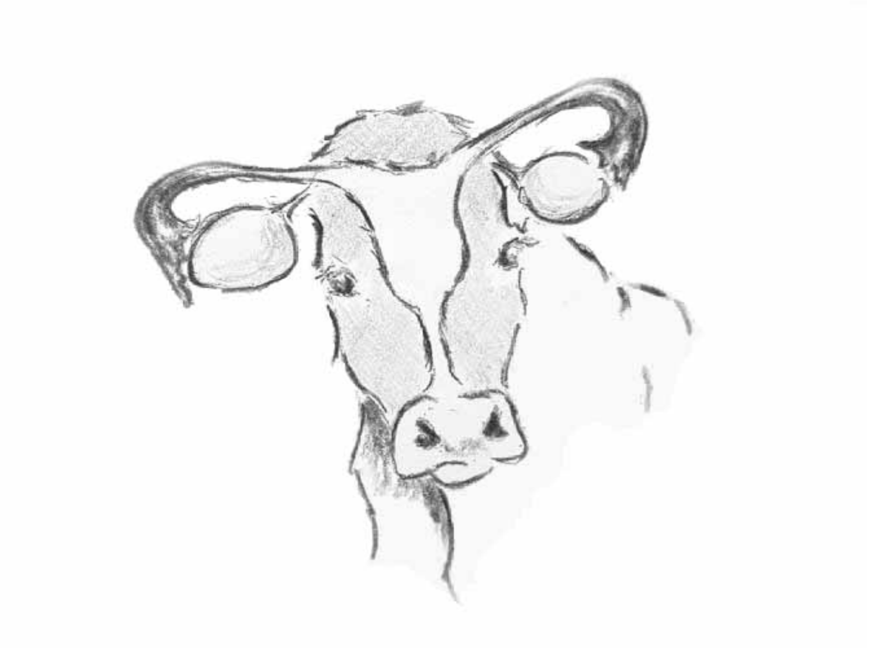
Progesterone Profiles, Oestrous Expression and Pregnancy in Dairy Cows

Sofia Nyman
Faculty of Veterinary Medicine and Animal Science
Department of Animal Breeding and Genetics
Uppsala



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Abstract

The cow's ability to reproduce is essential for milk production. Traditionally genetic evaluations for fertility have been based on measures on insemination- and calving dates, which in general are highly influenced by on-farm decisions. Unfortunately, the low heritability of classical fertility traits makes the genetic improvement slow. This thesis aims to provide information useful for a genetic evaluation utilising progesterone (**P4**) based fertility traits having higher heritability, and by using genomic information to try to identify genetic markers associated with fertility. The aim was also to investigate the characteristics of oestrous expression, pregnancy losses and their phenotypic relationships to P4 profiles as future potential traits included in breeding evaluation. Progesterone data from two data sets were used in the analyses: in Papers I and II from Swedish Red (**SR**) and Swedish Holstein (**SH**) dairy cows, and in Papers III and IV from Holstein-Friesian cows from four different countries. Swedish Red cows had higher conception rate, more intensive oestruses and longer oestrus durations compared to SH cows, irrespectively housing systems. Conception rate was found to increase with stronger oestrus intensity (**OI**), from 24% for with weaker and more uncertain oestrous symptoms, e.g. red and swollen vulva, to 54% for primary oestrous symptoms, e.g. standing. A total pregnancy loss of 65% was found for Swedish dairy cows, with an early embryonic loss of 29%. Swedish Red cows had significantly lower total pregnancy loss compared to SH cows (62 vs 68%). Early embryonic loss and total pregnancy loss had a tendency to decrease, while OI increased, with increasing cycle number. Cows with pregnancy losses had somewhat higher P4 levels at the day of insemination and lower P4 levels at some time points during gestation compared to pregnant cows. Heritability estimates were moderate for delayed cyclicity and commencement of luteal activity (**CLA**; 0.24 and 0.18 respectively) as well as the genetic correlation with milk yield in early lactation (r_g=0.57 and 0.45). This may imply deterioration in these traits if not considered in the breeding evaluation. A genome-wide association study identified 44 genetic markers associated with the seven endocrine fertility traits. Three chromosomes were further fine-mapped for delayed cyclicity, cessation of cyclicity, CLA and oestrous cycle length using imputed sequences. Five regions with several possible candidate genes related to reproductive functions were identified. However, due to the high linkage disequilibrium it was not possibly to pinpoint a specific causal mutation. In the future, emphasis should be put on how automated P4 registration and oestrus detection could be used to improve and increase the number of registrations for e.g. OI and CLA and how we beneficially can include these in the genomic breeding evaluation.

Keywords: dairy cattle, fertility, oestrus, oestrus intensity, pregnancy, progesterone, progesterone profile, embryonic loss, GWAS, imputation

Author's address: Sofia Nyman, SLU, Department of Animal Breeding and Genetics, P.O. Box 7023, SE-750 07 Uppsala, Sweden

Progesteronprofiler, brunstvisningsförmåga och dräktighet hos mjölkkor

Sammanfattning

Mjölkkornas fruktsamhet har en avgörande roll för mjölkföretagens ekonomi. Den traditionella avelsvärderingen baseras på fruktsamhetsmått som är beräknade med hjälp av registreringar av inseminerings- och kalvningsdatum och är därmed beroende av besättningsägarens skötselstrategier. Tyvärr har den låga arvbarheten hos de klassiska fruktsamhetsegenskaperna bidragit till ett långsamt genetiskt framsteg för dessa egenskaper. Syftet med denna avhandling var att ta fram användbar information om progesteronbaserade fruktsamhetsmått med högre arvbarheter och genom att nyttja genomisk information försöka identifiera genetiska markörer kopplade till fruktsamhet. Syftet var även att undersöka förekomsten av brunstsymtom och brunsstyrkans betydelse samt omfattningen av dräktighetsförluster hos våra svenska mjölkkor. Även sambandet mellan brunststyrka, dräktighetsförluster och atypiska progesteronprofiler studerades. Två olika material användes för att studera progesteron (**P4**); i studie I och II användes data från Svensk röd och vit boskap (**SR**) och Svensk Holstein (**SH**), och i studie III och IV användes data från Holstein-Friesiankor från fyra olika länder. SR-kor hade högre dräktighetsresultat och starkare och längre brunster jämfört med SH-kor, oberoende av stallsystem. Dräktighetsresultatet blev bättre med starkare brunst, från 24% (svaga och osäkra brunsttecken, t.ex. röd och svullen vulva) till 54% (primära brunsttecknen, t.ex. stå för upphopp). En total dräktighetsförlust på 65% observerades hos de svenska mjölkkorna, där tidig embryoförlust låg på 29%. SR-kor hade signifikant lägre totala dräktighetsförluster än SH-kor (62 vs. 68%). Tidiga embryoförluster och totala dräktighetsförluster hade en tendens att minska, medan brunststyrkan ökade, med ökande ägglossningsnummer. Kor med dräktighetsförluster hade något högre P4-värden vid insemineringstillfället och lägre P4-värden vid vissa tidpunkter under dräktigheten jämfört med dräktiga kor. Medelhöga arvbarheter skattades för försenad brunstcykelstart och intervallet från kalvning till start av luteal aktivitet (**CLA**; 0.24 och 0.18). Försenad brunstcykelstart och CLA var också genetiskt korrelerad med mjölkavkastning i tidig laktation (r_g=0.57 and 0.45) vilket kan tyda på att dessa två egenskaper kommer att försämras om de inte tas hänsyn till i avelsvärderingen. I en genomisk associationsstudie identifierades 44 signifikanta genetiska markörer associerade med de sju endokrina egenskaperna. Genom att använda imputerade sekvenser finmappades tre kromosomer för att analysera försenad brunstcykelstart, avbruten cyclicitet, CLA och brunstcykelns längd. Fem regioner med flertalet möjliga kandidatgener med reproduktionsfunktion identifierades. Dock kunde de specifika kausala mutationerna inte hittas på grund av att flera närliggande markörer var starkt kopplade till regionerna. I framtiden borde det läggas vikt på hur automatiska brunstregistreringar och P4 mätningar kan användas för att förbättra och öka mängden registreringar för t.ex. OI and CLA och hur vi på bäst sätt kan inkludera dessa i en genomisk avelsvärdering.

