Abstract

The objective of this study was to investigate whether monitoring progesterone concentrations in milk and blood plasma can be used to predict time of ovulation in dairy cattle. Whole milk was sampled twice daily and blood samples were collected once a day before the morning milking. Ovulation was assessed by trans-rectal ultrasonography at 4h intervals beginning from the end of oestrus. For a parameter to be useful as predictor for time of ovulation, it should be precise (i.e. variation between animals should not exceed 12h). In milk, progesterone concentration dropped <15ng/ml at 97.7±17.8h (range: 54-126h) before ovulation, to <5ng/ml at 79.7±11.2h (range: 54-98) before ovulation to decline further to <2ng/ml at 70.7±16.8h (range: 38-90h) before ovulation (n=20). In plasma, progesterone concentration dropped to <4ng/ml 90.5±19.6 h (range: 66-138h) before ovulation and to <2ng/ml at 75.0±12.2 h (range: 50-98) before ovulation. These intervals were not influenced by parity, milk production or days in milk. In conclusion, monitoring of progesterone alone is not sufficient to predict ovulation because of the large variation in timing of decrease of progesterone concentrations relative to ovulation between animals. At best the range is about 2 days.

Introduction

In ordinary dairy practice, time of insemination in relation to time of ovulation is variable and insemination is usually determined by standing heat signs. However, several studies have shown that in more than 50% of all oestrous periods, standing heat is not displayed (Van Eerdenburg et al. 1996, Heres et al. 2000, Roelofs et al. 2004, 2005c) and many insemination moments will be missed if the farmers look for standing heat specifically. Also other behavioural oestrous signs are not displayed by all animals in oestrus or are displayed in variable duration and often a very short time, so they are easily missed (Roelofs et al. 2004, 2005c).

A parameter which occurs in all cyclic cows and indicates the onset of the follicular phase and subsequent ovulation is the decline of the progesterone level. Confirmation of oestrus and ovulation by means of a period of low progesterone concentrations in milk or blood plasma, followed by a rise, has been widely used (Plotka et al. 1967, King et al. 1976, Walton & King 1986, Darwash et al. 1999). Unfortunately, actual time of ovulation was not assessed in these studies. If a good relationship exists between the decline of progesterone and subsequent time of ovulation, measurement of progesterone concentration could be a tool to improve fertilization results by predicting the time of ovulation.

The objective of this study was to investigate whether monitoring progesterone concentrations in milk and blood plasma can be used to predict time of ovulation in dairy cattle.

Materials and Methods

The experiment was conducted at the experimental dairy farm at Wageningen University and Research Centre, the Netherlands. The Ethical Committee for Experimentation with Animals (Wageningen, the Netherlands) approved the experimental protocol.

Animals, feed and housing
Data were collected from 20 lactating Holstein-Friesian cows from a herd of approximately 80 cows. The cows were housed in a free stall barn with slatted concrete floor and cubicles. Parity of the 20 cows varied between one and four and the cows were 140.9±95.2 (mean±SD) days in milk (range: 49 to 363 days). The cows were fed an ad lib mixture of grass silage, corn silage and mineral supplements, and concentrates according to production level (Central Animal Feed Bureau-standards, 2000). Average milk production during the sampling period was 25.0±5.6 litres/day (mean±SD) with 4.60% fat and 3.38% protein.

Milk and blood sampling
Starting 12 days after a detected ovulation, milk and blood samples were taken. Milk sampling continued until the next oestrus, which was on average 9.8±2.5 days (mean±SD). Droplets of whole milk were collected continuously during milking (twice a day; between 9 am-10 am and 8 pm-9 pm). The milk was thoroughly mixed and a sample (10ml) was immediately stored at -20ºC until assayed. Blood samples were collected once daily just before the morning milking until the next oestrus. Plasma samples were collected by coccygeal venipuncture into 10ml heparinized evacuated blood collection tubes (Venoject, Leuven, Belgium) and stored at -20ºC until analysis after centrifugation at 2,000 x *g* for 10 min at 4ºC. If after 11 days of sampling no oestrous signs had been displayed, blood sampling was stopped.

Progesterone assay
Progesterone concentration in milk and plasma was measured in triploid using a commercial ELISA kit (Ridgeway Science, Gloucester, UK). For milk samples with progesterone levels between 2 and 30ng/ml, the intra-assay and inter-assay coefficients of variation were 7.8% and 13.4%, respectively, and the sensitivity was 1.3ng/ml. For plasma samples with progesterone levels between 2 and 20ng/ml, the intra-assay and inter-assay coefficients of variation were 7.5% and 12.4%, respectively, and the sensitivity was 0.9ng/ml.

Ultrasonography
The ovaries of the cows were examined rectally using a scanner with a 7.5 MHz sector transducer (Scanner 200, Pie Medical, Maastricht, The Netherlands). The

reproductive tract was not manipulated or palpated before or during the examinations, that started approximately 18h after onset of oestrus. During the first examination, both ovaries were scanned to determine the location of the preovulatory follicle. Thereafter, this ovary was scanned every 4h until the disappearance of the follicle, marking ovulation time. Time of ovulation was defined as the first ultrasound the preovulatory follicle had disappeared minus 2.0h. Six days after oestrus, ultrasound examination was performed to determine if a corpus luteum had appeared thus confirming ovulation.

Statistics

The intervals between decline in progesterone concentration and time of ovulation different thresholds were defined as follows; for milk progesterone, three thresholds were chosen: the first sample with a concentration less than: 1) 15ng/ml (M15), 2) 5ng/ml (M5) and 3) 2ng/ml (M2). For plasma progesterone two thresholds were chosen: the first sample with a concentration less than: 1) 4ng/ml (P4) and 2) 2ng/ml (P2). Multivariate analysis (SAS system for Windows V8, 1999) showed no effect of parity, milk production or days in milk on progesterone concentrations and intervals to ovulation. Correlations between milk and plasma progesterone concentration were examined using Pearson correlation coefficients. Means and standard deviations are shown in the results section.

Results

Twenty complete profiles of milk progesterone concentration (i.e. maximum concentration until concentrations <2ng/ml, Figure 5.1A) and 18 complete profiles of plasma progesterone concentration (Figure 5.1B) were available. A large variability was found between progesterone profiles of individual animals (Figure 5.1A and 5.1B). In individual animals, the decline in progesterone concentration was rapid. For milk progesterone the decline from <15 to <5ng/ml occurred, on average, in 1.5 (0 to 4) consecutive samples, corresponding with 18.0h. The decline from <5ng/ml to <2ng/ml occurred, on average, in 0.8 (0 to 3) consecutive samples, corresponding with 9.0h. For plasma progesterone concentrations, the decline from <4ng/ml to <2ng/ml occurred, on average, in 0.7 (0 to 3) consecutive samples, corresponding with 16.0h.

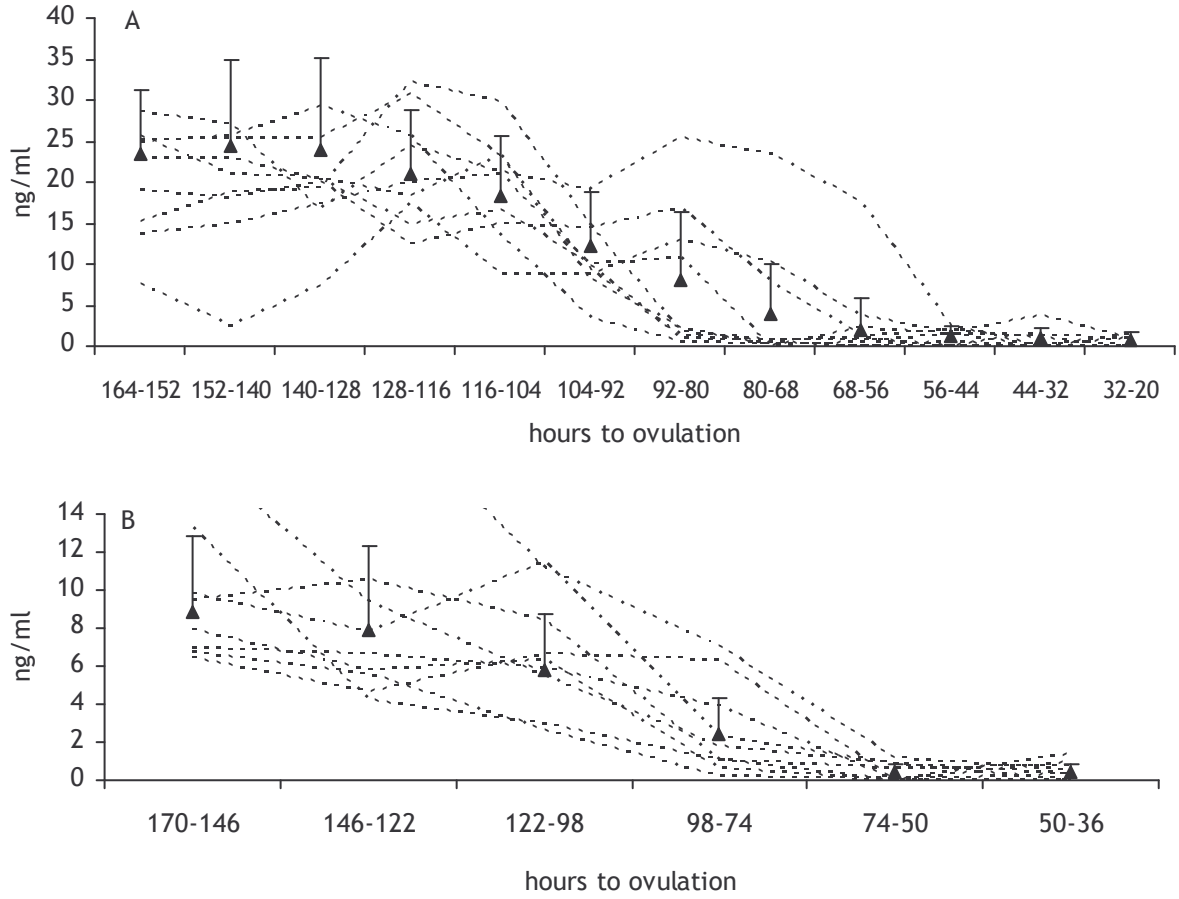

Figure 5.1. Individual (lines) and mean+SD (black triangles) profiles (ng/ml) in milk (A) and plasma (B) progesterone at different intervals before time of ovulation. For clarity, individual profiles from nine representative animals are shown, average is of all profiles.

The average intervals between progesterone decline and ovulation are presented for the different thresholds of progesterone concentration in milk and plasma (Table 5.1). The variation in these intervals was the smallest for the interval between milk progesterone concentrations <5ng/ml and time of ovulation (SD=11.2, Table 5.1) and highest for plasma progesterone concentrations <4ng/ml and time of ovulation (SD=19.6, Table 5.1).

Table 5.1. Intervals from selected thresholds of milk and plasma progesterone concentrations until time of ovulation

	ng/ml	mean±SD (h)	range (h)
Milk progesterone	<15	97.7±17.8	54-126
	<5	79.7±11.2	54-98
	<2	70.7±16.8	38-90
Plasma progesterone	<4	90.5±19.6	66-138
	<2	75.0±12.2	50-98

Figure 5.2 shows the distribution of the interval between declining progesterone concentrations in milk (Fig. 5.2A) or plasma (Fig. 5.2B) and ovulation. For example, in 70% of the cases, ovulation occurred between 96 and 72h after milk progesterone concentrations were <5ng/ml. Milk and plasma concentrations showed an overall correlation of r=0.43 (P<0.05). This correlation was better when progesterone concentration was below the threshold limits (plasma progesterone <4ng/ml: r=0.62, P<0.05; milk progesterone <15ng/ml: r=0.52, P<0.05).

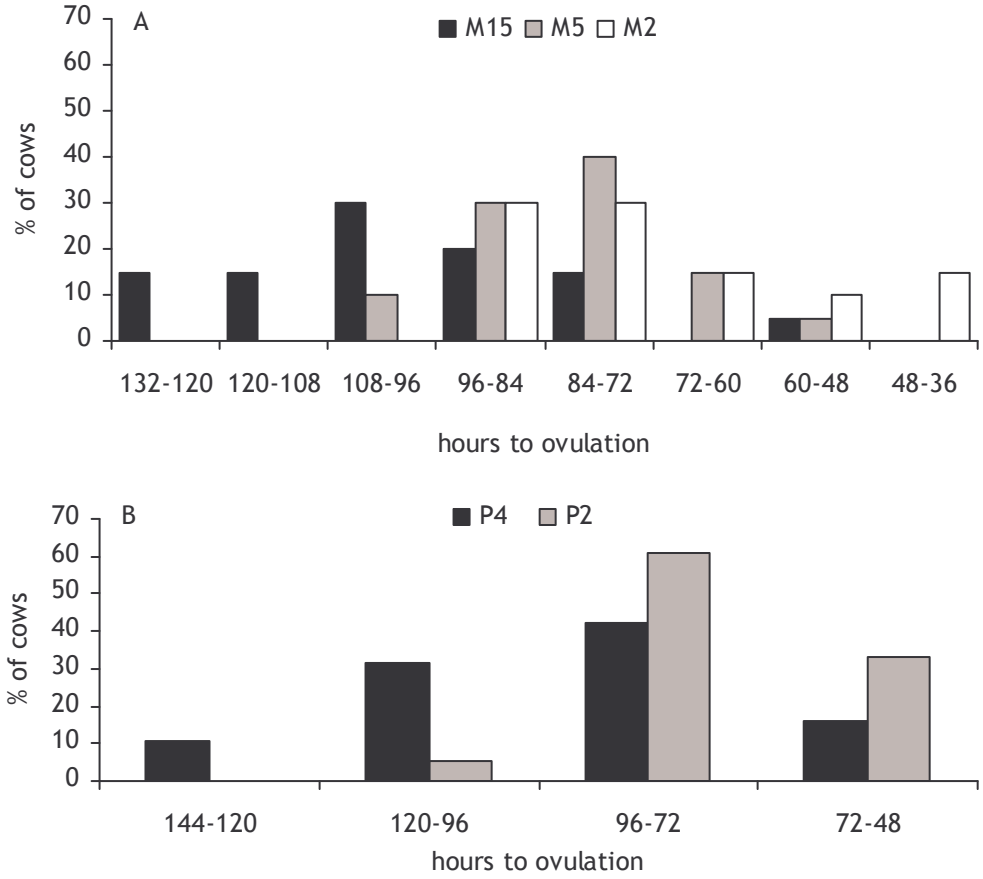

Figure 5.2. Distribution of progesterone concentration in milk (A, n=20) and plasma (B, n=18) at selected thresholds (M15=milk progesterone <15ng/ml, M5=milk progesterone <5ng/ml, M2=milk progesterone <2ng/ml, P4=plasma progesterone <4ng/ml and P2=plasma progesterone <2ng/ml) and time of ovulation. Milk was sampled at 12h intervals and plasma at 24h intervals.

Discussion

Close correlations between milk and plasma progesterone concentrations are found in most studies (Dobson & Fitzpatrick 1976 (r=0.88); Meisterling & Dailey 1987 (r=0.95)), justifying the use of milk progesterone values to monitor endocrine changes (Dobson & Fitzpatrick 1976) For a parameter to be useful as predictor for ovulation, several prerequisites are needed (e.g. sensitivity, automation, high accuracy etc.). Perhaps most importantly, a predictor for ovulation should be precise. Earlier studies (Trimberger 1948; Hall et al. 1959) found that insemination

should take place in a 12h window (between 6 and 18h before ovulation) to achieve high fertilization rates. Based on these intervals, an 'ovulation predictor' should have an accuracy of 12h. In the present study, a large variation in intervals from decline in milk or plasma progesterone concentrations to ovulation was found (standard deviations ranging from 11.2 to 19.6h). These intervals appeared not to be influenced by cow factors as parity, milk production or days in milk. A possible (physiological) explanation for the large variation in the interval between progesterone decline and ovulation comes from the work of Sirois and Fortune (1988). They found a shorter interval between the decline to basal progesterone concentrations in plasma (<1ng/ml) and the LH peak when a larger dominant follicle was present at the time of basal progesterone concentrations. Since the interval between LH peak and ovulation is quite constant (e.g. Roelofs et al., 2004), the size of the dominant follicle during luteolysis would also affect ovulation time and represent a source of natural variation in follicular phase duration. The large variability in ovulation time relative to the process of luteolysis also means that using the actual onset or end of progesterone decline instead of a quantitative threshold would most likely not improve prediction of ovulation.
The large variation in timing of progesterone decline to ovulation makes monitoring of progesterone concentrations unsuitable for prediction of ovulation time. However, monitoring of progesterone decline could be useful to increase sensitivity of other predictors of ovulation time. If, for example, the number of paces or mounting activity indicates oestrus/ovulation, while progesterone concentrations are above a certain threshold (e.g. 5ng/ml in milk), it is safe to assume that ovulation will not occur and animals should not be inseminated. This use of progesterone monitoring will be more feasible when concentrations can be measured on-line during each milking (Mottram et al. 2001). Currently, good ovulation time predictors in cattle are not yet available, although the number of paces taken by a cow during oestrus seems promising (Roelofs et al. 2005b).
In conclusion, monitoring of progesterone alone is not sufficient to predict ovulation time in ordinary dairy practice because of the high variation in profiles between animals. However, if progesterone concentrations can be assessed automatically by on-line measurements, it could be a helpful tool to assist in predicting time of ovulation by means of other parameters.