Nyckelord: mjölkko, fruktsamhet, brunst, brunststyrka, dräktighet, progesteron, progesteronprofiler, embryoförluster, GWAS, imputering

Dedication

To my beautiful daughters, Saga and Esther
I love you to the moon and back

"However difficult life may seem, there is always something you can do, and succeed at. It matters that you don't just give up"

-Stephen Hawking

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nyman, S., Malm, S.E., Gustafsson, H. and Berglund, B. (2016). A longitudinal study of oestrous characteristics and conception in tie-stalled and loose-housed Swedish dairy cows. *Acta Agriculturae Scandinavica Section A-Animal Science* 66(3), 134-144.

II Nyman, S., H. Gustafsson and B. Berglund. Extent and pattern of pregnancy losses and progesterone levels during gestation in Swedish Red and Swedish Holstein dairy cows (*submitted*).

III Nyman, S., Johansson, K., de Koning, D.J., Berry, D.P., Veerkamp, R.F., Wall, E. and Berglund, B. 2014. Genetic analysis of atypical progesterone profiles in Holstein-Friesian cows from experimental research herds. *J. Dairy Sci* 97, 7230-7239.

IV Nyman, S., S. Duchemin, D.J. de Koning and B. Berglund. Genome-wide Association Study for Normal and Atypical Progesterone Profiles in Holstein-Friesian Dairy Cows (*manuscript*).

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Abbreviations

50K	Illumina BovineSNP50 v1 BeadChip
AI	Artificial insemination
AMS	Automated milking system
AR2	Imputation accuracy
BCS	Body condition score
BTA	*Bos Taurus* autosome
CFH	Calving to first observed heat
CFS	Calving to first service
CI	Calving interval
CLA	Commencement of luteal activity
CR	Conception rate
CWAS	Chromosome-wide association study
DIM	Days in milk
EBV	Estimated breeding value
ECM	Energy corrected milk
EEL	Early embryonic loss
FL	Foetal loss
FLOS	The interval from first to last observed oestrous symptom
FLS	Interval from first to last service
FOO	Interval from calving to first ovulatory oestrus
GEBV	Genomic estimated breeding value
GS	Genomic selection
GWAS	Genome-wide association study
HF	Holstein-Friesian
ILI	Inter-luteal interval

IOI	Inter-ovulatory interval
LD	Linkage disequilibrium
LEL	Late embryonic loss
LPL	Luteal phase length
MAF	Minor allele frequency
Mbp	Megabase pair
NAV	Nordic cattle genetic evaluation
NEB	Negative energy balance
OI	Oestrus intensity
P4	Progesterone
PFS	Pregnancy at first service
QTL	Quantitative trait locus
SH	Swedish Holstein
SNP	Single nucleotide polymorphism
SR	Swedish Red
TAI	Timed artificial insemination
UK	United Kingdom
WGS	Whole genome sequencing

1 Introduction

The cow's ability to reproduce is essential for milk production and is a key factor for cost efficiency in the dairy industry. A general decline in fertility was observed in most populations until the early 2000s (Berry et al., 2014) as a negative effect from the concurrent increase in milk production over the same time period (Pryce and Veerkamp, 2001). There is an important relationship between fertility and milk yield. Fertility has an impact on milk yield through the effect of pregnancy. The basic assumption is that the average number of empty days is longer than the recommended of 50 days. Therefore it is assumed that a shorter calving interval will reduce the average number of empty days but not the average days in milk (NAV, 2018a). Genetics is known to contribute to the variation in cattle performance traits including milk production.

Reproductive and management factors contributing to the deterioration in the overall fertility trait may include feeding, poor return to cycling after calving, poor oestrous expression and detection, as well as inappropriate timing of insemination (Walsh et al., 2011). Dobson et al. (2008) documented that the percentage of animals that stand to be mounted and the duration of standing heat have decreased during the last decades, whereas the number of silent oestruses has increased with increasing milk production. The economic consequences of fertility are mostly due to changes in calving interval, since this has an effect on annual production per cow. There is also an effect of the artificial insemination (**AI**) costs and the labour it involves, and the cost of any labour involved in oestrus detection. An early resumption of ovarian activity accompanied by visual oestrous symptoms is therefore essential. Early resumption of ovarian cyclicity postpartum facilitates a greater number of oestrous cycles before insemination which, on average, increases the likelihood of subsequent conception (Darwash et al., 1997).

Impaired fertility results in additional inseminations, higher replacement rates and increased culling rate. In fact, fertility problems are cited as one of the most common reasons for industry culling (Ahlman et al., 2011). During 2016 about 18% of the dairy cows in Sweden were culled due to reduced fertility (Cattle statistics, 2017).

1.1 Swedish dairy production

Dairy cattle production has undergone considerable changes during the past decades, which is also true for Sweden. These structural changes have led to a decreased number of cows in fewer but larger herds. In the year of 1999 there were approximately 14,000 dairy herds in Sweden but that number have decreased to 3,900 in 2016 (Statistics Sweden, 2017). Correspondingly, in the year 1999 the average number of dairy cows per herd was 32 and had increased to 85 cows per herd in year 2016 (Statistics Sweden, 2017). The possibility for increasing the number of cows per herd is connected to the development of milking equipment and corresponding housing system. An increasing number of cows are kept in free-stalls compared to tie-stalls, which is an on-going trend, as farmers in Sweden have not been allowed to build new tie-stalls since 2007. In 2016, 68% of the cows were held in free-stalls, where half of them were milked in automated milking systems (**AMS**) and the other half in parlours.

The two most common breeds in Sweden were, in 2017, Swedish Holstein (**SH**; 55.2%) and Swedish Red (**SR**; 35.8%). The SH breed is increasing compared to the SR breed (Cattle statistics, 2018).

1.1.1 The Nordic breeding evaluation

Figure 1 show the genetic trend for female fertility in Sweden for Holstein and SR dairy cattle between 1996 and 2016. In Sweden fertility has been included in the Swedish genetic evaluation since 1974 (Lindhé et al., 1994). The SH dairy cows decreased in fertility until the early 2000s (Lindhé and Philipsson, 2001) where after it levelled out and started to slowly increase again in 2008, while for the SR dairy cows the genetic trend have remained stable (Figure 1).
In 2002 the Nordic Cattle Genetic Evaluation (**NAV**) was established, including Sweden, Denmark and Finland, and in 2005 the first estimated breeding values (**EBV**) for fertility together with conformation, milking speed and temperament was published. The fertility index in the Nordic breeding evaluation describes the genetic potential to start or resume oestrous cycle after calving, to show oestrus and to conceive at insemination. The fertility index includes breeding values for sub-indexes as interval from calving to first insemination (cows), interval from first to last insemination (heifers and cows),

and number of inseminations (heifers and cows). The fertility index is included in the Nordic Total Merit index (NTM). NTM is the breeding goal and aims for healthy, fertile, well producing and long-lasting cows with good conformation and describes the total economic potential determined by genetics (NAV, 2018b). In addition to the breeding values included in the NTM, breeding values for conception rate (heifers and cows) and oestrus intensity/heat strength (heifers and cows in Sweden) are estimated and used as indicator traits.

The first genomic prediction of breeding values in Sweden was performed for Holstein in 2008 and the first genomic estimated breeding values (**GEBV**) were published in 2011.

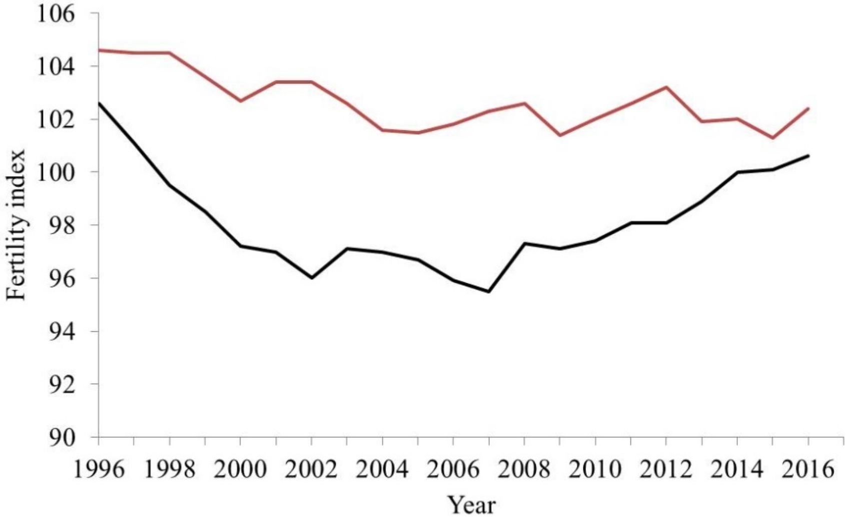

Figure 1. Genetic trends in female fertility for Holstein (black line) and Swedish red dairy cattle (red line) from 1996 to 2016 (NAV, 2018c).

1.2 Fertility traits in dairy cows

The female fertility is composed of a number of physiological stages and includes the ability of the cow to start an oestrous cycle after calving, express oestrous symptoms, become pregnant after insemination or breeding and to maintain the pregnancy. Depending on how the traits are recorded they are either described as continuous traits or as phenotypes expressed in distinct classes, i.e. threshold traits. Fertility traits used in breeding objectives are based on a number of recorded traits that characterize the female fertility (see chapter 1.1.1). These traits are sometimes criticized because they do not reflect the cow’s true physiological status instead they are more dependent on management factors such as the farmers’ decisions (e.g. poor oestrus detection and voluntary waiting period). Phenotypes for the classical fertility traits are based on information about insemination- and calving dates reported by the farmer.

Endocrine fertility traits, including progesterone (**P4**) based fertility measures, can offer a more objective and accurate measurement of the ovarian activity in dairy cows. Heritabilities for endocrine fertility traits determined by e.g. P4 profiles are generally higher than classical fertility traits. Previous studies of the heritability of commencement of luteal activity (**CLA**) have been reported to be between 0.13 and 0.28 (Darwash et al., 1997; Veerkamp et al., 1998; Berry et al., 2012; Tenghe et al., 2015). A higher heritability estimate could be due to a more effectively estimated genetic variance, as P4 based fertility traits are free from management influences, such as e.g. oestrus detection, and AI dates (Pryce and Veerkamp, 2001). Other reproduction phenotypes derived from P4 profiles in milk can include length of first luteal phase, persistency of corpus luteum, delayed ovulation and percentage of animals with a milk progesterone concentration greater or equal to 3 ng/ml in the first 60 days post-calving (Royal et al., 2000; Petersson et al., 2006b).

1.2.1 Progesterone and progesterone profiles

Luteal P4 is essential for the preparation of the uterus and oocyte before AI, as well as for the maintenance of an optimal uterine environment and supporting the uterus to develop the embryo/foetus during the gestation in the cow (Starbuck et al., 2004; Fair and Lonergan, 2012; Karen et al., 2014). To study P4 concentration and ovarian activity in dairy cows milk P4 is commonly used. Because of the high correlation between P4 concentration in milk and blood (0.88; Dobson and Fitzpatrick, 1976) P4 analysis of milk samples can be used to study the ovarium activity after calving.

The oestrous cycle averages 21 days (range 18-24 days) and consists of two phases: the follicular phase (3-5 days), and the luteal phase (16-18 days). Although, reports have demonstrated that variation in the cycle length has significantly increased (Kerbrat and Disenhaus, 2004). The oestrous cycle starts with a period when the reproductive organs prepare for oestrus and the sexual reproductive period (Hurnik, 1987). The ovarian follicles start to grow and high levels of oestrogen are produced. During this period P4 levels are low and the increased oestrogen levels will trigger sexual behavior, prepare the genital tract for copulation and facilitate the sperm transport (Hurnik, 1987; Sjaastad et al., 2004). When the cow enters oestrus, the P4 levels remain low and oestrogen levels are at a peak. At the end of the oestrus oestrogen decreases rapidly and ovulation and rupture of the follicles occurs. The corpus luteum (**CL**) is then formed and produces P4. Peak levels of P4 will be reached about eight days after ovulation and remain high for about 14 days during the luteal phase. After that the CL is degenerated and a new ovulation and follicular phase can occur, unless the cow becomes pregnant and the CL is maintained during the pregnancy (Forde et al., 2011).

Deviations from a normal oestrous cycle, also called atypical P4 profiles, have been associated with reduced fertility, e.g. longer calving to first service (**CFS**) and lower conception and pregnancy rates (Royal et al., 2002; McCoy et al., 2006; Petersson et al., 2006a). This will likely result in a decreased milk production per cow, and reduced herd profitability. Opsomer et al. (1999) studying primiparous dairy cows from Belgium reported that the most common deviations of the oestrous cycle were a late start of cyclicity and prolonged luteal phases, which together constituted 43% of all profiles and 88% of all atypical P4 profiles. Petersson et al. (2006a) studying SR and SH cows found a higher proportion of delayed cyclicity compared to prolonged luteal phase and cessation of cyclicity.

1.3 Oestrus detection

One of the underlying mechanisms of the decreased fertility rates is depressed oestrous behavior, which makes it difficult for the farmers to determine the optimal time for AI (Dobson et al., 2008). Failure to detect cows in oestrus or to inseminate cows that are not in oestrus results in delayed insemination and longer calving interval (**CI**) which may impact the economy for the farmers.

There are different methods to detect oestrus e.g. visual oestrus detection, activity transponders and mounting detectors. Visual detection of oestrus is labor intensive and challenging, and requires skilled observers. The use of activity transponders are increasing in herds with loose-housed cows and are useful since the herds, in Sweden and globally, are getting bigger and goes towards loose-housing systems. In Sweden and in many other countries, also tie-stall systems for housing still remain common (Ranasinghe et al., 2009; Cattle Statistics, 2017) where activity transponders are not suitable and visual detection is a better method for oestrus detection. There are methods, e.g. timed artificial insemination (**TAI**), to facilitate the timing of AI and avoid labor intensive oestrus detection. These methods are not an option in Sweden together with some other European countries since they use includes different exogenous hormones.

1.3.1 Behavior characteristics related to oestrus

Oestrus in cows is characterized by a period wherein the cow is receptive of being mounted by a bull or a herd mate. This period, referred to as the receptive phase of oestrus or the true oestrus, is regarded as the period when the cow expresses standing oestrus and she is not making any effort to be mounted by other cows (Hurnik et al., 1975; Sveberg et al., 2011). Standing oestrus is only shown by about 50% of cows in oestrus and lasts for a short period of time of about five to seven hours (Roelofs et al., 2005; Sveberg et al., 2011).