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6

Effect of time of insemination relative to ovulation on fertilization rates and embryo characteristics in spontaneous ovulating dairy cattle

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Submitted

Abstract

The objective of this study was to examine effects of the interval between insemination and ovulation on fertilization and embryo characteristics (quality scored as good, fair, poor and degenerate; morphology; number of cell cycles and accessory sperm number) in spontaneously ovulating dairy cattle. Time of ovulation was assessed by ultrasonography. Cows were inseminated once between 36h before ovulation and 12h after ovulation. In total 122 ova/embryos were recovered seven days after ovulation. Insemination-ovulation interval (12h intervals) had a significant effect on fertilization and the percentage of viable and good embryos. Fertilization rates were significantly higher when insemination was performed between 36 to 24h and 24 to 12h before ovulation (85.2 and 82.4%) when compared with insemination after ovulation (56.3% fertilization). Cows inseminated between 24 and 12h before ovulation yielded highest percentages of viable and good embryos (76.5% and 67.7%, respectively) when compared with insemination after ovulation (31.3% and 6.3%, respectively).
Insemination-ovulation interval had no effect on number of accessory sperm cells and number of cell cycles when corrected for embryo quality. This study showed that the insemination-ovulation interval in which fertilization can occur is quite long (from 36 to 12h before insemination), while the interval in which the fertilized ovum will develop into a good embryo is considerably shorter (only from 24 to 12h before insemination). Aging of spermatozoa resulting in DNA damage in the spermatozoon or aging of the ovum resulting in instability in the nuclear and cytoplasmic organelles might explain these negative effects on embryo development.

Introduction

The optimal time at which insemination should take place relative to ovulation (insemination-ovulation interval) depends mainly on the lifespan of fertile spermatozoa and on the viable lifespan of the ovum in the female genital tract. Despite the extensive use of artificial insemination (AI) in dairy cows, only limited data are available on the optimal time of insemination relative to ovulation. An estimate of the optimal time for AI relative to ovulation, obtained more than half a century ago, is 6-24h before ovulation (Trimberger 1948). In that study, time of ovulation was assessed by rectal palpation of the ovaries every 2h. More recent studies have focussed on the effect of different intervals between AI and the onset of oestrus, without assessing exact time of ovulation, although ovulation is quite variable relative to oestrous signs (Roelofs et al. 2005c). An optimal time of AI of 6 to 17h after an increase in walking activity (Maatje et al. 1997) or 4 to 12h following the first standing event associated with the onset of oestrus (Dransfield

et al. 1998) was found. In these studies conception was determined by means of pregnancy diagnosis, so no distinction could be made between fertilization failure and (early) embryonic death.
Data obtained from embryos/ ova recovered 6-7 days after a synchronised oestrus, suggested that AI at 12h after the onset of oestrus provided a compromise between a lower fertilization rate (found with AI at the onset of oestrus) and lower embryo quality (found with AI at 24h after onset of oestrus), but again, time of ovulation was not assessed in that study (Dalton et al. 2001).
No other information about the effects of insemination-ovulation interval is currently available. Therefore, the objective of the present experiment was to determine effects of time of AI relative to ovulation (assessed by ultrasonography) on fertilization rate, embryo quality, and accessory sperm number at Day 7 after ovulation in spontaneously ovulating dairy cattle.

Materials and Methods

The experiment was conducted at the experimental dairy farm of Wageningen University and Research Centre, the Netherlands. The Ethical Committee for Experimentation with Animals (Wageningen, the Netherlands) approved the experimental protocol.

Animals, feed and housing
Data were collected from a herd of about 80 lactating Holstein-Friesian cows over a period of 18 months. Parity of the animals varied between one and six and the animals were 77.0±20.1 (mean±SD) days in lactation at the time of observed ovulation, with a range of 43 to 131 days. The cows were fed an ad lib mixture of grass silage, corn silage and mineral supplements, and concentrates according to production level (Central Animal Feed Bureau-standards, 2000). They were housed in free stalls with a concrete, slatted floor. The cows were milked by an automated milking system (Liberty, Prolion, Vijfhuizen, The Netherlands). Estimated 305-day milk production was 7949±1178 (mean±SD) kg.

Insemination
Frozen-thawed semen doses ($15*10^6$ sperm cells) of two known bulls with proven comparable fertility, derived from 11 different ejaculates per bull, were used. Cows were inseminated at different times after onset of oestrus (assessed by pedometers (Roelofs et al. 2005b) or visual observation). Time of insemination was chosen in such a way that they were estimated to be performed from 36h before ovulation to 12h after ovulation (Roelofs et al. 2005b). The aim was to obtain 25 embryos in each interval of 12h (36 to 24h, 24 to 12h and 12 to 0h before ovulation and 0 to 12h after ovulation).

Some cows were used more than once in consecutive cycles. Cows that were used repeatedly were inseminated with different ejaculates from the same bull, and at different times relative to ovulation.
All artificial inseminations were performed by the same person. For every insemination the following parameters were recorded (modified after Loeffler et al. 1999): cow number, date, time, bull, charge number of the ejaculate, ease of cervical passage (difficult, moderately easy, easy), presence of uterus tone (present, absent) and presence of clear mucus at insemination (present, absent).

Detection of ovulation
The ovaries of the cows were examined rectally using a scanner with a 7.5 MHz sector transducer (Tringa 50S, Pie Medical, Maastricht, The Netherlands). The reproductive tract was not manipulated or palpated before or during the examinations, that started approximately 18h after onset of oestrus. During the first examination, both ovaries were scanned to determine where the preovulatory follicle was located. Thereafter, this ovary was scanned every 4h until the disappearance of the follicle, marking ovulation time. Each examination the diameter of the preovulatory follicle was measured. Time of ovulation was defined as the first examination the preovulatory follicle had disappeared minus 2h. About seven days after oestrus, an ultrasound examination was performed to determine if a corpus luteum had appeared, confirming ovulation.

Embryo recovery
About seven days (between 5.1 and 8.8 days) after ovulation, the ipsilateral uterus horn was flushed with sterile filtered PBS-ET (Bio Whittaker Europe, Verviers, Belgium), using the standard non-surgical method (Elsden et al. 1976). Embryo age was calculated in the following way: when the insemination was performed 8h or earlier before ovulation then embryo age (in h) was the hours between ovulation and the flushing procedure; when insemination was performed within 8h before, or after ovulation then embryo age (in h) was the hours between ovulation and the flushing procedure minus (8 hours+the insemination-ovulation interval). The 8h is the average time it takes for inseminated sperm to able to fertilize the ovum (Hunter & Wilmut 1984).
The initial search and evaluation of embryos and ova was performed with a stereomicroscope at magnifications of 10x to 64x. Morphology of the embryos was scored based on developmental stage (Lindner & Wright 1983). Quality of the embryos was scored based on the scoring system published by the International Embryo Transfer Society (Robertson & Nelson 1998). It consists of four quality codes: good quality embryos, fair quality embryos, poor quality embryos and dead or degenerating embryos. An unfertilized ovum was designated when there was no indication of cleavage. The same person classified all the embryos on morphology and quality. Embryos and ova were then subjected to hypotonic treatment (0.6%

w/v KCl, 0ºC, 15 min.) and subsequently placed on a fat-free glass slide. Small droplets of methanol/acetic acid (3/1 v/v) were added until disruption and spreading of the embryo. After drying and staining with 10% Giemsa in PBS, the nuclei (cells) and accessory sperm were counted using a microscope (magnification 200x) (Soede et al. 1995). Classification of unfertilized ova was confirmed if nuclei count was zero or one. The number of cell cycles was calculated as follows: number cell cycles = 2log(x), where x = the number of nuclei.

Additional measured parameters

Cycle number

Ultrasound examinations were performed weekly in all cows starting 14 days after parturition until the appearance of a corpus luteum. The cycle number was calculated as the number of days in lactation at the moment of ovulation minus the number of days in lactation at the moment of the appearance of the first corpus luteum divided by 21 days.

Body condition score

The body condition of all cows was scored every month by the same person using a 5-point system (Braun et al. 1986), where score 1 is an emaciated cow and score 5 is an extremely fat cow. This system is based on visual appearance of the cow and handling the backbone, loin and rump areas.

Duration of oestrus/increase in walking activity

Duration of oestrus and increase in walking activity was calculated by the increase in number of steps measured by pedometers. For details, see Roelofs et al.(2005b)

Progesterone concentration

Progesterone concentrations were assessed in blood plasma, sampled at the day of embryo recovery, by means of ELISA. For details, see Roelofs et al. (2005a).

Statistics

The following outcome variables were compared regarding exposure to a number of factors (see Table 6.1): ‘fertilization’, consisting of two classes (fertilized and unfertilized), ‘embryo quality’, consisting of three classes (good; fair and poor; degenerate), ‘viable embryos’, consisting of two classes (good, fair and poor embryos versus degenerate and unfertilized embryos) and ‘good embryos’, consisting of two classes (good embryos versus fair, poor, degenerate and unfertilized embryos).

Logistic regression was performed on these four outcome variables (fertilization, quality, viable embryos and good embryos) according to the method of Hosmer & Lemeshow (1989). First, all variables were subjected to univariate analysis (PROC LOGISTIC, SAS system for Windows V8, 1999). All variables with a P-value<0.25 based on -2 log likelihood (-2LL) entered multivariate analysis.

Table 6.1. Variables used in analyses and the number of recovered ova/embryos for each class

Variables	Class	Number	Variables	Class	Number
Parity[abc]	first	36	Clear mucus	present	23
	second	37	at insemination[abc]	absent	93
	third or more	49	Insemination-ovulation	-36h<IOI≤-24h	27
Days in lactation[a]	continuous	122	interval (IOI)[abc]	-24h<IOI≤-12h	34
Cycle number[1abc]	first+second	37		-12h<IOI≤0h	29
	third	39		0h<IOI≤+12h	32
	fourth	28	Flushing number[abc]	first	64
	fifth and more	17		second or more	58
Body condition[2abc]	<2.8	36	P_4-concentration at flushing (ng/ml)[4abc]	continuous	69
	2.8-3.3	50			
	>3.3	36	Embryo age (in h)[5bc]	continuous	89
Duration of oestrus[3abc]	0 h	16	Fertilization[c]	unfertilized	33
	≤10h	41		fertilized	89
	10-12h	27	Morphology	2 to 16-cell	14
	>12h	24		morula	48
Increase in walking activity[3abc]	<400%	24		blastocyst	25
	400-500%	29	Quality[bc]	good	48
	501-600%	18		fair	13
	>600%	18		poor	9
Size preovulatory follicle (mm)	continuous	121		degenerate	19
			Viable embryos[6]	yes	70
Bull[abc]	Bull A	63		no	52
	Bull B	59	Good embryos[7]	yes	48
Ease of cervical passage[abc]	easy	61		no	74
	normal	50	Number of cell cycles[8]	continuous	118
	difficult	11	Accessory sperm[b]	continuous	117
Uterus tone[abc]	present	110			
	absent	12			

[1]number of ovulations after calving assessed by weekly ultrasonography starting 14 days after calving until appearance of the first corpus luteum; [2]based on a scale of 1 to 5, where 1 is extremely skinny and 5 is extremely fat; [3]based on pedometer measurements (Roelofs et al., 2005b); [4]Progesterone concentrations measured in blood plasma with an ELISA (Ridgeway Science, Gloucestershire, United kingdom); [5]When IOI is -8 h or larger than embryo age (in h) is the hours between ovulation and the flushing procedure, when IOI is smaller than -8h than embryo age (in h) is the hours between ovulation and the flushing procedure minus (8h + IOI). The 8 h is the average time it takes for inseminated sperm to able to fertilize the ovum (Hunter & Wilmut, 1983); [6]yes=embryos of good to poor quality; no=unfertilized ova + degenerate embryos; [7]yes= embryos of good quality; no=embryos of fair to poor quality + degenerate embryos + unfertilized ova; [8]number of cell cycles= 2log(number of nuclei)

[a]variables included in initial univariate models for the outcome variables fertilization, quality, viable embryos and good embryos

[b]variables included in initial univariate models for the outcome variable number of cell cycles

[c]variables included in initial univariate models for the outcome variable log-transformed accessory sperm

Monitoring the change in -2LL of the model, variables were excluded one-by-one from the multivariate analysis by descending P-value, until all variables had a P<0.10. Within this backward-elimination procedure, exclusion of variables was checked for confounding by monitoring the change in regression parameters. Confounding was considered to be present if difference in parameter estimates exceeded 25% or exceeded 0.1 if the parameter estimate was between -0.4 and 0.4. Two-way interaction terms could not statistically be tested due to the small sample size. For the final model the Hosmer-Lemeshow goodness-of-fit statistic was computed (Hosmer & Lemeshow 1989).
The strength of association between a factor and the outcome variable is presented in terms of odds ratios (OR). An OR=1 denotes that there is no association between a factor and the outcome variable; if OR>1 then exposure might be a risk factor; if OR<1 then exposure might be a preventive factor (Noordhuizen et al. 2001).
Accessory sperm data were first transformed by the equation (10log(x+1)), in which x is the accessory sperm number. This step was performed to normalize the large and highly skewed variance in accessory sperm numbers (DeJarnette et al. 1992). Transformed accessory sperm data and the number of cell cycles were analyzed for differences related to the variables presented in Table 6.1. First univariate analyses were performed using the GLM-procedure (The SAS-System for Windows V8, 1998). All variables with a P-value<0.25 entered multivariate analysis. Variables were excluded one-by-one from the multivariate analysis by descending P-value, until all variables had a P<0.10.

Results

Effects on fertilization rate and percentage of viable and good embryos
In total, 122 embryos/ova were recovered from 187 flushing-procedures. Thirty-two cows were used once and 35 cows were used more than once for embryo recovery. Of the 122 embryos/ova, 33 were unfertilized (27%), 16% were degenerate embryos, 7% were poor embryos, 11% were fair embryos and 39% were good embryos. The smallest interval in which the results can be presented is 4h, because time of ovulation was assessed with an accuracy of 4h. Therefore, the distribution of unfertilized ova, degenerate, poor, fair and good embryos in 4h intervals is presented in Figure 6.1. The highest percentage of good embryos (89%) was recovered when insemination was performed between 16 and 12h before ovulation. Earlier inseminations and later inseminations up to 4h before ovulation resulted in 33 to 64% good embryos, whereas inseminations performed later than 4h after ovulation did not result in good embryos at all.
Table 6.2 shows the variables which were included in the final logistic model for fertilization. Insemination-ovulation interval (IOI) and the presence/absence of clear mucus at the time of insemination were the only two factors that had a

significant effect on fertilization rate. Fertilization rates were significantly higher when AI was performed between 36-24h and 24-12h before ovulation (85.2 and 82.4%, respectively) when compared to AI after ovulation (56.3% fertilization). In 20% of the inseminations, clear mucus was present at the time of AI.

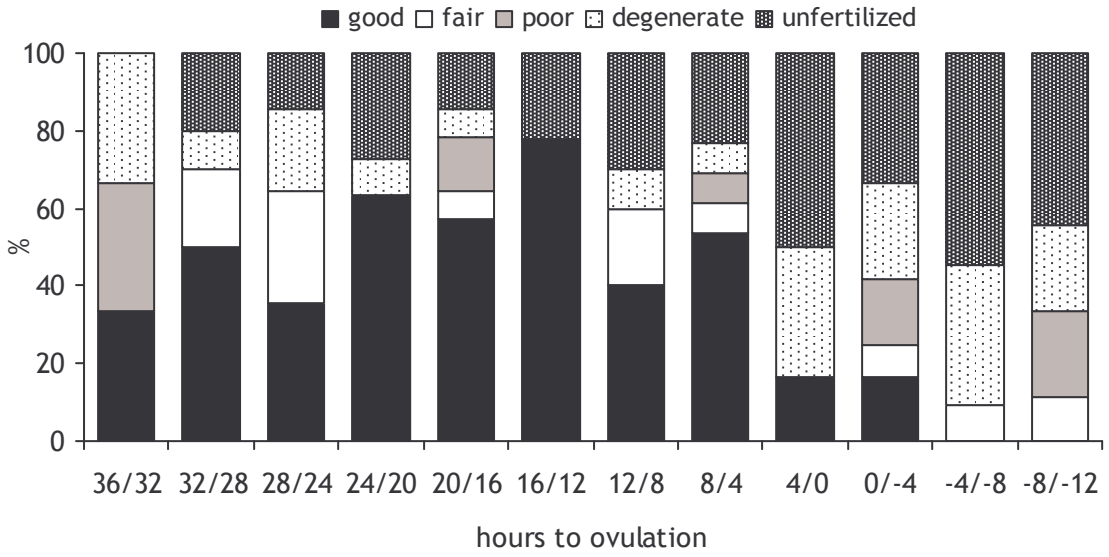

Figure 6.1. Cumulative percentages of unfertilized ova and different quality embryos for different insemination-ovulation intervals.

When clear mucus was present, significantly more inseminations resulted in fertilized ova (96%) compared to 67% fertilized ova when clear mucus was absent (Table 6.2). The presence or absence of clear mucus seemed not related with time of insemination.

Table 6.2. Fertilization rates with odds ratios (ORs) and 95%-CI for variables included in the final logistic model[1]

		Frequency		Prevalence			
Variable	Category	n	%	fertilization	OR	95% CI	Pwald
IOI	-36h<IOI≤-24h	27	22	85.2	4.7	1.3-18.0	0.02
	-24h<IOI≤-12h	34	28	82.4	4.0	1.2-13.0	0.02
	-12h<IOI≤0h	29	24	69.0	2.4	0.8-7.6	0.13
	0h<IOI≤+12h	32	26	56.3	1		
Clear mucus	absent	93	80	66.7	1		
	present	23	20	95.7	9.7	1.2-78.1	0.03

[1]The Hosmer-Lemeshow goodness-of-fit statistic was 0.43 (P=0.98; d.f.=4). There was no evidence of lack of fit in this model.

Percentage of viable embryos (i.e. embryos of good to poor quality) was influenced by insemination to ovulation interval and the presence of clear mucus at the time of insemination (Table 6.3). Overall percentage of viable embryos was 57.4%. When cows were inseminated between 24 and 12h before ovulation highest percentage of

viable embryos were found (76.5%) in comparison to insemination after ovulation where the percentage of viable embryos decreased dramatically (31.3%, see also Figure 6.1). Presence of clear mucus at the time of ovulation resulted in a higher percentage of viable embryos (78.3%) compared to absence of mucus (51.6%, Table 6.3).

Table 6.3. Viable embryo rate with odds ratios (ORs) and 95%-CI for variables included in the final logistic model[1]

		Frequency		Prevalence			
Variable	Category	n	%	viable embryo	OR	95% CI	Pwald
IOI	-36h<IOI≤-24h	27	22	66.7	4.8	1.5-15.2	0.009
	-24h<IOI≤-12h	34	28	76.5	8.1	2.6-25.8	0.0004
	-12h<IOI≤0h	29	24	55.2	3.4	1.1-10.7	0.04
	0h<IOI≤+12h	32	26	31.3	1		
Clear mucus	absent	93	80	51.6	1		
	present	23	20	78.3	3.0	0.9-9.5	0.06

[1]The Hosmer-Lemeshow goodness-of-fit statistic was 1.73 (P=0.88; d.f. = 5). There was no evidence of lack of fit in this model.

Percentage of good embryos was influenced by insemination to ovulation interval and the presence of clear mucus at the time of insemination (Table 6.4). Overall percentage of good embryos was 39.3%. When cows were inseminated between 24 and 12h before ovulation higher percentages of good embryos were found (67.7%) compared to earlier (40.7%, P<0.05) and later (41.4%, P<0.1) inseminations. Insemination after ovulation decreased the percentage of good embryos dramatically (6.3%, see also Figure 6.1). Presence of clear mucus at the time of ovulation resulted in a higher percentage of good embryos (73.9%) compared to absence of mucus (44.1%, Table 6.4).

Table 6.4. Good embryo rate with odds ratios (ORs) and 95%-CI for variables included in the final logistic model[1]

		Frequency		Prevalence			
Variable	Category	n	%	good embryo	OR	95% CI	Pwald
IOI	-36h<IOI≤-24h	27	22	40.7	8.3	1.6-43.1	0.01
	-24h<IOI≤-12h	34	28	67.7	27.5	5.4-139.7	<.0001
	-12h<IOI≤0h	29	24	41.4	11.3	2.1-59.2	0.004
	0h<IOI≤+12h	32	26	6.3	1		
Clear mucus	absent	93	80	44.1	1		
	present	23	20	73.9	2.8	0.9-8.5	0.06

[1]The Hosmer-Lemeshow goodness-of-fit statistic was 0.90 (P=0.97; d.f. = 5). There was no evidence of lack of fit in this model.

Embryo characteristics

Development of the embryos in terms of number of cell cycles was on average 5.6±1.7 (range: 1.0-7.8). Number of cell cycles was not different for the various insemination-ovulation intervals when quality of the embryo was included in the analysis (Table 6.5). Degenerate embryos had fewer cell cycles compared to good, fair and poor embryos, independent of insemination-ovulation interval (Figure 6.2 and Table 6.6).

Table 6.5. Number of cell cycles and accessory sperm number per embryo for various insemination-ovulation intervals

		Cell cycles	Accessory sperm	
IOI	N	Lsmeans[1]±se	mean±se	median
-36h<IOI≤-24h	23	5.1±0.2	14.1±7.2	4.0
-24h<IOI≤-12h	25	5.4±0.2	23.2±6.3	15.0
-12h<IOI≤0h	20	5.1±0.2	21.3±7.4	9.5
0h<IOI≤+12h	16	5.6±0.2	44.3±21.8	8.5

[1] Mean corrected for Quality (P<0.05)

Table 6.6. Accessory sperm number for different quality embryos and unfertilized ova presented as the mean and median values

fertilization or		cell cycles	accessory sperm number[1]			embryo/ova with
embryo quality	n	mean±SD	mean±SD	median	range	accessory sperm (%)
good	46	6.5±0.6[a]	21.3±31.8[a]	10	0-129	80
fair	12	6.1±0.5[a]	22.5±46.9[a]	3.5	0-168	83
poor	8	5.7±0.7[a]	17.5±29.7[a]	9.5	0-89	75
degenerate	19	3.0±1.6[b]	35.3±80.4[a]	6	0-320	79
unfertilized	33	-	7.9±43.7[b]	0	0-251	6

[1] Statistics for accessory sperm number based on 10log-transformed values

[a,b] Different superscripts within a column are significantly different (P<0.05)

Developmental stage of the embryos was related to quality of the embryo and the number of cell cycles; embryos that were not developed up to a morula (n=14) were all degenerate and of the 25 embryos that were blastocysts, 21 were good and only two were of fair quality, one was of poor quality and one was degenerate. Of the embryos that were morulae (n=48), 52% were of good quality, 23% of fair quality, 15% of poor quality and 10% was degenerate. In morulae, the number of cell cycles was higher for good quality embryos (6.3±0.5, n=25), compared to degenerate embryos (5.2±0.9, n=5, P<0.05). The number of cell cycles of fair (6.0±0.4, n=10) and poor (5.7±0.7, n=6) morulae was intermediate (P>0.1).

The average number of accessory sperm found in the zona pellucida of embryos and ova was 19.7±47.5 (range: 0-320, median: 3.0). Only two of the 33 unfertilized

ova had accessory sperm (9 and 251 sperm cells, Table 6.6, Figure 6.3). Thus a lower number of accessory sperm was seen in unfertilized ova compared to embryos ($P<0.05$, Table 6.6). The number of accessory sperm of embryos did not differ between the different insemination-ovulation intervals ($P>0.1$, Table 6.5). Quality of the embryos was not related to accessory sperm number ($P>0.1$, Figure 6.3 and Table 6.6).

Embryos/ova recovered from cows inseminated with bull A tended to have more accessory sperm (29.7±62.6, median=7.0, n=61) compared to bull B (8.8±15.8, median=1.5, n=56, $P<0.1$).

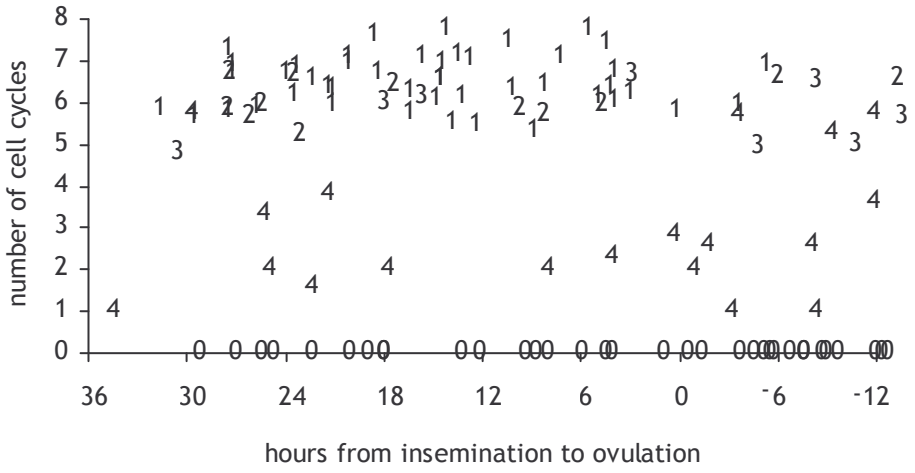

Figure 6.2. Relationship between insemination time relative to ovulation and the number of cell cycles, shown for the different embryo quality classes (0=unfertilized, 1=good, 2=fair, 3=poor, 4=degenerate)

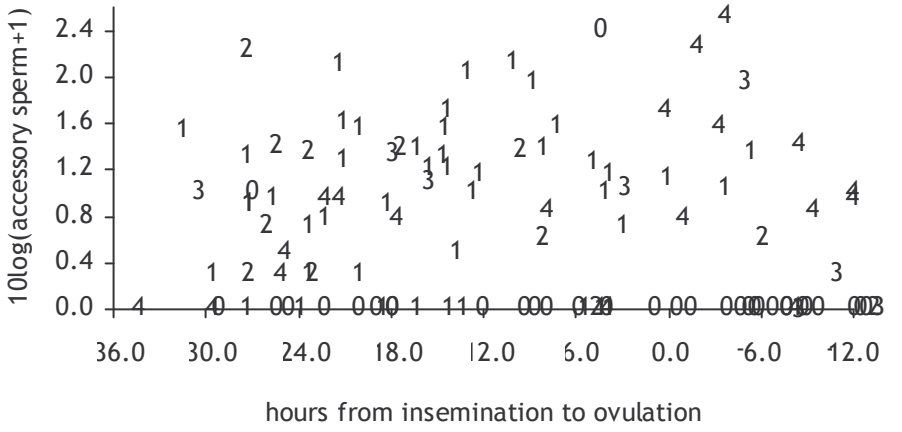

Figure 6.3. Relationship between insemination time relative to ovulation and the number of of accessory sperm cells (10log-transformed), shown for the different embryo quality classes (0=unfertilized, 1=good, 2=fair, 3=poor, 4=degenerate)

Discussion

The optimal time at which AI should take place relative to ovulation depends mainly on the lifespan of spermatozoa to ensure fertilization and on the viable lifespan of the ovum. When AI is performed early, spermatozoa have to reside a prolonged period of time in the female reproductive tract. The question is if this prolonged period has a negative effect on the fertilizing ability of the spermatozoa. Studies with polytocous animals, like rabbits (Tesh 1969) and pigs (Soede et al. 1995), have found a reduction in the percentage of fertilized ova when AI was performed early (more than 29 and 32h before ovulation, respectively). In cattle, most studies have looked at conception rates (mostly around 35 days after insemination) and the effects of early AI on conception rates are ambivalent. Some studies did not find effects on conception rate with early AI (Gwazdauskas et al. 1986, Maatje et al. 1997), whilst other studies found lower conception rates (Trimberger & Davis 1943, Pursley et al. 1997, Dransfield et al. 1998). In those studies, it is not known if the lower conception rates are caused by fertilization failure, or are caused by (early) embryonic death. Our results suggest that the fertilizing ability of spermatozoa is not diminished when AI is performed up to 36h before ovulation, as fertilization rates did not differ when AI was performed between 36 and 24h before ovulation compared to AI closer to ovulation (Table 6.2). This agrees with the findings of Dalton et al. (2001). They found no significant difference in fertilization rates in non-lactating cattle when AI was performed 2h after the onset of oestrus (66%) compared to AI performed 12 or 24h after the onset of oestrus (74 and 82%, respectively).

In pigs, the decreased fertilization was attributed to the limited number of fertile sperm cells present at the site of fertilization as indicated by the low numbers of accessory sperm cells in the embryos (Hancock & Hovell 1962, Soede et al. 1995). In cattle, the number of accessory sperm was not affected when AI was performed early compared to AI closer to ovulation (our study, Dalton et al. 2001). This suggests that not only the ability to fertilize, but also the number of spermatozoa able to penetrate the ovum is not affected when AI is performed early in cattle.

The combination of unaffected fertilization rates (our study, Dalton et al. 2001) but lowered conception rates (Trimberger & Davis 1943, Pursley et al. 1997, Dransfield et al. 1998) with early AI implies that the development of the fertilized ovum is impaired with early AI. In rabbits, aging of spermatozoa reduced not only fertilization but also cleavage rates and the proportion of embryos that developed into blastocysts (Maurer et al. 1969). The results of our study show a slight decrease in the percentage of viable embryos (Table 6.3) and a marked decrease in the percentage of good embryos (Table 6.4) with AI performed 36 to 24h compared to 24 to 12h before ovulation. This would mean that early AI does not affect motility of the spermatozoa and the ability to capacitate, bind to the zona

pellucida, penetrate and activate the ovum, but that early AI does affect the ability of the fertilizing spermatozoon to result in normal development of the fertilized ovum.
An explanation for the altered development of the fertilized ovum could be that aging of the spermatozoa leads to DNA damage of the spermatozoa. An experiment in which sperm DNA was damaged artificially using irradiation demonstrated that sperm with damaged DNA did not affect fertilization *in vitro*, but did affect the development of the embryo (Fatehi et al. 2004). The mechanisms involved in effects of sperm aging *in vivo* are not known.
Surprisingly, Dalton et al. (2001) found a higher percentage of good embryos when AI was performed 2h after the onset of oestrus compared to later. The authors suggested that the duration of sperm residence in the female reproductive tract might allow further selection pressure favouring competent sperm, thus optimizing embryo quality at that early insemination. However, no other results support these suggestions. Dalton et al. (2001) inseminated about 2h after the onset of oestrus based on mounting detectors. This would correspond with about 26h before ovulation according to the study of Walker et al. (1996), who found an interval between first mount and time of ovulation of 28h. In our study, we found a decline in percentage of good embryos with AI more than 24 hours before ovulation. Maybe Dalton et al. (2001) did not inseminate early enough to find negative effects on embryo quality. More research is required to assess which mechanisms are involved in sperm aging and how these affect embryonic development.
When AI is performed after ovulation, there is a chance that fertilization or development of the embryos is compromised because of aging of the ovum. Numerous researches found lower conception rates when AI was performed a long time after the onset of oestrus or, in other words, around the time of ovulation (Trimberger 1943, Maatje et al. 1997, Dransfield et al. 1998, Martinez et al. 2004). Based on experiments with various species, the problems with aging of the ovum seem mainly due to instability in the nuclear and cytoplasmic organelles (reviewed by Hunter, 1985). Fertilization failure could occur because of a loss of chromosomes from the metaphase plate. Even if the ovum is penetrated, normal fertilization (activation of the ovum) or formation of a viable zygote cannot ensue (Hunter, 1985). AI performed after ovulation resulted in decreased fertilization rates in our study (Table 6.2). To our knowledge, no other information about fertilization failure in aging cattle ova is available. Chian et al. (1992) found that aging of the ovum *in vitro* resulted in lower fertilization rates; 25% of 2- to 4 cell embryos were found when ova were cultured for 44 to 48h before fertilization compared to 57-44% ($P<0.05$) in ova that were cultured for 20 to 40h before fertilization. Although *in vitro* aging of the ovum is not the same as *in vivo* aging, the processes affected might be similar.
Our results show not only low fertilization rates, but also low numbers of viable (Table 6.3) and good (Table 6.4) embryos when AI was performed after ovulation.