The primary oestrous symptoms stand to be mounted and lowering of the back are accompanied by secondary symptoms such as mounting, anogenital sniffing, chin resting, anxiety, licking and rubbing, all of which are regarded as less accurate because they can occur during other periods of the oestrous cycle than the receptive phase (Diskin and Sreenan, 2000; Kerbrat and Disenhaus, 2004; Roelofs et al., 2010). Moreover, there are a number of local genital symptoms, such as swelling and redness of the vulva as well as vulvar discharge, that are highly associated with oestrus (Stevenson et al., 1983; Roelofs et al., 2010).

Behavioral expressions are more apparent when the cows are loose, but this impedes observation of local symptoms. Cows that are tied cannot exhibit standing or mounting behaviors, and farmers must rely on the detection of secondary and local oestrous symptoms (Ranasinghe et al., 2009). Tied cows express sexual receptivity by lowering their backs and raising their tails upon contact with a neighbor animal or with the herdsman (Gustafsson, 1984; Hurnik, 1987).

1.4 Pregnancy losses

Pregnancy losses are a major cause of infertility in dairy cows and are probably the major source of economic wastage in the modern dairy system (Diskin et al., 2012). By diagnosing pregnancy losses at an early stage open cows can be identified and reduced which may lead to an increased profitability.

Despite the high fertilisation success rate in cattle after AI (~90%), calving rates are significant lower (30-50%) indicating the occurrence of extensive embryonic and foetal losses during the pregnancy (e.g. Aylon et al. 1978; Santos et al., 2004; Diskin et al., 2012). The Committee on Bovine

Reproductive Nomenclature (1972) established the embryonic period from the fertilisation to the differentiation stage, at approximately 42 days after insemination, and the foetal period from day 42 to calving. Moreover, embryonic losses are often categorized as early or late before and after day 25, respectively.

Embryonic mortality appears to be greater in modern high-yielding dairy cows and more embryos dies before day seven after AI compared with in low-producing dairy cows and heifers (e.g. Diskin et al., 2012). Although the majority of pregnancy loss occurs during the early embryonic period, the extent of late embryonic and foetal loss causes higher and more serious economic losses to producers, especially in seasonal production systems, because it is often too late to rebreed the cow (Walsh et al., 2011; Diskin et al., 2012).

1.4.1 Progesterone and pregnancy losses

Progesterone concentrations during both the cycle preceding and following insemination affect embryo survival. There is strong evidence that both excessive and insufficient P4 concentrations at specific time points are negatively associated with pregnancy results (for review, see Diskin et al., 2012). Changes in P4 during the luteal phase immediately before oestrus and after AI can cause losses of embryos during days four to eight after oestrus, during maternal recognition of pregnancy on days 14 to 17 after oestrus, and during the late embryonic period (between day 28 to 42-50 (Inskeep and Daily, 2005). Atypical P4 profiles have a deviating P4 concentration pattern compared to a normal oestrous cycle, and have in earlier studies been associated with longer calving to first service, calving intervals, and reduced conception rates (Darwash and Lamming, 1998; Royal et al., 2002).

For the SR and SH dairy cows the incidence and pattern of embryonic and foetal losses after AI in spontaneous oestrus have not been investigated in earlier studies. Although, many reports have described pregnancy losses in a limited number of HF cows with synchronized oestruses under pastoral conditions or in loose housing systems (for review see Diskin et al., 2012)

1.5 Gene mapping

Fertility is a complex trait which means that it is influenced by many genes with small effects and also to a large extent by environmental factors.

It is of interest to find regions, also known as quantitative trait locus (**QTL**), across the cattle genome that are responsible for some of the genetic variation in fertility traits. QTL are stretches of DNA containing a gene or a group of genes that explain a measurable part of the variation of a trait. To find these regions, the genome is screened by using genetic markers such as e.g. single-nucleotide polymorphism (**SNP**) markers with known locations. The aim of genome-wide association studies (**GWAS**) is to identify specific genes or chromosomal regions related to the specific trait.

By using whole-genome sequences (**WGS**) causal variants underlying QTL could be identified more effectively using GWAS. Whole-genome sequences should contain the polymorphisms that are causing the genetic differences between individuals. Daetwyler et al. (2014) who were the first to implement WGS data in cattle, and Druet et al. (2014) found higher precisions in detecting QTL if the data used includes the causal variants. It is possible to increase the power and precision of QTL mapping further by increasing the numbers of markers with imputed sequences (Duchemin et al., 2016; Höglund et al., 2014; Sahana et al., 2014). In addition to revealing the architecture structure that underlies the physiological and biological process of female reproduction, this information could in practice be applied to genomic selection (**GS**) schemes.

Most of the previous GWAS have been performed on classical fertility traits, e.g. calving to first service (Höglund et al., 2009, Olsen et al., 2011, Berry et al., 2012), pregnancy rates (Berry et al., 2012), and non-return rate (Olsen et al., 2011). Only a few GWAS have been performed on endocrine fertility traits. Tenghe et al. (2016) found 17 QTL regions spread on eight *Bos Taurus* autosomes (**BTA**) for six endocrine fertility traits. Berry et al. (2012) found clear associations with CLA for two SNPs, one on BTA2 (130.1 Mbp) and one on BTA21 (9.38 Megabase pair (**Mbp**)). Tenghe et al. (2016) reported regions on chromosome 2 and 3 (among others) that are likely to include mutations associated with the CLA, inter-ovulatory interval, luteal phase length and CFS amongst other traits. The high costs associated with data collection in larger populations are reflected in the low number of genomic studies on fertility traits, and especially for endocrine fertility traits. Large data of classical fertility traits based on calving and insemination dates are relatively easier to get.

2 Aims of the thesis

The overall aim of the thesis was to study how we can use oestrus observations, progesterone profiles and classical fertility traits in an improved breeding for dairy cattle fertility. More specifically, the aims were to investigate:

- if the oestrous expression have changed with increased milk production and if there are any differences between Swedish Red and Swedish Holstein dairy cows and between tie-stalled and loose-housed cows (*Paper I*)

- the extent and pattern of pregnancy losses in Swedish Red and Swedish Holstein dairy cows and if there are associations between oestrous expression, progesterone profiles and pregnancy losses (*Paper II*)

- genetic parameters for measures of atypical progesterone profiles and to investigate if the information could be useful in an improved genetic evaluation for fertility (*Paper III*)

- the associations between genomic regions and the normal and atypical progesterone profiles to identify possible marker variants or candidate genes associated with atypical progesterone profiles (*Paper IV*).

3 Overview of Materials and Methods

The thesis is mainly based on data from a semi-commercial herd in Sweden collected by the Department of Animal Breeding and Genetics at the Swedish University of Agricultural Sciences. Paper III and IV are also based on British (from the Scotland's Rural College, United Kingdom), Dutch (from Wageningen UR Livestock Research, the Netherlands) and Irish (from Teagasc, Moorepark, Ireland) material.

A summary of data and traits used in Papers I-IV is can be found in Table 1. Records of calving data, services, heat observations and pregnancy diagnosis were used to calculate the more classical fertility measures including calving to first observed heat (**CFH**), calving to first service (**CFS**), pregnancy at first service (**PFS**), interval from first to last service (**FLS**) and calving interval (**CI**).

3.1 Animals and management

3.1.1 Swedish material

The data was collected in a semi-commercial herd in Uppsala by the Department of Animal Breeding and Genetics, the Swedish University of Agricultural Sciences. Data for SR and SH dairy cows between January 1992 and December 2008 was used in Papers I and II, and data for SH dairy cows between 1987 and 2011 was used in Papers III and IV (here named HF cows instead of SH cows). The cows were in their 1st to 10th lactation. The average milk production at day 60 after calving was for SR cows 1,911 kg ECM and for SH cows 2,044 kg ECM. The experimental herd was kept at Jälla agricultural collage and the cows were in either a tied or a loose housing system in the same building. From 1994 the cows were subject to a calving interval trial, in which they were inseminated for expected calving intervals of either 12 or 15 months. During this trial, 50% of the cows had a pre-planned voluntary waiting period of 140 days.