An explanation for these findings could be the increased chance of polyspermy when aged ova are fertilized (Hunter, 1985). On one hand, it is possible that the zona block is not instigated after fertilization of an aged ovum, which increases the chance on multiple fertilizations. On the other hand, sperm transport could be altered when AI is performed after ovulation, resulting in more sperm transported to the site of fertilization, increasing the risk of polyspermy (Hunter & Greve 1997). Polyspermy might result in abnormal fertilization and inhibition of the zygote to develop to a blastocyst as found after *in vitro* fertilization (Chian et al. 1992, Long et al. 1994). From our data, with assessments at Day 7 after ovulation, it is not possible to know for sure if polyspermy has occurred. If AI after ovulation results in more sperm at the site of insemination, more accessory sperm would be expected, because accessory sperm are thought to represent the number of sperm competing for fertilization during the time the ovum is receptive (Saacke et al. 2000). Degenerate embryos resulting from AI after ovulation tended to have more accessory sperm (73.1±116.2, n=8) compared to good, fair and poor embryos (15.5±30.6, n=8, P=0.1) in the same interval. So, a question that cannot be answered in our study is whether the degenerate embryos in our study were indeed polyspermic. Further, in the study of Dalton et al. (2001) a higher number of accessory sperm was found when AI was performed 24h after the onset of oestrus (median: 4) compared to AI 2h after the onset of oestrus (median: 1). The percentage of viable embryos in their study was also lower with late AI compared to early AI (66% and 92%, respectively). The higher number of accessory sperm might indicate a higher chance of polyspermy, although it is not known whether a relationship exists between the number of accessory sperm and the chance of polyspermy in aging ova.

Not only polyspermy causes abnormal fertilization in aged ova. Chian et al. (1992) concluded in their study that aged ova after *in vitro* fertilization have a lower ability to cleave, even though normal monospermic fertilization may occur. Which mechanisms are responsible for this diminished ability to cleave *in vitro* is not known, but it is feasible that it also occurs *in vivo*.

Because it takes about 8h after AI before spermatozoa are capacitated and capable of fertilization (Hunter & Wilmut 1983), it is feasible that fertilization and embryonic development are already impaired when AI is performed shortly before ovulation. Our results suggest that fertilization rate and percentage of viable embryos already decrease when AI is performed less than 4h before ovulation (Figure 6.1) and the percentage of good embryos seems already lowered when AI is performed less than 12h before ovulation (Figure 6.1). This corresponds with the results of studies where low conception rates were found when AI was performed 16 or 24h after the onset of oestrus based on mounting detectors and pedometers (Maatje et al. 1997, Dransfield et al. 1998), which corresponds with AI around 12 and 6h before ovulation (based on Walker et al. 1996 and Roelofs et al. 2005b, respectively). Dalton et al. (2001) did not find a lower fertilization rate when AI

was performed 24h after the onset of oestrus (corresponding with about 4h before ovulation, Walker et al. 1996), but did find a higher percentage of degenerate embryos. Our findings and the results of these other studies imply that the period in which fertilization can occur is longer than the period in which normal development of the embryo is possible, thus affecting embryo survival rate.

The presence of clear mucus at the time of AI was positively related to fertilization rate (Table 6.2), and the percentage of viable and good embryos (Table 6.3 and 6.4). This finding is in agreement with other studies, in which a higher conception rate was found when clear mucus was present during AI compared to when clear mucus was absent (Hahn 1969, Stevenson et al. 1983). Contrary to these findings, Mahmoudzadeh et al. (2001) did not find a lowered conception rate when clear mucus was absent during AI. The prevalence of presence of clear mucus was in our study not related with insemination-ovulation interval ($P>0.1$). It is not clear why the presence of clear mucus at the time of AI results in a higher percentage of fertilization, especially because the spermatozoa are deposited intrauterine by-passing the cervix with AI. An explanation could be that when clear mucus is present during insemination, this indicates a good uterine environment and an optimal hormonal status, possibly resulting in better fertilization rates, irrespective of time of insemination.

In conclusion, the results of this study show that the insemination-ovulation interval in which fertilization can occur is quite long, the interval in which the fertilized ovum develops into a good embryo is considerably shorter. Questions which mechanisms are responsible for diminished fertilization of aged ova and which mechanisms affect embryo development by aged ova or aged sperm remain an interesting area of research.

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7

Effect of time of artificial insemination on embryo sex ratio in dairy cattle

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Abstract

The objective of the present study was to examine whether different intervals between insemination and ovulation have an influence on the sex of seven-day-old embryos in dairy cattle. Cows were inseminated once with semen of one of two bulls of proven fertility between 36h before ovulation and 12h after ovulation. Time of ovulation was assessed by ultrasound at 4h intervals. In total, 64 embryos were determined to be male or female. Of these 64 embryos, 51.6% were female. The sex ratio in the various insemination-ovulation intervals (early: between 36 and 20h before ovulation; intermediate: between 20 and 8h before ovulation; late: between 8h before and 12h after ovulation) did not significantly differ from the expected 1:1 sex ratio (50%, 50% and 55% females, respectively). Bull (Bull A and B) and Parity (primiparous and multiparous) had no influence on the expected 1:1 sex ratio either. The number of cell cycles was similar for male and female ($P=0.23$) embryos when quality of the embryo ($P<0.0001$) was included in the model.
The results of this study indicate that, in cattle, the interval between insemination and ovulation does not influence the sex ratio of seven-day-old embryos.

Introduction

Numerous efforts have been made to alter the sex of calves by varying time of insemination (Pursley et al. 1998, Martinez et al. 2004). It has been suggested that early inseminations (i.e. far before ovulation) would result in more female calves whereas late inseminations (i.e. close to ovulation) would result in more male calves, due to different timing of capacitation and survival time of the X- and Y-chromosome bearing spermatozoa in the female reproductive tract (Martinez et al. 2004). However, several other studies offer contradicting explanations for potential effects of varying insemination time on sex ratio (Rorie et al. 1999). Previous studies have varied insemination time relative to visually observed oestrus (Ballinger 1970, Gebicke Harter et al. 1977, Martinez et al. 2004), using mounting behaviour (Rorie et al. 1999), and intravaginal conductivity (Wehner et al. 1997) all of which probably do not predict time of ovulation very accurately (Roelofs et al. 2003, Roelofs et al. 2005c). In those studies, sex ratio was assessed in the calves born. It is not possible to differentiate between differences in fertilization or (early) embryonic death by X- or Y-chromosome bearing spermatozoa by using calves born. If indeed timing of capacitation and hence survival time of the X- and Y-chromosome bearing spermatozoa is different, the interval between insemination and ovulation could alter the sex ratio. Therefore, to study effects of insemination time on embryo sex ratio, it would be better to inseminate according to actual time of ovulation.

The objective of the present study was to investigate whether different intervals between insemination and ovulation (assessed by using repeated ultrasound) have an effect on the sex of seven-day-old embryos in dairy cattle.

Materials and Methods

Animals, feed and housing

Data were collected from a herd of about 80 lactating Holstein-Friesian cows over a period of one and a half years. Parity of the animals varied between one and six and the animals were 76.4±19.8 (mean±SD) days in lactation, with a range of 43 to 120 days. The cows were fed an ad lib mixture of grass silage, corn silage and mineral supplements, and concentrates were given according to production level (Central Animal Feed Bureau-standards, 2000). The animals were housed in free stalls with a concrete slatted floor. The cows were milked by an automated milking system (Liberty, Prolion, Vijfhuizen, The Netherlands). 305-day milk production was 8024±1285 (mean±SD) kg.

Insemination

Frozen-thawed semen doses of two bulls with proven fertility, derived from 11 ejaculates per bull, were used. Cows were inseminated once at different times after onset of oestrus (assessed by pedometers (Roelofs et al. 2005b) or visual observation). Time of insemination was estimated to be from 36h before ovulation to 12h after ovulation. All artificial inseminations were performed by the same person.

Ultrasonography

The ovaries of the cows were examined rectally using a scanner with a 7.5 MHz sector transducer (Tringa 50S, Pie Medical, Maastricht, The Netherlands). The reproductive tract was not manipulated or palpated before or during the examinations, that started approximately 18h after onset of oestrus. During the first examination, both ovaries were scanned to determine where the preovulatory follicle was located. Thereafter, this ovary was scanned every 4h until the disappearance of the follicle, marking ovulation time. Time of ovulation was defined as the first time the follicle had disappeared minus 2.0h. About seven days after oestrus, an ultrasound examination was performed to confirm the presence of a corpus luteum and thereby ovulation.

Embryo recovery

About seven days after ovulation, the ipsi-lateral uterine horn was flushed with sterile filtered PBS-ET (Bio Whittaker Europe, Verviers, Belgium), using the standard non-surgical method (Elsden et al. 1976). The initial search and

evaluation of embryos and ova was performed with a stereomicroscope at magnifications of 10x to 64x.
Quality of all embryos was scored, by the same person, based on the scoring system published by the International Embryo Transfer Society (Robertson and Nelson, 1998). An ovum was described as unfertilized when there was no indication of cleavage. Embryos and ova were then spread by the method described by Soede et al. (1995) and the number of nuclei and accessory sperm were counted using a microscope (magnification 200x). The number of cell cycles was calculated as follows: number cell cycles = 2log(x), where x = the number of nuclei. After the nuclei and accessory sperm were counted, the slides were decoloured with ethanol and further processed for assessment of the sex of the embryo.

Assessment of sex of the embryo
For the X-chromosome, the BAC clone Texas 101 labelled with biotin-14-dUTP was used and for the Y-chromosome, the BAC clone Texas 28 labelled with digoxigenin-11-dUTP was used (Cai et al. 1995). Labelling was done by a standard Nick Translation reaction (Rigby et al. 1977). Fluorescence in situ hybridization (FISH) was performed as described by Viuff et al. (1999). An embryo was designated as female when two signals were visible, consistent with the X-chromosomes (n=33). An embryo was designated as male when only one X-chromosome signal was visible (n=13) or when one X-chromosome and one Y-chromosome signals were visible (n=18).

Statistics
Time of insemination relative to ovulation was divided into three insemination-ovulation intervals: early (insemination between 36 and 20 hours before ovulation, n=23), intermediate (insemination between 20 and 8 hours before ovulation, n=20) and late (insemination between 8 hours before and 12 hours after ovulation, n=22). Ratios of male/female embryos within each insemination time class (early, intermediate and late), embryo quality class (good, fair, poor and degenerate) and parity class (primiparous and multiparous) and for each bull (A and B) were compared to the expected sex ratio 1:1 using the Chi-square test (The SAS-System for Windows V8, 1998). The effect of sex (male or female) on the number of cell cycles was analyzed using the GLM procedure (The SAS-System for Windows V8, 1998), with the class-variable Quality (good, fair, poor and degenerate) included in the model.

Results

The sex of 65 of 78 embryos could be determined. One embryo was aneuploid (XXY) and therefore excluded from the analysis. The percentage of embryos in which the

sex could be determined was lower for degenerate embryos (53.9%) compared to good (90.9%), fair (83.3%) and poor (87.5%) embryos (P<0.05). Of the remaining 64 embryos, 48.4% were male and 51.6% were female. Table 7.1 shows the sex ratios of the embryos for various variables. The sex ratio for the early, intermediate and late insemination-ovulation intervals did not differ from the expected 1:1 ratio.
Degenerate embryos will not result in the birth of a calf. When the embryos classified as degenerate were excluded from the analysis, still no differences from the expected 1:1 sex ratio were found for the insemination-ovulation intervals (% male embryos for inseminations early: 52.4%; intermediate: 52.6% and late: 52.9%). Bull and Parity had no influence on the expected 1:1 sex ratio either (Table 7.1).
When sex ratio is analyzed in the different embryo quality categories, there appears to be a skewed sex ratio for the degenerate embryos. Of the seven embryos designated as degenerate, 85.7% are female (P=0.06, Table 7.1).
The number of cell cycles was similar for male (5.8±0.2 (s.e.)) and female (5.6±0.2 (s.e.), P=0.23) embryos when quality of the embryo (P<0.0001) was included in the model.

Table 7.1. Sex ratio of seven-day-old bovine embryos for inseminations performed early, intermediate or late relative to ovulation

Parameter		Male embryos	Female embryos	Ratio Male:Female (%)	X^2	P-value
Insemination time	Early[1]	11	11	50.0:50.0	0.00	1.00
	Intermediate[2]	10	10	50.0:50.0	0.00	1.00
	Late[3]	10	12	45.5:54.5	0.18	0.67
Quality	Good	21	19	52.5:47.5	0.10	0.75
	Fair	7	3	70.0:30.0	1.60	0.21
	Poor	2	5	28.6:71.4	1.29	0.26
	Degenerate	1	6	14.3:85.7	3.57	0.06
Bull	A	17	16	51.5:48.5	0.03	0.86
	B	14	17	45.2:54.8	0.29	0.59
Parity	Primiparous	10	8	55.6:44.4	0.22	0.64
	Multiparous	21	25	45.7:54.3	0.35	0.56

[1]Inseminations performed between 36 and 20h before ovulation; [2]Inseminations performed between 20 and 8h before ovulation; [3]Inseminations performed between 8h before and 12h after ovulation

Discussion

In our study there was no effect of different insemination-ovulation intervals on sex ratio of seven-day-old cattle embryos. In the literature, conflicting results of insemination time on sex ratio in cattle are described. Martinez et al. (2004) found that early insemination resulted in more female calves and late inseminations resulted in more male calves, another study found more females calves with early and late inseminations after synchronization (Pursley et al. 1998), whereas several

studies did not find any effect on sex ratio with different insemination times (Ballinger 1970, Foote 1977, Rorie 1999). In a study where cows were inseminated according to conductivity of the cervical mucus, inseminations performed during declining conductivity resulted in more female calves, whereas inseminations performed during rebounding of conductivity resulted in more male calves (Wehner et al. 1997).
Although a strong dependence of the sex ratio on the time of insemination relative to ovulation is suggested, time of ovulation was not assessed in those studies.
The underlying physiology for skewed sex ratios with different insemination times in relation to ovulation is not well understood. It is possible that a preferential selection of sperm bearing the X- or Y-chromosome at fertilization exists, because of differences in motility (Goodall & Roberts 1976), survival time (Rohde et al. 1973), or capacitation rate (Wehner et al. 1997) of X- and Y-spermatozoa. These differences would favour Y-spermatozoa when insemination is performed close to ovulation and X-spermatozoa when insemination is performed far before ovulation. Our results do not support these suggestions, because sex ratio was not skewed in the various insemination-ovulation intervals. It is also possible that differences in sex ratio at birth are caused by sex-specific death of embryos after fertilization (Hardy 1997). Previous studies assessed sex ratio of calves between days 60-80 of pregnancy or after birth, therefore no distinction could be made between differences in fertilization or differences in (early) embryonic death of X- and Y-spermatozoa. In our study sex ratio was assessed in seven-day-old embryos. Overall, 51.6% of the embryos were female. Looking at the sex ratio in the viable embryos (good, fair and poor) and the number of cell cycles of both sexes in our study, there is no indication that frequency of embryonic mortality is higher in female compared to male embryos. The percentage of females tended to be higher in the degenerate embryos. However, because of the low number of degenerate embryos in which the sex could be determined, firm conclusions cannot be drawn. Foote et al. (1977) also suggested that the sex ratio found at birth is similar to the ratio at fertilization.
In conclusion, our results indicate that, in cattle, varying the interval between insemination and ovulation does not influence the embryo sex ratio, neither by dispersion of the sex ratio at fertilization nor by sex-selective embryonic death.

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8

General Discussion

To come to an optimal insemination strategy achieving high calving rates for dairy cattle, parameters are required that can predict time of ovulation, and the optimal insemination-ovulation interval should be assessed. In this thesis (Chapters 3, 4 and 5) several possible predictors of ovulation time are discussed. In this chapter a discussion is presented on applicability and quality of the various ovulation time predictors. In Chapter 6 of this thesis fertilization rates and embryonic characteristics of seven-day-old embryos are presented dependent on the timing of insemination relative to ovulation. In practice, calving rates are of more interest than fertilization rates. Therefore, an attempt is made to translate the fertilization rates into calving rates. Subsequently, the best predictors of ovulation time are combined with these estimated calving rates to come to a practical insemination strategy which maximizes calving rates.

Possible predictors of ovulation time

In this paragraph, the practical application of various parameters to predict time of ovulation will be discussed. Because ovulation is always preceded by oestrus (i.e. a recurring period of sexual receptivity), the possibility of a parameter to predict time of ovulation may, among other things, depend on the possibility to detect oestrus. Therefore, this paragraph will not only deal with prediction of ovulation time but also with detection of oestrus.
For a parameter to be useful as a predictor of ovulation time, several prerequisites need to be met. The parameter should have a small variation in time to ovulation, and the measurements should be easy to carry out, repeatable and preferably be automated. It should also be present in a high proportion of the animals. Various parameters were studied as possible predictors for time of ovulation and evaluated on their practical applicability (Table 8.1). As shown in Table 8.1, measurements of body temperature and conductivity of the vaginal mucus are unsuitable as predictors of ovulation time. In our experiments the measurements within an animal were so variable that it was not possible to assess an actual change before ovulation in either of the parameters. Measurements of conductivity of the vaginal mucus are also not practical since labour input is high and there is no possibility to automate the measurements. For experimental measurements it is possible to place internal electrodes to measure vaginal conductivity (Smith et al. 1989) or body temperature (Mosher et al. 1990) automatically, but this is not feasible in practice. Gil et al. (1997) found a high correlation between milk temperature and body temperature, so measurements of temperature could be automated. However, whether changes in milk temperature would be a good predictor for ovulation time in a high proportion of animals needs further investigation.

Table 8.1 Overview of the practical applicability of various parameters to predict time of ovulation in dairy practice

Parameter	Variation to ovulation time[2]		Labour	Automation	Proportion
	S.D. (h)	Range (h)	input	possible	of animals[2]
Behaviour (onset of showing)					
All signs[1]	5.1	20	high	no	100%
Standing heat	5.2	27	high/low	yes	58%
Mounting	5.3	30	high	no	90%
Walking activity increase	3.9	17	low	yes	83%
Progesterone decline	11.2	44	high/low	no/yes	100%
LH-peak	2.0	6	high	no	100%
Oestradiol-decline	3.9	16	high/low	no/yes	100%
Body temperature rise	-	-	high/low	no/yes	-
Vaginal conductivity increase	-	-	high	no	-

[1]All behavioural signs as described by van Eerdenburg et al. (1996), which include sniffing, flehmen, chin resting, mounting, being mounted, standing heat and restlessness; [2]Based on the results of the experiments described in Chapters 3, 4 and 5

An advantage of monitoring hormone concentrations to predict time of ovulation is that the changes in LH, oestradiol and progesterone occur in all animals before ovulation (Table 8.1). However, monitoring of hormones to predict time of ovulation is not yet applicable in practice. Although the surge in LH shows a very good relationship with time of ovulation, it is not possible to monitor this hormone easily. Efforts have been made to monitor LH in milk of dairy cattle and buffaloes, but up to now these efforts have not been successful (Batra & Pandey 1983, Johnson & Reeves 1988). Even when LH could be measured in milk, it would probably not be an appropriate tool to predict time of ovulation because of the short duration of the LH-surge in dairy cattle (on average 9.5±1.6h, Chapter 2).
Oestradiol was measured in blood plasma in our experiment, which is not feasible for practice but oestradiol concentrations can also be assessed in milk (Meisterling & Dailey 1987). However, ovulation time can probably not be predicted with monitoring oestradiol concentrations alone, because oestradiol concentrations not only increase before ovulation but may also increase during the oestrous cycle (Dieleman et al. 1986), probably reflecting the growth of a dominant (but not preovulatory) follicle. Monitoring of the progesterone decrease before ovulation is not suitable because of the large variation with time of ovulation (Table 8.1). When oestradiol and progesterone could be measured automatically during milking, perhaps monitoring both hormones simultaneously could be used to predict time of ovulation. This depends on the relationship between the peak or decrease of oestradiol and time of ovulation. In our experiment (Chapter 2) the decrease in oestradiol relative to time of ovulation was assessed in 12 animals, taking blood samples for oestradiol at 3h intervals. The range from oestradiol decrease to time of ovulation was 16h (Table 8.1), whether this range is similar when oestradiol would be assessed twice a day in milk, needs further investigation. When the

decrease in milk oestradiol could serve as a predictor for ovulation time, simultaneous measurements of milk progesterone concentrations could be helpful to define false positive alerts that occur due to increased oestradiol during the oestrous cycle. When progesterone concentrations are high, it is obvious that ovulation will not occur.
Observation of behavioural oestrous signs and changes in walking activity (measured by the number of steps in 2h periods) seem applicable predictors of ovulation time because of the good relationship with time of ovulation (Table 8.1). However, observation of behavioural signs every 3h for 30 min as was done in our experiment (Chapter 3) is too labour intensive to be carried out in practice. Effects of less frequent observations or limiting the number of behavioural oestrous signs will be discussed in light of their usefulness as predictor of ovulation time. Measurements of walking activity can be automated and thus are easy to carry out; this parameter will also be further discussed as predictor of ovulation time.

Behavioural oestrous signs
Ovulation occurred on average 30.6±5.1h after the onset of behavioural oestrus when observations were done for 30 min every 3h and all behavioural oestrous signs according to Van Eerdenburg et al. (1996) were included (Chapter 3). In practice, sniffing and chin resting may often not be noticed as oestrous behaviour. When only mounting behaviour and standing heat are taken into account, ovulation occurred on average 28.7±5.3h and 26.4±5.2h, respectively, after first display of these behavioural signs. Other studies, that assessed the interval between onset of oestrus based on standing heat and time of ovulation, found similar intervals (e.g. Walker et al. 1996, Lopez et al. 2002, Chapter 1). Observation of the behavioural signs as carried out in our experiments is very labour intensive. Therefore, in practice, the observation frequency of oestrous behaviour will be less frequent. In Table 8.2 the percentages of detected oestrous periods are presented when in our dataset (Chapter 3), observations would have been done three times daily (8.00h, 17.00h and 23.00h) or twice daily (8.00h and 17.00h) compared to 8 times daily. A distinction is made between all oestrous periods and oestrous periods in which at least two animals are seen in oestrus at the same time because in the latter case, animals show more oestrous behaviour, which affects the detection rate (Chapter 3). The variation to ovulation time, as presented in Table 8.1, is not much affected by less frequent observations, however, the results in Table 8.2 show that many oestrous periods will be missed when observations are carried out less frequently as also found by Van Vliet & Van Eerdenburg (1996). When all behavioural signs are observed, 90 and 77% of all oestrus periods would be detected when observations were performed 3 and 2 times daily, respectively. The percentage of detected oestrous periods decreases further to 61 and 48%, respectively, when only mounting behaviour is observed and to 30 and 19%, respectively, when only standing heat is observed.

Table 8.2 Detection rates for various observation frequencies (30 min per observation period) per day.

	all oestrous periods			at least 2 animals in oestrus		
	observation frequency[1]			observation frequency[1]		
	8	3	2	8	3	2
All behavioural signs[2]	100	90	77	100	90	83
Mounting behaviour[3]	89	61	48	95	71	65
Standing heat[3]	57	30	19	71	41	35

[1]observation frequency: 8 times per day (at 8.00, 11.00, 14.00, 17.00, 20.00, 23.00, 2.00 and 5.00h), 3 times per day (at 8.00, 17.00 and 23.00h), 2 times per day (at 8.00 and 17.00h) for 30 min each time; [2]at least 50 points scored based on van Eerdenburg et al. (1996); [3]a cow has to mount another cow or display standing heat at least once during an observation period of 30 min

Excluding oestrous periods in which only one animal is in oestrus results in higher detection percentages for all observation frequencies, especially for observations of mounting behaviour and standing heat twice daily (Table 8.2). The explanation for this is obvious: when at least two cows are in oestrus at the same time they will interact with each other, therefore being able to mount more and show standing heat more often, increasing the chance that this behaviour is detected during an observation period. Van Vliet & Van Eerdenburg (1996) found that observations before milking resulted in lower detection percentages than observations after milking. Because the cows in our experiment were milked using an automated milking system, there are no standard milking times. Therefore, in our dataset, detection percentages will probably not be affected much by timing of the observation periods. It is obvious that prediction of ovulation time based on visual observations is time consuming and requires much dedication. When all the behavioural oestrous signs described by Van Eerdenburg et al. (1996) are observed three or two times per day, respectively 90% and 77% of the animals would have been detected in oestrus in our experiment. However, a study of Heres et al. (2000) showed that, in practice, only 47% of the animals in oestrus were detected by the farmers using the same scoring system twice a day. This demonstrates that this system is not easily implemented in dairy practice to predict ovulation time. The detection of standing heat could be automated by means of mount detectors (Williamson et al. 1972). Mount detectors record when a cow displays standing heat. Different systems are used in practice, but in all systems a mount detector is placed on the sacrum of a cow which detects when a cow has received one or more mounts (Rorie et al. 2002). However, in our dataset only in 57% of all oestrous periods and in 71% of the oestrous periods in which at least two cows were in oestrus at the same time, the cows displayed standing heat during the observations (when observations were carried out for 30 min every 3h). Several studies have reported oestrus detection efficiencies of over 90% for cows equipped with a mount detector (At Taras & Spahr 2001, Rorie et al. 2002, Cavalieri et al. 2003). However,

in those studies most of the time at least two cows were in oestrus at the same time because of oestrus synchronization or large herd sizes. The average herd size in The Netherlands is about 60, which means that, most of the time, only one cow will be in oestrus. This decreases the efficiency of mount detectors. If cows are in oestrus alone, they will mount cows that are not in oestrus, which will result in false positive attentions of mount detectors. Furthermore, the presence of brushes in the barn for the cows will complicate the use of mount detectors.
Mounting behaviour (mounting, or trying to mount another cow) is displayed in a large number of oestrous periods. It is observed in 89% of all oestrous periods with observations every 3h. Unfortunately, this behaviour cannot (yet) be recorded automatically. This makes mounting behaviour a promising but not yet practical predictor of ovulation time.
In conclusion, the relationship between the display of behavioural oestrous signs and time of ovulation is good (Table 8.1). However, observation of all the behavioural signs except for standing heat cannot be automated and requires high labour inputs. Decreasing observation frequencies result in lower detection percentages (Table 8.2). Standing heat can be assessed automatically, but is displayed in a low proportion of the animals (Table 8.1). All together, prediction of ovulation time based on behavioural oestrous signs is not feasible in dairy practice.

Activity measurements
In contrast with behavioural oestrous signs, prediction of ovulation time by an increase in walking activity (measured by the number of steps taken in 2h periods) can be automated and requires little time and effort (Table 8.1). The detection rates for activity measurements depend highly on the algorithms and thresholds that are used to define an increase in activity. In our experiment, the best detection rate was 87% (Chapter 4). Depending on the threshold, the number of false pedometers oestrus alerts ranged from 1 to 17 in 5% to 40% of all oestrous periods (Chapter 4). In our experiment, the number of steps was stored in 2h periods. However, many pedometers used in practice store the number of steps in 12h periods. Liu & Spahr (1993) concluded from their experiment that activity counts that were stored in 12h periods identified the increased activity during oestrus as well as did the activity counts stored in 2h periods. When we convert the data from our experiment (Chapter 4) to number of steps in 12h periods (from 0.00h to 12.00h and from 12.00h to 0.00h) the percentage of correct detected oestrous periods became 92% (using a threshold of 3 times the standard deviation of the preceding 10 days for the same 12h period). This detection rate is higher compared to storage of steps in 2h periods. However, the number of false oestrus alerts increased; 1 to 2 false oestrus alerts were found in 26% of the oestrous periods compared to the 6% for storage of steps in 2h periods using the same threshold. Although detection percentage increased with storage of steps in 12h periods (compared to 2h periods), prediction of ovulation time becomes less

accurate. The interval between onset of pedometer oestrus based on 12h periods and time of ovulation is 33.3±5.9h with a range of 16 to 46h, while the interval between onset of pedometer oestrus based on 2h periods and ovulation is 29.3±3.9h with a range of 22 to 39h (Chapter 4). This larger variation could be a problem, because AI has to be performed 24 to 12h before ovulation (see next section) which implicates that insemination should be performed 5 to 17h after the onset of increase in activity. When it takes at least 12h before the increase in activity is observed, this leaves almost no time for optimal timed insemination. More frequent reading of the pedometers seems therefore a logical approach to solve this problem.

It has to be noted that the activity measurements in our study were performed at only one farm. Although it is known that basal activity levels differ between cows and farms (Nebel et al. 2000), the question is whether the relative increase in activity at oestrus is also different between farms. In other words, the question is whether the high detection rate and the high correlation between increase in activity and ovulation time found in our experiment (Chapter 4) will be similar for other farms. López-Gatius et al. (2005) studied the activity of two herds kept on different farms but under the same housing, management and milking conditions. They found no difference in increase in activity at oestrus between the farms. However, activity levels were affected by parity, milk production and season. Each additional lactation and each 1 kg increase in milk yield per day were associated with decreases of 21.4 and 1.6% walking activity at oestrus, respectively. Nothing was mentioned about activity levels between farms outside oestrous periods. Another study found similar increases in activity at oestrus when cows were kept in a covered straw yard compared to cows kept in cubicles (Schofield et al. 1991). These findings suggest that prediction of ovulation time by measurement of activity, as found in our study, should be applicable on other farms as long as the threshold for an increase in activity is based on the individual activity pattern of the cow.

In conclusion, monitoring the number of steps measured by pedometers meets all the prerequisites for ovulation time prediction; a good relationship exists between increase in activity and time of ovulation, the labour input for measurement of activity is low and the measurements can be automated, also a high proportion of the animals show an increase in their activity before ovulation. Therefore, prediction of ovulation time based on an increase in the number of steps seems useable in dairy practice. However, if this is true for all farms needs to be investigated.

Improvement of oestrus expression

As already mentioned, the success of a parameter to predict time of ovulation is partly based on the ability of the parameter to detect the recurring period of sexual receptivity (i.e. the oestrous period) preceding ovulation. When oestrus

symptoms are expressed for a longer period of time or more intensely, e.g. more events of standing heat displayed by more animals or a larger increase in activity, they will be more easily detected. The expression of oestrous signs (behavioural oestrous signs and also the increase in activity) is influenced by many factors. These factors can be related to social interactions, management, environment, nutrition, age and physiological status of the cow, genetics or presence of a bull (reviewed by Orihuela 2000). In our experiment (Chapter 3), it was found that the number of points assigned for behavioural oestrous signs increased when more cows were in oestrus at the same time. In other words, cows displayed more behavioural oestrous signs (especially chin resting and mounting behaviour) when more cows were in oestrus at the same time. It was also shown that more cows mounted other cows and more cows displayed standing heat when more than one cow was in oestrus at the same time. Also the number of steps taken by cows in oestrus increased when more cows were in oestrus at the same time, although this increase was not statistically significant. Various other studies also found an increase in the expression of oestrous behaviour (especially in the number of mounts) or an increase in the number of steps (Hurnik et al. 1975, Helmer & Britt 1985, Varner et al. 1994) when more cows were in oestrus at the same time. So, it is apparent that oestrous behaviour can be stimulated and intensified. As mentioned earlier, the average herd size and calving pattern in The Netherlands is such that, in many cases, only one cow will be in oestrus and therefore oestrous behaviour will not be stimulated by other herd mates. An interesting question that arises is whether oestrous behaviour can be stimulated in another way.