Table 1. *Summary of data and endocrine fertility traits used in Paper I-IV*

Trait[1]	Breed[2]	No. of cows	No. of ins.	In Paper
Oestrous traits				
FLOS	SR + SH	417	1,016	I
FOO	SR + SH	280	363	II
Oestrus intensity	SR + SH	568	2,082	I-II
Pregnancy traits				
Conception rate	SR + SH	571	2,130	I
Early embryonic loss	SR + SH	156	619	II
Late embryonic loss	SR + SH	71	273	II
Foetal loss	SR + SH	36	171	II
Total pregnancy loss	SR + SH	263	1,063	II
Atypical progesterone profiles				
Cessation of cyclicity	SR + SH	37	72	II
Cessation of cyclicity	HF	53	94	III-IV
Delayed cyclicity	SR + SH	23	43	II
Delayed cyclicity	HF	138	178	III-IV
Prolonged luteal phase	SR + SH	94	222	II
Prolonged luteal phase	HF	170	240	III-IV
Progesterone level traits				
P4day0	SR + SH	449	1,790	II
P4day10	SR + SH	488	1,854	II
P4day21	SR + SH	389	1,456	II
P4day30	SR + SH	249	957	II

[1] FLOS=the interval from first to last observed oestrous symptom, FOO=interval from calving to first ovulatory oestrus, P4day0=Progesterone concentration at insemination, P4day10=Highest progesterone concentration days 7-13 after insemination, P4day21=Highest progesterone concentration days 18-24 after insemination, P4day30= Highest progesterone concentration days 27-33 after insemination.

[2] SR=Swedish Red, SH= Swedish Holstein and HF=Holstein Friesian

Milk sampling for P4 analysis started in the second week after calving. Milk was sampled twice weekly until ovarian activity was detected. Sampling was then reduced to once a week until first AI. Thereafter milk P4 was analysed at every AI and at day 10 and 21 after AI. Milk P4 concentration was determined in whole milk. In Sweden the following four different kits were used to determine P4 concentrations; from the start of the collection of data until 1995, the Farmose kit (Orion Diagnostica, Espoo, Finland) was used; between the years 1995 and 1998, the Spectra kit (Orion Diagnostica) was used; from 1998 to the start of December 2007 the Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA) was used; and from December 2007, the Ridgeway kit was used (Ridgeway Science Ltd., Alvington, UK). The threshold values for the start of the luteal phase, which is the period where the corpus luteum secretes high P4, were for these kits 25.4, 9.5, 4.1 and 5.0ng/ml respectively.

In Papers I and II oestrus observations were studied. After calving, cows in both housing systems were visually observed, for approximately 20 min, for oestrous symptoms by the experienced research herd staff at three fixed times per day (07:30, 11:30 and 17:00). At each oestrus observation, one or more oestrous symptoms were recorded and scored according to a scoring system modified from Van Eerdenburg et al. (1996) and used by Växa Sverige (Table 2). The oestrous symptoms included in this thesis were, stand to be mounted, mounting other cows, lowering of the back, anxiety, mooing, licking, swelling and redness of vulva, discharge and discharge color. Data regarding P4 levels were not available for the herd staff at the time of oestrus detection. The decision to proceed with AI was made by the herd staff. A maximum of five AIs were allowed per breeding period and the AI period were restricted to a maximum of 130 days; thereafter the cows' were culled due to infertility.

Table 2. *Definition of an oestrus intensity scoring system based on that used by Växa Sverige*

Score		Definition
1	Very weak	Very weak uncertain symptoms (e.g., symptoms of dried vulvar discharge)
2	Weak	Weak uncertain symptoms (e.g., discharge, a red and swollen vulva, and anxiety)
3	Normal	More evident symptoms (e.g., lowering of the back when touched, clear and stringy discharge, mounting other cows and occasionally standing to be mounted)
4	Strong	Spontaneous lowering of the back, plentiful stringy discharge, several recorded mountings and standing to be mounted
5	Very strong	Very strong sexual activity, spontaneous lowering of the back, very frequent mountings and standing to be mounted

3.1.2 British material

The British material in Paper III and IV was from a research herd at Scotland's Rural College, United Kingdom (**UK**). The data was collected between 2003 and 2005 and comprised 238 lactations from 148 HF dairy cows. The average milk production at day 60 after calving was 1,306 kg ECM. Milk P4 was sampled and analysed three times per week until the first 140 days in the lactation. Milk P4 concentration was measured in whole milk and analysed using the Ridgeway kit (Ridgeway Science Ltd, Rodmore Mill Farm, Alvington, UK). The threshold value for the start of the luteal phase was 5.0ng/ml.

3.1.3 Dutch material

The Dutch material in Paper III and IV was from a research herd at Wageningen UR Livestock Research, the Netherlands. The data was collected between 1991 and 1998, and between 2003 and 2004, and comprised 672 lactations from 666 HF dairy cows. The average milk production at day 60 after calving was 1,912 kg ECM. Milk P4 was sampled and analysed twice weekly for the first 100 days of lactation. Milk P4 concentration was measured in whole milk and analysed using the Ridgeway kit (Ridgeway Science Ltd, Rodmore Mill Farm, Alvington, UK). The threshold value for the start of the luteal phase was 5.0ng/ml.

3.1.4 Irish material

The Irish material in Paper III and IV was from a research herd at Teagasc, Moorepark, Ireland. The data was collected between 2001 and 2004 and comprised 280 lactations from 168 HF dairy cows. The average milk production at day 60 after calving was 1,266 kg ECM. Milk P4 was sampled and analysed three times per week until 26 days after first AI. Milk P4 concentration was measured in whole milk and analysed using the Ridgeway kit (Ridgeway Science Ltd, Rodmore Mill Farm, Alvington, UK). The threshold value for the start of the luteal phase was 5.0ng/ml.

3.1.5 Marker data

In Paper IV, DNA extracted from blood samples was used for genotyping purposes. Genotyping was performed with the Illumina BovineSNP50 v1 BeadChip (Illumina Inc., San Diego, US; **50K**). A total of 1,735 cows with genotypic information were included in the analyses.

A reference population of 547 HF cows and bulls from the 6th run (Run6) of the 1000 Bull Genomes Consortium was available (Daetwyler et al., 2014). The information from the reference population was used to impute the genotypes of our experimental population from 50K to sequence data. All positions of the SNPs on the 50K SNP genotypes and variants on the sequences were aligned to the bovine genome assembly UMD3.1 (Zimin et al., 2009; SNPchiMp, 2018).

3.2 Fertility traits

3.2.1 Progesterone based fertility traits

Progesterone concentrations were plotted against days postpartum to create individual lactation P4 profiles. The generated P4 profiles were used to derive early P4 based fertility measurements and to classify the profiles. The P4 profiles were classified into four different categories (Figure 2). These four categories included: (*i*) normal oestrous cycle, (*ii*) delayed cyclicity, (*iii*) prolonged luteal phase, and (*iv*) cessation of cyclicity.

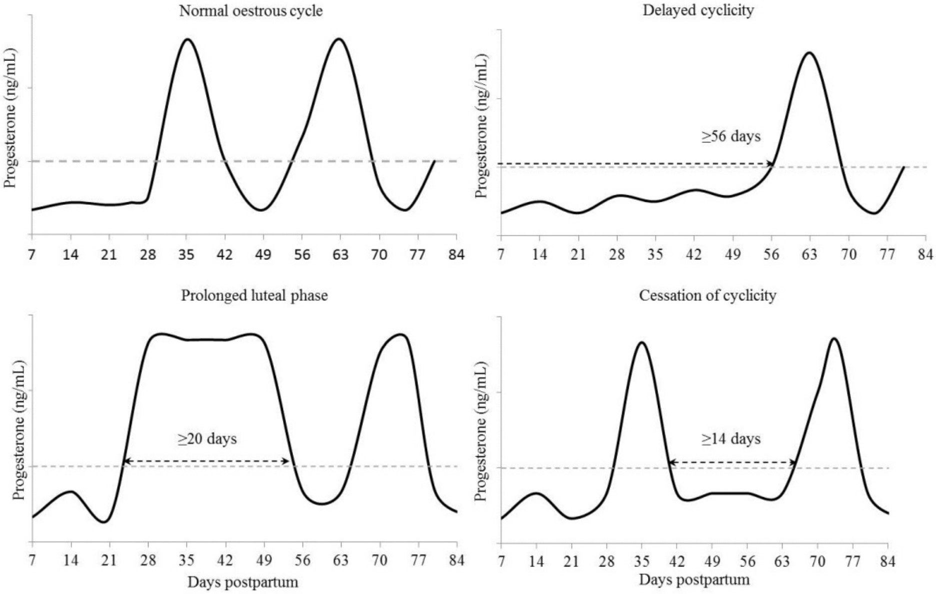

Figure 2. Illustration of the four categories of progesterone profiles; normal oestrous cycle, delayed cyclicity, prolonged luteal phase and cessation of cyclicity. The grey dotted line represent the predefined threshold for luteal activity and black dotted lines represent the atypical pattern.

Each normal P4 profile was divided into four cycle length traits (Figure 3). The cycle length traits included; (*i*) commencement of luteal activity (**CLA**), defined as days from calving to the first rise in P4 concentration above the predefined threshold, (*ii*) luteal-phase length (**LPL**) defined as the period between the rise in P4 concentration above the threshold to the diminish in P4 concentration below the threshold, (*iii*) inter-luteal interval (**ILI**) defined as the period of time following ovulation in which the corpus luteum secretes P4 levels below the threshold, and (*iv*) inter-ovulatory interval (**IOI**), also referred as the oestrous cycle length, defined as the interval between the rise in P4 above the threshold in one oestrous cycle to the P4 rise above the threshold in the next oestrous cycle (Figure 3).

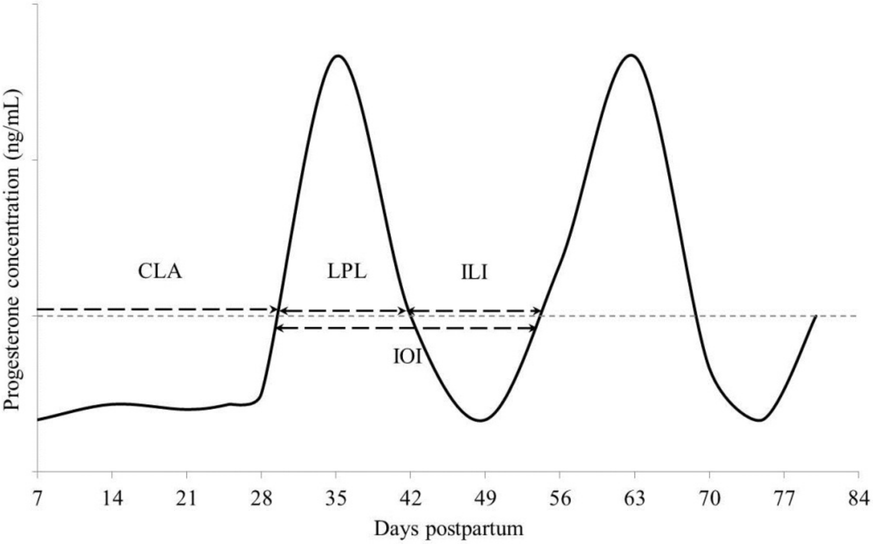

Figure 3. Illustration of the cycle length traits; commencement of luteal activity (CLA), luteal phase length (LPL), inter-luteal interval (ILI) and inter-ovulatory interval (IOI). The grey dotted line represents the predefined threshold for luteal activity and the black dotted lines represent the cycle length traits.

In Paper II the P4 concentration at the day of AI, and the highest P4 concentration between days 7-13 (**P4day10**), between days 18-24 (**P4day21**) and between days 27-33 (**P4day30**) were analysed to compare the levels of P4 for pregnant cows and cows with pregnancy losses.

3.2.2 Oestrous traits

Recordings of oestrus observations were used, together with the P4 measurements to define three oestrus traits; the interval from first to last oestrous symptoms **(FLOS)**, the interval from calving to first ovulatory oestrus (**FOO**), and oestrus intensity (**OI**).

The interval from first to last oestrous symptoms constituted the whole oestrus period and was defined as the duration (in hours) between the first and last detected oestrous symptom of one oestrus resulting in an AI.

The interval from calving to first ovulatory oestrus was defined as the first ovulatory oestrus after calving which was estimated as two days after the day of oestrus and confirmed by a P4 concentration below the pre-defined threshold value. The day of oestrus was defined as the day with the strongest oestrus intensity confirmed by a P4 concentration below the pre-defined threshold value for luteal activity.

Oestrus intensity was determined using a cumulative score based on an overall scoring system evaluating the oestrus observations, including one or more oestrous symptoms, recorded for each animal by the herd staff. The scoring system was based on the oestrous symptoms and defined in a five point scale, where the lower scores were based on local visual symptoms regarded as more uncertain and the higher scores were based on more classical symptoms, such as stand to mounted, lowering of the back, and mounting (Table 2).

3.2.3 Pregnancy traits

The conception rate (**CR**) was defined as the percentage of AIs resulting in a confirmed pregnancy at day 60 after AI.

Pregnancy losses were estimated using data from P4 values combined with AI information. A schematic figure over the time from AI to calving and the extent and pattern of pregnancy losses are shown in Figure 4.

Early embryonic loss was defined as the proportion of cows that lost their pregnancy between day 1 and 24 after AI, based on the number of cows with low P4 concentrations or repeated inseminations up to day 24, divided by the total number of inseminated cows at AI. At day 25, a total of 619 inseminations were defined as early embryonic loss.

Late embryonic loss was defined as the proportion of cows that lost their pregnancy between day 25 and 60 after AI, based on the number of cows with low P4 concentrations or repeated inseminations from day 25 up to day 60, divided by the total number of cows that remained pregnant at day 25. Six cows were culled due to fertility failure between day 25 and 60 after AI and considered as LEL. At day 61, a total of 273 inseminations were defined as late embryonic loss.

Foetal loss was defined as the proportion of cows that lost their pregnancy from day 61 to parturition, based on the number of cows with low P4 concentrations or repeated inseminations from day 61 to calving, divided by the total number of cows that remained pregnant at day 61. Eleven cows were culled due to abortion and 102 cows due to fertility failure, between day 61 in gestation and calving, and considered as FL. At the day of calving, 171 inseminations were defined as foetal loss.

Total pregnancy loss was defined as the proportion of cows that lost their pregnancy between day 1 after AI and calving, divided by the total number of cows that remained pregnant at calving. During the observed periods for pregnancy losses 341 cows were excluded because of culling reasons not related to fertility. The inseminations from these culled cows were excluded immediately after the period in where the cows were culled and not included in the total pregnancy loss. At the subsequent day of calving, 1,789 inseminations remained in the dataset and 1,063 of these were defined as total pregnancy loss.