From research in pigs, it is known that the presence of a boar influences oestrous behaviour of sows. Oestrus detection rates were higher and duration of oestrus was longer when oestrus was detected with fence-line contact with a boar compared to oestrus detection without a boar (Langendijk et al. 2000). Not much research has been done regarding the effect of bull exposure on oestrous behaviour in lactating dairy cattle. Shipka & Ellis (1998) found no effect of fence-line bull exposure twice a day compared to no bull exposure on oestrous behaviour in high producing Holstein cows that were housed in tie stalls. However, Kilgour et al. (1977) found, that cow to cow behaviour changes in the presence of a bull. An interesting question would be if a bull housed near the cows would attract cows in oestrus and/or would intensify the oestrous behaviour between cows.

Combination of parameters

It is possible that a higher (oestrus) detection rate can be achieved and therefore more ovulations can be predicted when a combination of parameters is used (reviewed by Firk et al. 2002). Peralta et al. (2005) compared the efficiencies of oestrus detection for inseminations performed based on visual observations of standing heat (7.30h, 13.30h and 1.00h), mount detectors (continuously) and activity measurements (continuously), and various combinations of the three

systems. They found that visual observations detected the highest number (49.3%) of oestrous periods as compared to activity measurements (37.2%) and mount detectors (48.0%). The total percentage of oestrous periods detected by the three systems combined was 80.2%. The low oestrus detection percentages found in their study were said to be due to heat stress. The study showed that a combination of parameters increased oestrus detection rates. The question is whether a combination of parameters will also increase detection percentages when they are already at the high level as found in our experiments.
In our experiments the 'golden standard' for oestrus and subsequent ovulation was the expression of behavioural oestrous signs (Chapter 3). Therefore, it is not known if ovulations have occurred without display of any behavioural oestrous sign. However, because not only mounting behaviour and standing heat but also sniffing and chin resting were observed, it is not likely that many ovulations have occurred without notice, especially not when the distribution of oestrous duration is examined (Figure 8.1). Only a small percentage of the oestrous periods lasted only 3h, which indicates that probably few or no oestrous periods were missed because of an even shorter duration.

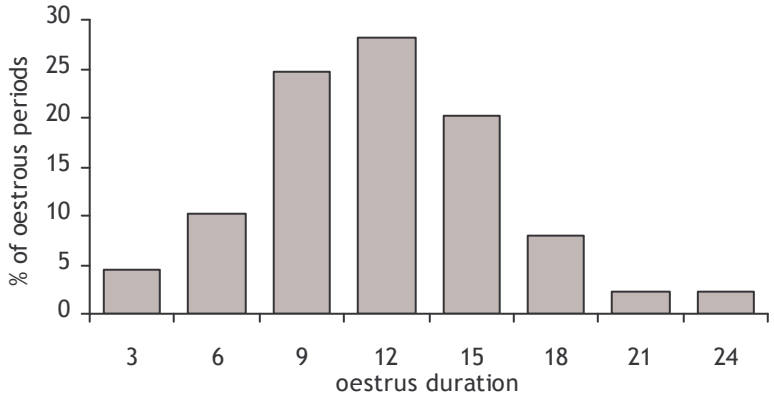

Figure 8.1 Duration of oestrus based on behavioural oestrous signs (Chapter 3)

This is substantiated by a study in which observations were carried out every 2h for 30 min and progesterone concentrations were used to assess whether ovulation had occurred (Van Eerdenburg et al. 1996). In their study, in which the same scoring system for oestrous behaviour was used, oestrus detection rate was 100% and average duration of oestrus was 13.7±6.5h.
In our study, the detection rate using activity measurements was 83% (Chapter 4). This detection percentage would not have increased when activity measurements would have been combined with visual observations of standing heat three times a day, because the animals that did not show an increase in activity, also did not display standing heat during the observation periods. However, if also the other behavioural signs would be observed three times per day (especially sniffing and

chin resting) in combination with activity measurements, the percentage of detected oestrous periods would increase to 95%.
Another way to increase the detection rate by activity measurements is to use other definitions for the increase in activity. As the results described in Chapter 4 show, detection percentages increased when thresholds were defined with 2.5 or 2 times the standard deviation (detection rates of 87%). Also when not two but one 2h period of increased activity would be used to define pedometer oestrus, the detection rate would increase to 97%. The problem with achieving higher detection rates is that the accuracy decreases, i.e. the number of false positives will increase. Although measurements of progesterone concentrations in the milk did not prove useful as predictor of ovulation time because of the high variation in profiles between animals, it could be a helpful tool to assist in predicting time of ovulation. Numerous studies have shown that oestrus can be confirmed by a period of low progesterone concentrations in milk (Walton & King 1986, Friggens & Chagunda 2005), therefore anticipating subsequent ovulation (Chapter 5). If progesterone concentrations could easily be measured online, false positive alerts based on activity measurements could be recognized by the milk progesterone concentration. If progesterone concentrations in the milk are high at the time the activity is increased, it is apparent that the increase in activity will not be followed by an ovulation. So, automated milk progesterone measurements could improve the detection efficiency and accuracy of activity measurements. It should be noted that progesterone is low from approximately 3 days before to 3 days after ovulation. Therefore, the use of progesterone will not eliminate all false positive alerts.

Expected calving rates based on insemination-ovulation interval

In our experiment we have assessed fertilization rates and embryo quality seven days after ovulation for various insemination-ovulation intervals (Chapter 6). Insemination in an optimal interval should ultimately result in a high chance on the birth of a calf. The question is which embryos would have resulted in a born calf if we would not have flushed them from the uterus seven days after ovulation. It is certain that degenerate embryos will not result in the birth of a calf. Therefore, the optimal intervals in which fertilization rates (assessed seven days after ovulation) are high are not of interest for dairy practice. Likely, the use of optimal intervals in terms of viable embryos (excluding degenerate embryos) is of more use. The results described in Chapter 6 show that inseminations performed between 36 and 0h before ovulation yielded high numbers of viable embryos. However, not all embryos that seem viable seven days after ovulation are of similar quality and may therefore result in the birth of a calf. The question that arises is:

what are the chances of embryos of different qualities (good, fair and poor) to result in the birth of a calf? In experiments using embryo transfer of fresh embryos derived from super-ovulated cattle, a large variation in pregnancy rates is found when good, fair and poor quality embryos are transferred. Transfer of good quality embryos resulted in 45 to 76% pregnancies; transfer of fair quality embryos resulted in 27 to 67% pregnancies and transfer of poor quality embryos resulted in 20 to 33% pregnancies (Schneider et al. 1980, Wright 1981, Lindner & Wright 1983, Hoogenkamp 1984, Donaldson 1985, Hasler 1998). Pregnancy in those studies was assessed between 40 and 90 days after insemination. Foetal death can occur later in pregnancy, but the incidence of foetal mortality normally does not exceed 5% (Makarechian & Arthur 1990, Xu & Burton 1999, Chagas e Silva et al. 2002). Averaging the pregnancy rates of the various studies mentioned above, means that embryo transfer resulted in calving rates of 60, 45 and 30% for good, fair and poor embryos, respectively. If the probability to result in a calf is the same for non-transferred embryos, this means that the expected calving rate for various 12h intervals in our experiment would be as presented in Figure 8.2; inseminations performed between 36 to 24h, 24 to 12h and 12 to 0h before ovulation would result in a 36, 42 and 31% calving rate, respectively. Inseminations after ovulation decrease calving rates dramatically, to only 11% (Figure 8.1). However, it seems likely that survival of embryos is higher for non-transferred embryos compared to transferred embryos.

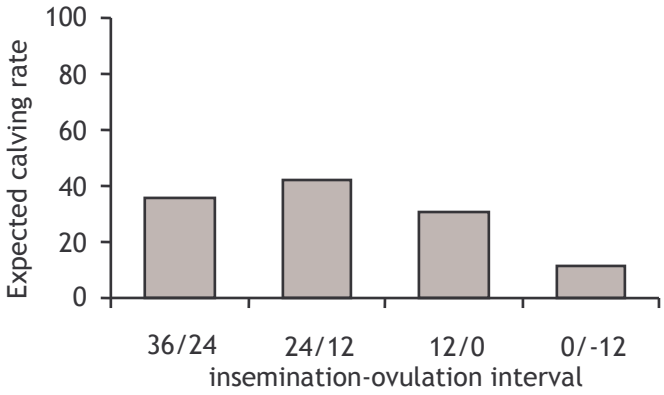

Figure 8.2. Expected calving rates (%) per 12h insemination-ovulation interval for chances of good, fair and poor quality embryos to result in a calf of 60, 45 and 30%, respectively. (For numbers of good, fair and poor embryos, see Chapter 6)

Figure 8.3 shows the expected calving rates for various 12 and 4h intervals with different chances of good, fair and poor quality embryos to result in a calf. In the best case scenario (100% survival of all quality embryos, Figure 8.3A), calving rates over 75% are realised in the optimal period of insemination.

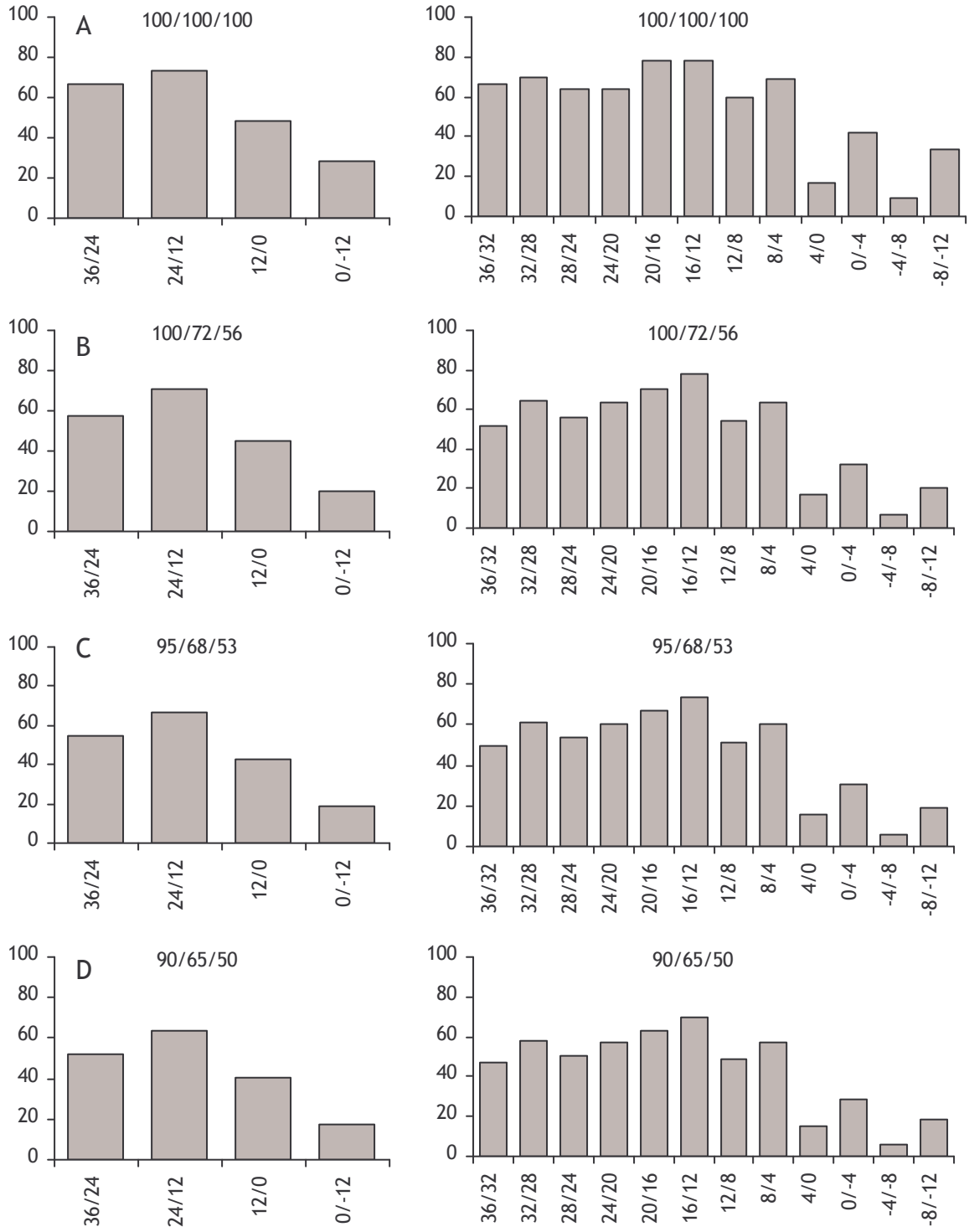

Figure 8.3. Expected calving rates (%) per 4 and 12h insemination-ovulation intervals (0=time of ovulation) for different chances of good, fair and poor quality embryos to result in a calf. Numbers above the graph represent the estimated calving rates for good, fair and poor embryos, respectively (for numbers of good, fair and poor embryos, see Chapter 6).

However, scenarios with less than 100% embryo survival seem more realistic. The scenarios in Figure 8.3B, C and D are arbitrarily chosen in such a way that the survival rates for the different embryo qualities have the same ratio as found in studies that used embryo transfer. The ratio for survival of good, fair and poor

embryos in those studies was on average 1.8:1.3:1, respectively (Schneider et al. 1980, Wright 1981, Lindner & Wright 1983, Hoogenkamp 1984, Donaldson 1985, Hasler 1998).
In the scenarios with less than 100% embryonic survival, insemination between 12 and 24h before ovulation results in calving rates between 64 and 71%. Inseminations performed earlier than 24h before ovulation results in all scenarios in better calving rates (52 to 67%) compared to inseminations performed between 12 and 0h before ovulation (40 to 49%) or inseminations performed after ovulation (18 to 28%). A question that arises is whether the optimal interval for insemination relative to ovulation might be smaller than 12h. In Figure 8.3 estimated calving rates for 4h insemination-ovulation intervals are also presented. It appears that inseminations 20 to 12h before ovulation (Figure 8.3A) or 16 to 12h before ovulation (Figure 8.3B, C and D) result in the highest calving rates. However, the number of embryos recovered in 4h intervals is too small to draw firm conclusions. Especially when AI is performed less than 4h before ovulation and after ovulation, calving rates decrease dramatically.
Data on the actual calving rate after first insemination in practice are difficult to find. Average non-return rate at 56 days after insemination is 68% in The Netherlands (Jaarstatistieken 2004). However, this is an overestimation of the actual calving rate, because all cows that are not recorded for re-insemination are included in this percentage. It is also possible that cows are not recorded for re-insemination because they have been culled or because they have eventually been mated by a bull. Therefore, the actual calving rate will be lower than this 68%. Actual calving rates after first AI were found to be only 42% in a survey conducted at 17 farms in The Netherlands (Poelarends & Smolders 2004). This percentage shows a high variation between farms; the range was 31% (from 28% to 59%). Many factors can be responsible for differences in actual calving rates between farms. They may affect either fertilization rate or embryo survival rate or both and can be related to e.g. nutritional influences, genetics, lactation number and -stage of the cows (De Kruif 1978, Stevenson et al. 1983, López-Gatius et al. 2002, Sreenan et al. 1996). Beside these factors, also differences in timing of insemination relative to ovulation could be a cause for variation in calving rate after first AI. As shown in Figure 8.2, optimal calving rates are expected when inseminations are performed between 12 and 24h before ovulation (64 to 73%). A smaller range in interval between insemination and ovulation might result in even higher calving rates. Maatje et al. (1997) found calculated conception rates of almost 90% in a small range of intervals between onset of oestrus (measured by pedometers) and time of insemination (inseminations needed to be performed between 10 and 13h after onset of oestrus). From these data, it is clear that calving rates in practice may be improved by optimizing insemination strategies.

In the next paragraph the most promising predictors of ovulation time (all behavioural signs, mounting behaviour, standing heat and walking activity) are combined with the expected calving rates of the various the insemination-ovulation intervals to come to an optimal insemination strategy.

Insemination based on prediction of ovulation time

In 1948 the a.m.-p.m. guideline for time of insemination was established. This guideline recommends that cows observed in oestrus in the morning should be submitted for AI in the afternoon, and cows observed in oestrus during the afternoon should be submitted for AI the following morning (Trimberger 1948). Since then several studies have examined the optimal time for insemination relative to the onset of oestrus (Table 8.3).