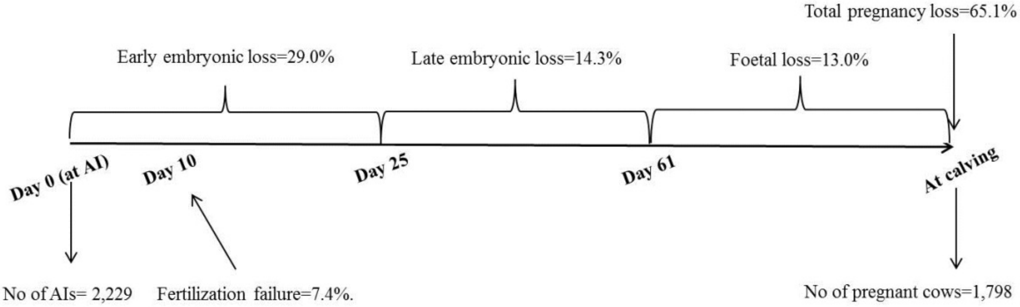

Figure 4. Schematic figure over the extent and pattern of pregnancy losses from insemination (AI) to calving.

3.3 Methods

For Paper I and II statistical analyses were carried out using the MIXED procedure in SAS (SAS Institute Inc., 2015). The categorical traits OI, the atypical P4 profiles and all pregnancy loss traits were also analysed using GLIMMIX procedure, but because there were no differences in the results between the two methods, the results from the MIXED procedure were presented. All models in Papers I and II included a random effect of cow nested within breed.

In Paper I the associations between breed, parity, housing, insemination year and insemination season (independent variables), and oestrus intensity, interval from first to last oestrous symptom and conception rate (dependent variables) were analysed using linear mixed models.

In Paper II the associations between breed, parity, calving interval group, cycle number, insemination year and insemination season (independent variables), and oestrus intensity, the interval from calving to first ovulatory oestrus, early embryonic loss, late embryonic loss, foetal loss and total pregnancy loss (dependent variables) were analysed using linear mixed models.

For the genetic analysis in Paper III, a linear mixed sire model for the atypical P4 profiles were used and for the cycle length traits a linear mixed animal model were used. Estimations of (co)variance were performed using the DMU-package for analysis of multivariate models (Madsen and Jensen, 2007). Univariate models were used for all heritability estimations and bivariate models were used for all estimations of genetic correlations.

The final model for the genetic analysis in Paper III and for the GWAS in Paper IV of the P4 profiles and cycle length traits included the fixed effects of parity within country, calving year within country and calving season within country.

The frequency distributions for the atypical progesterone profiles were normal but the distributions of the cycle length traits were skewed. Therefore, the natural logarithm (**ln**) of CLA, ILI, LPL, and IOI was used for these variables as the dependent variables in the statistical models.

3.3.1 GWAS and Imputations

In Paper IV, a genome-wide association study (**GWAS**) was performed with the GenABEL package RepeatABEL in R 2.15.0 software (Rönnegård, 2015, R Code Team, 2015) to identify significant SNP associations. The quality criteria applied was a minimum call rate of 95%, and minor allele frequency (**MAF**) above 1%. After filtering for poor quality data, 43,582 markers and 1,661 cows passed the quality criteria and remained in the study. The information on the reference population was used to impute the genotypes of our experimental population from 50K SNP genotypes to sequence level. This imputation was done by using Beagle version 4.1 (Browning and Browning, 2016). Imputation started by checking the inconsistency between 50K and the WGS reference population of 547 HF bulls, using the Conform-gt software (http://faculty.washington.edu/browning/conform-gt.html). After this check, the cows were imputed from 50K to sequence level. The imputation accuracy (**AR2**) of each marker was provided by Beagle as the bi-allelic r^2. The average accuracy of imputation was considered as a well-imputed variant when an accuracy above 0.7 was achieved, and therefore, this variant was retained for further analyses. The imputed genotypes for the three target chromosomes were analysed for association with the fertility traits using the same tools and models as the 50K GWAS.

4 Summary of results

4.1 A longitudinal study of oestrous characteristics and conception in tie-stalled and loose-housed Swedish dairy cows (*Paper I*)

In Figure 5 the prevalence of oestrous symptoms, based on visual observations 20 minutes three times per day, for SR and SH cows and cows in loose housing and tie-stall systems are shown.

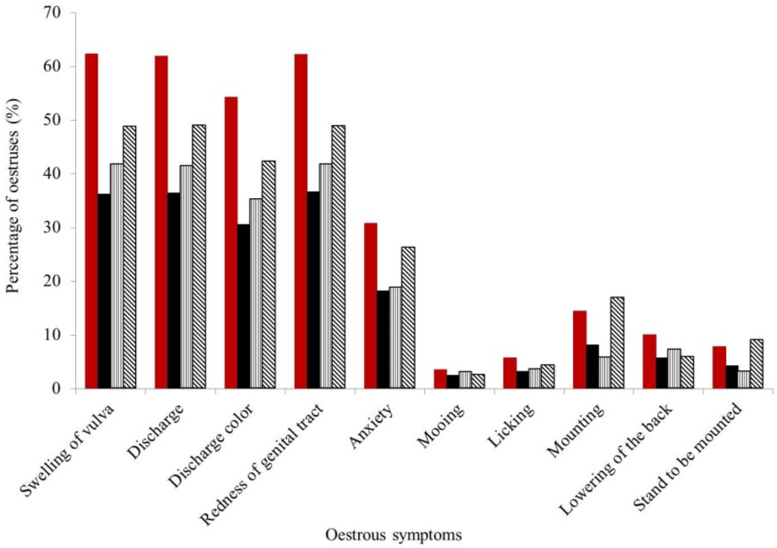

Figure 5. The prevalence of oestrous symptoms out of total oestruses per breed (Swedish Red, red bar, and Swedish Holstein, black bar) and housing system (tie-stall, vertical pattern bar and loose-housing, diagonal pattern bar).

The most frequently recorded oestrous symptoms were the local symptoms swelling and redness of the vulva and vulvar discharge for both SR and SH dairy cows.

The oestrous symptoms; mounting, stand to be mounted and lowering of the back, were seldom expressed in the present study and more expressed by SR cows compared to SH cows. Most oestrous symptoms (except from mooing and lowering of the back) were expressed more in loose housed cows compared to cows in tie-stall.

We found that the duration of the pre-receptive phase was long and included both secondary and local oestrous symptoms which imply that these could be used as predictors of an upcoming oestrus, especially the local symptoms that were more frequently observed and also expressed earliest in the pre-receptive phase.

Negative trends over the studied years for both conception and oestrous expression were found. Swedish Red cows had a more intensive oestrus compared to the SH cows in loose housing system and a decreasing trend in OI by year was found for both breeds.

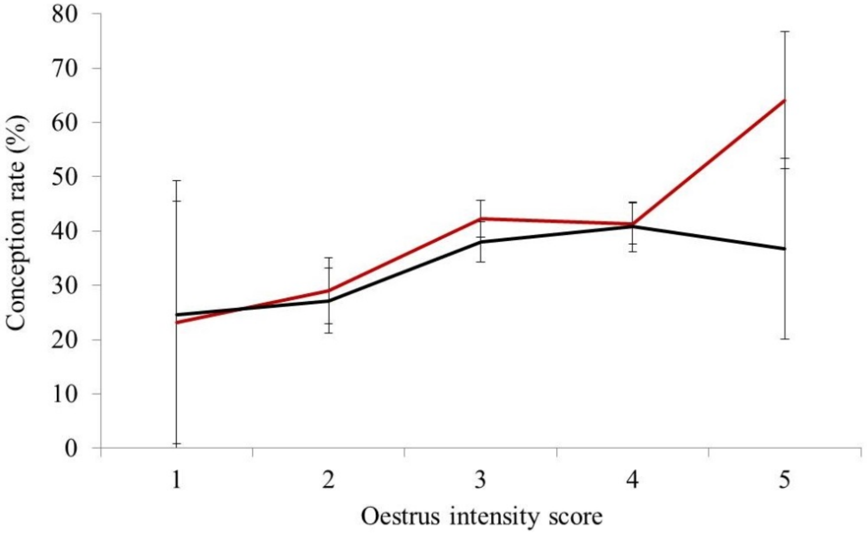

Figure 6. The conception rate (%) sorted by oestrus intensity scores one to five (one = very weak and five = very strong) for Swedish Red (red line) and Swedish Holstein (black line) dairy cows.