Table 8.3 Overview of optimal insemination time relative to onset of oestrus based on various parameters

Oestrus based on	Optimal insemination interval after onset of oestrus (h)	Conception rate (%)	Reference
discharge, nervousness, interest in herd mates, mounting, standing heat	7-12	55	Hall et al. 1959
walking activity	6-17	83	Maatje et al. 1997
standing heat	4-12	51	Dransfield et al. 1998
standing heat	12-18	73	Xu et al. 1998
standing heat	12	-	Dalton et al. 2001
behavioural, clinical and gynaecological symptoms	8-18	66	Martinez et al. 2004
Our results			
all behavioural signs	3-15	62[1]	This thesis, Chapter 3
Mounting	3-15	63[1]	This thesis, Chapter 3
standing heat	0-12	63[1]	This thesis, Chapter 3
walking activity	5-17	65[1]	This thesis, Chapter 4

[1]Estimated calving rate when the survival rate of good, fair and poor quality embryos is 95, 68 and 53%, respectively

If visual observations had been performed three times a day for 30 min in our experiment, the interval between the onset of behavioural oestrus (including all behavioural signs), first observed mount and standing heat and time of ovulation would be 27.1±5.4h, 26.7±5.1h and 24.3±5.1h, respectively (Chapter 3, this discussion). The interval between increase in activity (using 3 times SD) and time of ovulation was 29.3±3.9h (Chapter 4). The results in Chapter 6 have shown that the optimal time to inseminate is 24 to 12h before ovulation. Combining this optimal interval with the interval between onset of behavioural oestrus and ovulation time

suggests that insemination should be performed 3 to 15h after the observation of onset of behavioural oestrus (assessing all behavioural signs for 30 min, three times daily). Using the same observation frequency, insemination should be performed 3 to 15h after the first observed mount or 0 to 12h after first observed standing heat. This is in the same range as suggested by other authors (Table 8.3). Inseminations based on activity would mean that AI should be performed 5 to 17h after the increase in activity. This optimal insemination interval is the same as reported by Maatje et al. (1997) (Table 8.3).
A practical advice for Dutch farmers is to inseminate 12 to 20h after the first observed mount based on three or four observation periods of 10 to 15 min per day (Vink & Wolbers 1997). Another practical advice is to inseminate 6 to 16h after the first observed standing heat based on three observation periods of 20 min per day (CR-Delta 2001). Because of the variation in ovulation time, one insemination advice cannot ensure optimal insemination time for all animals. Table 8.4 shows the percentages of inseminations for the various 12h insemination-ovulation intervals, when cows are inseminated according to the different advices mentioned above.

Table 8.4. The percentage of inseminations for various insemination-ovulation intervals (IOI, 0=time of ovulation) when cows are inseminated based on first observed mount, first observed standing heat, observation of onset of behavioural oestrus (including all behavioural oestrous signs) or increase in activity.

IOI	Mounting		standing heat		all signs	activity
	practice[1]	Chapter 3[2]	practice[3]	Chapter 3[4]	Chapter 3[5]	Chapter 4[6]
36<AI≤24	2	11	4	7	14	10
24<AI≤12	33	78	74	81	72	84
12<AI≤0	65	11	22	11	14	6
0<AI≤-12	0	0	0	0	0	0

With three observations periods of 30 min per day, mounting behaviour was observed in 61% of all oestrous periods and standing heat in 30% and behavioural oestrus (including all signs) was observed in 90% of the oestrous periods. In 83% of all oestrous periods an increase in activity was observed.
[1]insemination 16h after first observed mount (Vink & Wolbers 1997); [2]insemination 9h after first observed mount; [3]insemination 11h after first observed standing heat (CR-Delta 2001); [4]insemination 6h after first observed standing heat; [5]insemination 9h after first observed behavioural sign; [6]insemination 11h after first increase of activity

As can be seen, most inseminations are performed too late when the timing is based on the practical advice for mounting behaviour; 65% of the inseminations would be performed 12 to 0h before ovulation. Insemination based on an increase in activity results in the highest percentage of inseminations in the optimal interval of 24 to 12h before ovulation (84%). From these results we can calculate expected calving rates using the different chances of good, fair and poor embryos to result in a calf (Figure 8.3). Figure 8.4 shows that expected calving rates increase with approximately 12% when inseminations based on first observed mount are

performed according to our results compared to the practical advice (Vink & Wolbers 1997).

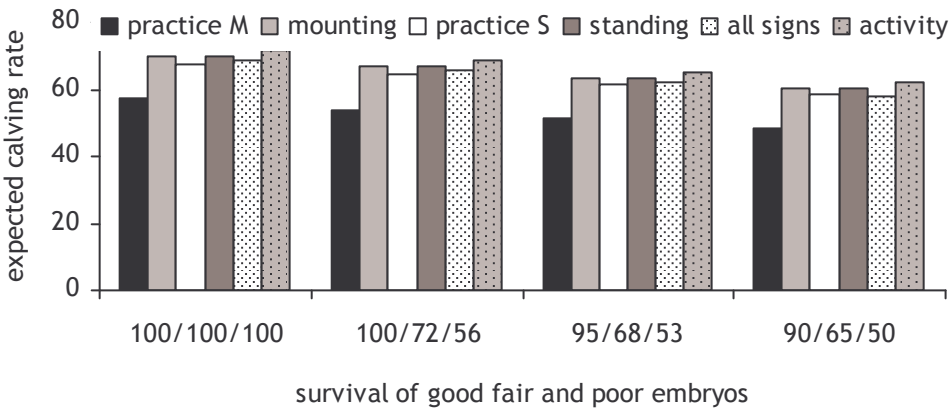

Figure 8.4. Expected calving rates when insemination are performed following practical advices (on average 16h after the first observed mount (practice M, Vink & Wolbers 1997) or 11h after first observed standing heat (practice S, CR-Delta 2001) or following our results (on average 9h after the first observed mount (mounting), 6h after the first observed standing heat (standing), 9h after the onset of behavioural oestrus (all signs) or 11h after increased activity (activity)) when visual observations are carried out three times daily for 30 min or activity is measured in 2h periods for different survival rates of good, fair and poor embryos to result in a calf.

Expected calving rates are about 2% higher when insemination time is based on the first observed standing heat according to our results (Chapter 3) compared to the practical advice (CR-Delta 2001). Surprisingly, these expected calving rates are almost 20% higher than the actual calving rates after first AI of about 42% (Poelarends & Smolders 2004). This either means that, in practice, embryonic survival is lower than assumed here or it indicates that in practice inseminations are not performed according to the advice. A survey done at 170 farms in The Netherlands seems to indicate that the average interval between detection and insemination on these farms varied from 3h to 24h. On almost half of the farms, the majority of inseminations were performed more than 15h after detection of oestrus (Hensen et al. 1992). Thus, it seems that insemination is performed too late on many farms. It has to be noted that only in 30% of all oestrous periods, standing heat is observed by three times daily observations (Table 8.2). It is possible that when standing heat is observed, timing of insemination is quite accurate, while in other oestrous periods with less clear behavioural oestrous signs, insemination is often wrongly timed. A reason for suboptimal timing of inseminations in absence of standing heat could be that a farmer is not sure about the oestrous status of a cow. Therefore, the decision to inseminate the cow is delayed, resulting in decreased calving rates because of inseminations performed to close or even after ovulation, especially when the cows are not inseminated by the farmer himself. In all scenarios of expected embryonic survival rates, it seems

better to inseminate (too) early than (too) late when time of ovulation is not known.

For inseminations based on an increase in activity, expected calving rates of almost 70% would be obtained when cows are inseminated 11h after the first increase in activity (Figure 8.4). As can be seen in Figure 8.4, expected calving rates are similar after inseminations based on onset of behavioural oestrus (assessing all behavioural signs), mounting behaviour and standing heat compared to an increase in activity. However, when cows are inseminated based on mounting behaviour, only 60% of the cows in oestrus will be inseminated because mounting behaviour is not observed in other cows (Table 8.2). For insemination based on standing heat, the percentage of cows inseminated is even lower, only 30%. When insemination is based on onset of behavioural oestrus, 90% of the animals in oestrus will be inseminated. However, as already mentioned, accurate observation of onset of behavioural oestrus is time consuming and not easy to implement in dairy practice (Heres et al., 2000). Insemination after an increase in activity is possible in 79-87% of the oestrous periods, depending on the definition of the thresholds for increased activity. The question is how these differences in detection rate affect the number of calves born. To answer this question, the detection rates (Table 8.2) and the estimated calving rates (Figure 8.4) are combined. When the cows that are detected in oestrus based on the first display of mounting, standing heat, behavioural oestrus or increase in activity, are inseminated according to the optimal insemination strategies for these parameters, this would result in, respectively, 38%, 19%, 55% and 54% of the cows in oestrus calving (Figure 8.5)

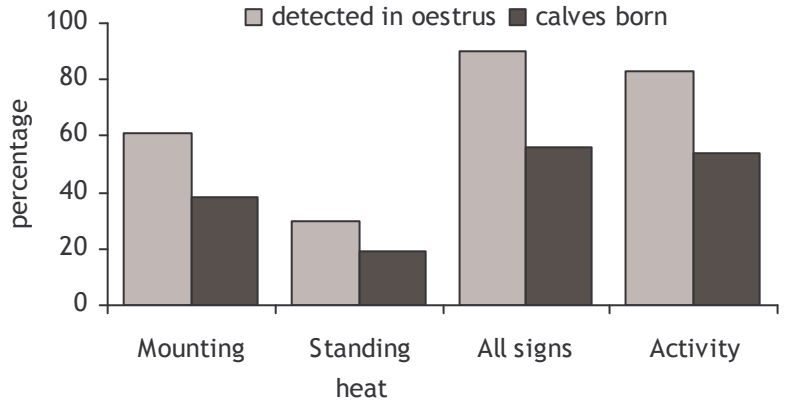

Figure 8.5. Percentage of cows detected in oestrus and percentage of calves born when oestrus is detected based on observations of mounting, standing heat or all signs three times a day or on an increase in activity. Inseminations are performed on average 9h after the first observed mount, 6h after the first observed standing heat, 9h after the onset of behavioural oestrus (all signs) or 11h after the increase in activity (based on Table 8.2 and Figure 8.4). The assumed calving rates for survival of good, fair and poor embryos of are 95, 68 and 53%, respectively.

In conclusion, expected calving rates are similar when a cow is inseminated either 3 to 15h after the onset of behavioural oestrus, 3 to 15h after first observed mount or 0 to 12h after first observed standing heat based on observations three times per day, or 5-17h after first increase in activity. When detection rates are taken into account, the best calving rates are realised when inseminations are performed according the onset of behavioural oestrus (assessing all behavioural signs) or according to an increase in activity. However, insemination based on an increase in activity seems the best strategy, because it requires low labour input and is easily implemented in dairy practice.

In conclusion

The first aim of the thesis was to establish the relationship between various oestrus characteristics and ovulation time in order to investigate whether these oestrus characteristics could predict ovulation time. It can be concluded that:

- All behavioural oestrous signs (including sniffing and chin resting) can predict time of ovulation accurately. However, observation of most of these behavioural signs cannot be automated and therefore, requires high labour input. Assessment of standing heat can be automated (using mount detectors), but standing heat is displayed in only a low proportion of the oestrous periods. Therefore, prediction of ovulation time solely based on behavioural oestrous signs is not (yet) feasible in dairy practice.
- An increase in activity measured by pedometers can predict time of ovulation accurately and is seen in a high proportion of the animals. The labour requirements for measurement of activity are low. Therefore, prediction of ovulation time based on an increase in the number of steps seems useable in dairy practice. This has to be validated on other farms.
- Monitoring of progesterone concentrations alone is not suitable as predictor of ovulation time because of the high variation in decrease of progesterone concentrations and time of ovulation.

The second aim of the thesis was to study the consequences of variation in the interval between insemination and ovulation on the success of fertilization and on embryonic characteristics. It can be concluded that:

- Timing of insemination relative to ovulation does not only affect fertilization, but also affects early embryonic development.
- The highest percentage of good quality embryos are found when inseminations are performed between 12 and 24h before ovulation.
- The interval between insemination and ovulation does not affect the sex of the embryos.

For the ultimate goal of the project, to come to an optimal insemination strategy that can be used in practice to maximize calving rates, it can be concluded that:

- Inseminations should be performed either 5 to 17h after first increase in activity, 3 to 15h after first observed mount, 0 to 12h after first observed standing heat or 3 to 15h after the onset of behavioural oestrus. Timing of insemination according to the increase in activity seems the best strategy, because this increase in activity is observed in most of the oestrous periods and requires low labour input.

It has to be stressed that the experiments were conducted on one experimental farm. Whether oestrus detection rates of the studied parameters and the relationship between the parameters and time of ovulation are the same on farms with different housing system, management, etc. needs to be investigated. My expectation is that detection rates might differ from farm to farm, but when oestrus is detected, the relationship with time of ovulation and the optimal insemination time will be similar as found in our experiments.

A last remark about the optimal insemination strategy:
I would advice to combine activity measurements with visual observations. When the pedometer readings show an increase in activity for a certain cow, you should observe that cow for the display of oestrous behaviour (including sniffing and chin resting). When she shows any of these behavioural signs, inseminate her 3 to 15h after the increase in activity. On the other hand, when a cow shows behavioural oestrous signs, check the pedometer measurements and again base insemination time on the pedometer readings.

Whether the optimal insemination strategy established in this thesis will indeed increase calving rates in dairy practice, has to be studied.

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Summary/Samenvatting

Summary

For conception to occur, insemination should be performed at the correct stage of the cow's oestrous cycle. Successful fertilization highly depends on the interval from insemination to ovulation. The optimal time at which insemination should be performed depends on the life span of fertile spermatozoa in the female tract and on the viable life span of the ovum after ovulation. However, in dairy practice, the timing of ovulation is not known; insemination time is based on oestrous behaviour. Unfortunately, the relationship between oestrous behaviour and ovulation time is not clear. Calving rates after first insemination are low, in The Netherlands on average below 50%. An important question is which part of this low calving rate is related to wrongly timed inseminations.
Therefore, the aim of the research described in this thesis was two-folded. Firstly, to establish the relationship between various oestrous characteristics and time of ovulation in order to investigate whether these oestrous characteristics could predict ovulation time and secondly, to study effects of different intervals between insemination and ovulation on success of fertilization and embryo quality. The ultimate goal of the project was to create an optimal insemination strategy that can be used in dairy practice.

To be able to study time of ovulation, time of ovulation needs to be assessed without influencing either the time of ovulation or other processes/parameters around ovulation. It is known that rectal ultrasound examinations can be used to assess time of ovulation. However, to determine the time of ovulation accurately, ultrasound examinations should be performed repeatedly. Therefore, an experiment was performed that investigated whether frequent rectal ultrasound examinations affected behavioural oestrous signs and peri-ovulatory hormone profiles (Chapter 2). If so, it would not be an appropriate tool for assessment of ovulation time in our studies. For this, oestrus was synchronised in two consecutive cycles. In half of these cycles, time of ovulation was assessed by rectal ultrasound examinations every 3h (UG), the other half served as control and no ultrasound examinations were performed (CG). There were no significant differences between the onset and duration of oestrus or intensity of oestrous behaviour between UG and CG. Furthermore, LH, oestradiol and progesterone profiles were similar between UG and CG. It was demonstrated that repeated rectal ultrasound examinations did not alter behavioural oestrus or peri-ovulatory hormone profiles. Therefore, it was concluded to be an appropriate tool to determine ovulation time in experiments, to study predictors of ovulation time (Chapters 3, 4 and 5) and consequences of insemination time relative to ovulation (Chapters 6 and 7).

The relationship between various oestrus characteristics and time of ovulation was studied in two experiments (Chapters 3, 4 and 5). The investigated parameters were: behavioural oestrous signs (Chapter 3), walking activity (Chapter 4) and progesterone profiles (Chapter 5).

Different behavioural oestrous signs were observed for 30 min at 3h intervals and walking activity was measured by pedometers that stored the number of steps in 2h periods. The relationship between behavioural oestrous signs, activity and time of ovulation (assessed by rectal ultrasound examinations every 3h) was investigated.

Oestrous behaviour In all oestrous periods, sniffing and chin resting were displayed, while mounting was displayed in 90% and standing heat was displayed in 58% of the oestrous periods. Ovulation occurred 30.0±5.1h after onset of behavioural oestrous signs. In practice, sniffing and chin resting may often not be noticed as oestrous behaviour. When only mounting behaviour and standing heat are taken into account, ovulation occurred on average 28.7±5.3h and 26.4±5.2h, respectively, after first display of these behavioural signs. However, as already mentioned, standing heat was displayed in only a limited number of oestrous periods, thereby limiting the practical use as predictor of ovulation time. Mounting behaviour cannot yet be assessed automatically, which also limits its practical use as ovulation predictor.

Walking activity Walking activity increases during oestrus, therefore, pedometer oestrus alerts (significant increases in the number of steps) were defined using different algorithms and thresholds, described in Chapter 4. The highest percentage of oestrous periods detected by pedometer measurements was 87%. Ovulation occurred at a rather stable 29.3±3.9h after the increase in activity. Because of the ease of measurements, the stable relationship with ovulation time and the high detection percentages, pedometer measurements seem an applicable ovulation predictor.

Progesterone Progesterone concentrations in blood and milk decrease before ovulation. In Chapter 5, an experiment is described that investigated whether monitoring progesterone concentrations in milk and blood plasma could be used to predict ovulation time. The interval between decreasing progesterone and time of ovulation (assessed by ultrasound examinations at 4h intervals) were investigated. The standard deviations of these intervals varied between 11.2 and 19.6h; the range in time to ovulation was at best about two days.

In conclusion, although informative, behavioural oestrous signs are not (yet) sufficient to predict ovulation time in practice because of the high labour requirements. Although pedometers measurements already seem an accurate tool to predict ovulation time in practice, the applicability on farms with e.g. different management or housing conditions still needs to be investigated. Further, monitoring progesterone profiles alone is not sufficient to predict time of ovulation, because of the large variation in timing of decrease of progesterone

concentrations relative to ovulation time. However, if progesterone concentrations can be assessed automatically by on-line measurements in milk, it could, for example, aid in detecting false positive oestrus alerts resulting from pedometer readings (Chapter 8).

Effects of the interval between insemination and ovulation on success of fertilization and embryonic characteristics are described in Chapters 6 and 7. For this study, inseminations were deliberately performed in a wide time range, resulting in inseminations between 36h before to 12h after ovulation. Seven days after ovulation the ovum/embryo was flushed from the uterus. Fertilization status and embryo quality were assessed. Fertilization rates were significantly higher when inseminations were performed between 36 to 24h and 24 to 12h before ovulation (85 and 82%) compared with inseminations after ovulation (56%). Inseminations performed between 24 and 12h before ovulation resulted in the highest percentages of viable (poor, fair and good quality) embryos (77%). The sex ratio in the various insemination-ovulation intervals did not differ from the expected 1:1 sex ratio.
In conclusion, the insemination-ovulation interval in which high fertilization rates are observed is quite long (from 36 to 12h before ovulation), while the interval in which the majority of fertilized ovum will develop into a good quality embryo is considerably shorter (24 to 12h before ovulation); the interval between insemination and ovulation did not influence the sex ratio of seven-day-old embryos.

In Chapter 8, these results are discussed in the light of their implementation in practical dairy farming in The Netherlands and to create an optimal insemination strategy. Based on the quality of recovered embryos, estimates were made of the effects of insemination-ovulation interval on calving rate. These estimates depend heavily on expected survival rates of embryos of good/fair/poor quality and varied from 63% to 73% of calves born in the optimal interval from insemination to ovulation. Even though true calving rates can not be reliably estimated, the highest chance of an insemination to result in a born calf is always found with inseminations between 12 and 24h before ovulation. To achieve this, inseminations should be performed at 3 to 15h after the onset of behavioural oestrus (based on all behaviours, including sniffing and chin resting), at 3 to 15h after first observed mount, or at 0 to 12h after first observed standing heat if 3 times daily accurate assessment of oestrus signs is done, or at 5 to17 h after first increase in walking activity (using a pedometer with 2-hourly storage of number of steps). Timing of insemination according to the increase in activity seems the best strategy, because this increase in activity is observed in most of the oestrous periods and the pedometers are easy to implement in dairy practice.

Samenvatting

Om een koe drachtig te krijgen moet inseminatie op het juiste moment plaatsvinden. Succesvolle bevruchting is in hoge mate afhankelijk van het interval tussen inseminatie en ovulatie (= de eisprong). Het optimale tijdstip waarop geïnsemineerd zou moeten worden hangt aan de ene kant af van de vruchtbare levensduur van sperma in het vrouwelijke geslachtsapparaat na inseminatie en aan de andere kant van de vruchtbare levensduur van de eicel na ovulatie. In de praktijk is het tijdstip van ovulatie echter niet bekend; het inseminatietijdstip wordt gebaseerd op tochtgedrag (= het gedrag dat koeien laten zien voor ovulatie). Helaas is de (exacte) relatie tussen tochtgedrag en het tijdstip van ovulatie niet duidelijk. Het afkalfpercentage na eerste inseminatie is vrij laag, in Nederland ligt dit gemiddeld onder de 50%. Een belangrijke vraag is welk deel van dit lage afkalfpercentage toe te schrijven is aan het insemineren op een verkeerd (niet optimaal) tijdstip.
Het doel van het onderzoek dat is beschreven in dit proefschrift is tweeledig. Het eerste doel is het vaststellen van de relatie tussen verschillende tochtkenmerken en het ovulatietijdstip om zo te onderzoeken of/welke tochtkenmerken het ovulatietijdstip kunnen voorspellen. Het tweede doel is het bestuderen van effecten van verschillende intervallen tussen inseminatie en ovulatie op bevruchtingskans en embryo kwaliteit om zo een optimaal inseminatietraject te kunnen definiëren. Het uiteindelijke doel van het project is om een optimale inseminatiestrategie te ontwikkelen voor de praktijk.

Om relaties met het ovulatietijdstip te kunnen bestuderen, moet het ovulatietijdstip bepaald kunnen worden zonder het moment of andere processen rondom ovulatie te beïnvloeden. Het is bekend dat rectaal scannen gebruikt kan worden om het ovulatietijdstip te bepalen. Om het tijdstip nauwkeurig te kunnen bepalen moet het scannen regelmatig plaatsvinden. Daarom is een experiment uitgevoerd waarin bestudeerd werd of regelmatig rectaal scannen invloed heeft op tochtgedrag en hormoonprofielen rondom tocht en ovulatie (Hoofdstuk 2). In dit experiment werd de tocht gesynchroniseerd in twee opeenvolgende cycli. In de helft van de tochten werd het ovulatietijdstip bepaald door middel van frequent rectaal scannen (elke 3 uur, UG), de andere helft fungeerde als controle waarbij niet gescand werd (CG). Er waren geen significante verschillen tussen UG en CG in begin van de tocht (uren na synchronisatie), de duur van de tocht of de intensiteit van het tochtgedrag. Ook waren de profielen van LH, oestradiol en progesteron vergelijkbaar tussen UG en CG. Het experiment toonde aan dat regelmatig rectaal scannen geen invloed had op tochtgedrag en hormoonprofielen. De conclusie was dat scannen een bruikbaar middel is om het ovulatietijdstip in experimenten te bepalen om zo voorspellers voor het ovulatietijdstip (Hoofdstukken 3, 4 en 5) en

consequenties van inseminatietijdstip in relatie tot ovulatie (Hoofdstukken 6 en 7) te bestuderen.

De relatie tussen verschillende tochtkenmerken en het ovulatietijdstip is bestudeerd in twee experimenten die beschreven zijn in Hoofdstukken 3, 4 en 5. De volgende parameters zijn onderzocht: tochtgedrag (Hoofdstuk 3), aantal stappen (Hoofdstuk 4) en progesteron profielen (Hoofdstuk 5).
Tochtgedragingen werden elke 3u gedurende 30 min geobserveerd en het aantal stappen (activiteit) werd gemeten door middel van stappentellers die het aantal stappen per 2-uurs periode weergeven.
Tochtgedragingen De gedragingen 'sniffen' (ruiken aan de vulva van een andere koe) en kinrusten (het plaatsen van de kop op de rug van een andere koe) kwamen in alle tochten voor, terwijl springen (het bespringen van andere koeien) in 90%, en staande tocht (het doodstil blijven staan wanneer een koe besprongen wordt) in 58% van de tochten voor kwam. Ovulatie vond plaats 30.0±5.1u na het begin van de tocht. In de praktijk worden sniffen en kinrusten vaak niet opgemerkt als tochtgedrag. Wanneer alleen naar springgedrag of staande tocht gekeken wordt, vond ovulatie 28.7±5.3u na de eerste sprong en 26.4±5.2u na de eerste staande tocht plaats. Echter, zoals al vermeld kwam staande tocht maar in 58% van de tochten voor, waardoor het praktische gebruik van staande tocht als voorspeller van het ovulatietijdstip beperkt wordt. Springgedrag kan (nog) niet automatisch vastgesteld worden, wat ook beperkend werkt voor het gebruik van springgedrag als voorspeller van het ovulatietijdstip in de praktijk.
Activiteit Het aantal stappen dat een koe zet is hoger gedurende de tocht dan daarbuiten. In het huidige onderzoek zijn tocht attenties op basis van de stappenteller (significante verhogingen in het aantal stappen dat een koe zet) gedefinieerd, waarbij gebruik is gemaakt van verschillende algoritmes en drempelwaarden (Hoofdstuk 4). Het hoogste percentage van tochten dat gedetecteerd werd door de stappentellers was 87%. Ovulatie vond plaats 29.3±3.9u na de eerste verhoging in activiteit. Doordat de metingen eenvoudig uitvoerbaar zijn, er een goede relatie is tussen verhoging van de activiteit en het ovulatietijdstip en omdat een hoog percentage van de tochten als zodanig gedetecteerd wordt, lijken stappenteller-metingen een goede manier om het ovulatietijdstip te voorspellen.
Progesteron profielen Progesteronconcentraties in het bloed en de melk dalen voor de ovulatie in cyclische dieren. In Hoofdstuk 5 is een experiment beschreven waarin onderzocht werd of het verloop van progesteronconcentraties in melk en bloed gebruikt kan worden als voorspeller van het ovulatietijdstip. Het interval tussen verlaagde progesteronconcentraties en het ovulatietijdstip (bepaald met behulp van scannen elke 4u) zijn onderzocht. De standaarddeviaties van deze intervallen varieerde tussen 11.2 en 19.6u; de range van de verschillende metingen tot ovulatie was in het beste geval ongeveer twee dagen.

Voor wat betreft het eerste doel van het project, het voorspellen van het ovulatietijdstip, kan geconcludeerd worden dat de verschillende tochtgedragingen wel informatief zijn, maar (nog) niet geschikt om in de praktijk het ovulatietijdstip te voorspellen, vooral door de hoeveelheid arbeid die het vraagt om het gedrag nauwkeurig te observeren. Hoewel stappentellers wel een goede manier lijken om in de praktijk het ovulatietijdstip te voorspellen, moet nog onderzocht worden of het ook werkt op bedrijven met verschillend management, huisvesting etc. Het volgen van de progesteronconcentraties is niet geschikt om het ovulatietijdstip te voorspellen door de te grote variatie in het tijdstip van daling van progesteron en het ovulatietijdstip, maar wanneer progesteron concentraties automatisch gemeten zouden kunnen worden tijdens het melken zou dit een goed hulpmiddel kunnen zijn om vals-positieve tocht attenties van bijvoorbeeld stappentellers (er wordt een verhoging in het aantal stappen gemeten terwijl de koe niet tochtig is) te kunnen detecteren (Hoofdstuk 8).

In Hoofdstuk 6 en 7 zijn effecten van het interval tussen inseminatie en ovulatie op bevruchtingskans en embryokenmerken beschreven. In het kader van dit experiment zijn de inseminaties bewust uitgevoerd in een groot tijdsinterval, resulterend in inseminaties tussen 36u voor ovulatie en 12u na ovulatie. Zeven dagen na ovulatie werd het embryo/de eicel uitgespoeld en werd bepaald of bevruchting had plaatsgevonden en zo ja, wat de kwaliteit van het embryo was. Bevruchtingspercentages waren significant hoger bij inseminaties uitgevoerd tussen 36 tot 24u en 24 tot 12u voor ovulatie (85% en 82%) vergeleken met inseminaties na ovulatie (56%). Inseminaties uitgevoerd tussen 24 en 12u voor ovulatie resulteerde in het hoogste percentage levensvatbare embryo's (77%). De sexe ratio in de verschillende inseminatie-ovulatie intervallen was niet afwijkend van de verwachte 1:1 sexe ratio.
Ten aanzien van het tweede doel van het project, het vinden van een optimaal inseminatietraject, kan geconcludeerd worden dat het inseminatie-ovulatie interval waarin hoge bevruchtingspercentages behaald worden vrij lang is (van 36 tot 12u voor ovulatie), terwijl het interval waarin het grootste deel van de bevruchte eicellen zich ontwikkeld tot een embryo van goede kwaliteit aanzienlijk korter is (24 tot 12u voor ovulatie).

In Hoofdstuk 8 worden allereerst de mogelijke voorspellers van het ovulatietijdstip bediscussieerd op grond van hun toepasbaarheid in de praktijk. Vervolgens worden optimale inseminatie strategieën geformuleerd op basis van deze voorspellers. Gebaseerd op de kwaliteit van de uitgespoelde embryo's worden schattingen gegeven van het afkalfpercentage voor de verschillende 12-uurs inseminatie-ovulatie intervallen. Deze schattingen van het afkalfpercentage zijn sterk afhankelijk van de verwachte overlevingspercentages van embryo's van goede, gemiddelde en slechte kwaliteit en variëren uiteindelijk van 63% tot 73% voor

inseminaties uitgevoerd tussen 12 en 24u ovulatie. Hoewel dus het uiteindelijke afkalfpercentage niet betrouwbaar geschat kan worden, bleek de kans dat een inseminatie leidt tot de geboorte van een kalf in alle gevallen het grootst wanneer inseminaties uitgevoerd worden tussen 24 en 12u voor ovulatie. De consequentie hiervan voor de praktijk is dat koeien geïnsemineerd zouden moeten worden tussen 3 en 15u na de aanvang van tochtgedrag (gebaseerd op alle tochtgedragingen), tussen 3 en 15u na de eerst geobserveerde sprong of tussen 0 en 12u na de eerst geobserveerde staande tocht, mits 3 maal daags nauwkeurige gedragsobservaties uitgevoerd worden, koeien moeten worden geïnsemineerd tussen 5 en 17u na verhoging in de activiteit (gebruikmakend van stappentellers die het aantal stappen registreren in periodes van 2u). Insemineren op basis van de verhoging in activiteit lijkt de beste strategie, omdat deze verhoging in activiteit in de meeste tochten voorkomt en stappentellers eenvoudig te implementeren zijn in de praktijk.

Curriculum Vitae

Personalia

Judith Bernardine Roelofs werd geboren op 13 maart 1975 in Arnhem en groeide op in Nijmegen. In juni 1993 behaalde zij haar VWO-diploma aan het Dominicus College te Nijmegen. In september 1993 begon zij aan de Hogere Agrarische School in Den Bosch aan de studie Veehouderij, deze studie werd in juni 1998 afgerond. In september 1998 begon ze aan de studie Zoötechniek in Wageningen, met als oriëntatie Veehouderij. Deze studie werd in september 2000 afgerond. Van juni t/m december 2000 vervulde zij een functie bij het Institute for Pig Genetics in Beuningen. In januari 2001 werd zij aangesteld als toegevoegd onderzoeker bij de vakgroep Adaptatiefysiologie aan de Wageningen Universiteit om vervolgens in september 2001 te starten met een promotie-onderzoek, bij dezelfde vakgroep, waarvan dit proefschrift het resultaat is.

List of publications

Roelofs J.B., Bouwman E.G., Soede N.M. and Kemp B. (2002). Influence of repeated ultrasound on oestrus and ovulation in cattle. Reprod In Dom Anim 37:249 (abstract)

Roelofs J.B., Van Eerdenburg F.J.C.M., Soede N.M. and Kemp B. (2003). Possibilities to predict time of ovulation in cattle. 19th Meeting Association Européenne de Transfert Embryonnaire, Rostock, Germany 19:83-91

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Measuring Behaviour, Amsterdam, The Netherlands (2002)
19th Scientific Meeting Assocation Européenne de Tranfert Embryonnaire, Rostock, Germany (2003)-oral presentation
15th International Congress on Animal reproduction, Porto Seguro, Brazil (2004)- poster presentation
9th Conference of the European Society for Domestic Animals, Murcia, Spain (2005)-two poster presentations
Regiomiddag ET, Deventer, the Netherlands (2004)-oral presentation
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