Conception rate increased with a more intensive oestrus (Figure 6). Cows with lower OI scores (one and two) based on only local symptoms (i.e. discharge and red and swollen vulva) and secondary symptoms (i.e. anxiety) had a relatively high CR (24 and 28% respectively), indicating that these symptoms should not be neglected in an oestrus detection. Conception rates were substantially higher for cows with an OI score of three, four and five (40, 41 and 54%). Conception rate was also found to decrease with increasing milk yield levels (22-5 days before AI).

4.2 Extent and pattern of pregnancy losses and progesterone levels during gestation in Swedish Red and Swedish Holstein dairy cows (*Paper II*)

Our results showed that the expected outcome from 100 inseminations performed in dairy cows in spontaneous true oestrus (low P4 levels) is a calving rate of around 35-40%.

Table 3. Least square means differences (Class A- Class B) for early embryonic loss (EEL), late embryonic loss (LEL), foetal loss (FL) and total pregnancy loss (TPL)

Class A	Class B	EEL	LEL	FL	TPL
Swedish Red	Swedish Holstein	0.3	-1.7	-1.3	-5.5
Parity 1	Parity 2	2.5	0.8	-0.1	-5.4
Parity 1	Parity 3	2.1	-0.4	-3.6	-13.9
Parity 2	Parity 3	-0.4	-1.2	-3.5	-8.5
Calving interval 12 months	Calving interval 15 months	3.6	-1.8	3.7	2.3
Ovulation number 1	Ovulation number 2	2.7	-3.4	5.7	0.6
Ovulation number 1	Ovulation number 3	7.1	-3.4	5.2	3.4
Ovulation number 1	Ovulation number 4	7.9	-2.2	3.3	4.6
Ovulation number 1	Ovulation number 5	11.5	-1.3	1.6	4.1
Ovulation number 2	Ovulation number 3	4.4	0.0	-0.5	2.8
Ovulation number 2	Ovulation number 4	5.2	1.2	-2.4	4.0
Ovulation number 2	Ovulation number 5	8.8	2.1	-4.1	3.5
Ovulation number 3	Ovulation number 4	0.8	1.2	-1.9	1.2
Ovulation number 3	Ovulation number 5	4.4	2.1	-3.6	0.7
Ovulation number 4	Ovulation number 5	3.6	0.9	-1.7	-0.5
Tie stall	Loose housing	2.0	-0.4	1.4	1.8
January-April	May-September	5.5	-0.6	-7.1	-3.8
January-April	October-December	-0.8	-0.5	-1.1	2.1
May-September	October-December	-6.3	0.1	6.0	5.9

Total pregnancy loss from AI to the day of calving was 65% (Figure 4). Early embryonic loss was estimated to 29%, late embryonic loss 14% and foetal loss 13%. Out of 619 inseminations used to calculate the pregnancy loss between days 1 to 24 after AI, P4 concentration was low at day 10 after AI in 171 oestrous cycles indicating a very early embryonic loss of 7.4% as a part of the early embryonic loss. Different systematic factors affected the pregnancy loss traits (Table 3). Swedish Red cows had 5.5% point lower total pregnancy loss compared to SH cows. Total pregnancy loss was found to increase with increasing parity and first parity cows had longer interval from calving to first ovulatory oestrus compared to cows in later parities.

Early embryonic loss decreased and total pregnancy loss tended to decrease with increasing ovulation number (Figure 7). We also found that oestrus intensity was stronger at later ovulations. Cows inseminated at ovulation number five and higher had significantly lower EEL compared to cows inseminated at first and second ovulation (11.5 and 8.8% points respectively). Cows inseminated at ovulation number three and higher had significantly stronger oestrus compared to cows inseminated at first or second ovulation.

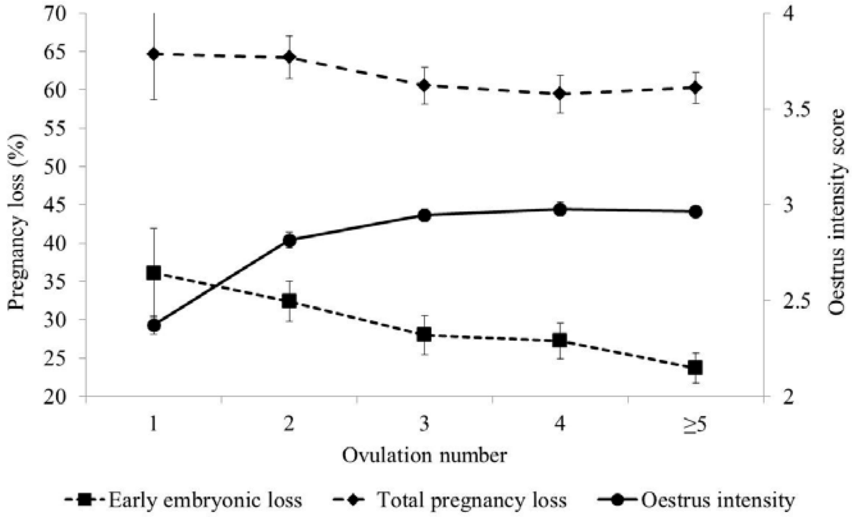

Figure 7. Least square means (SE) for oestrus intensity (solid line and circles; right hand y-axis, 2 = weak to 4 = strong), early embryonic loss (dotted line and square markers; left hand y-axis) and total pregnancy loss (dotted line and triangle markers; left hand y-axis) by ovulation number for Swedish dairy cows.

Early embryonic loss was found to increase with weaker oestrous expression which could be a result of an insemination at an inappropriate time for the cow.

Cows with delayed cyclicty had stronger OI, longer oestrus duration and longer start to first ovulatory oestrus compared to cows with a normal P4 profile in the first cycle.

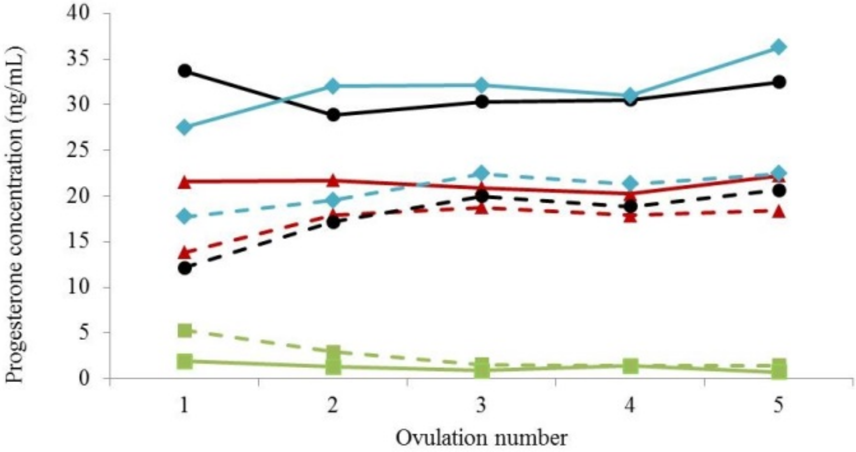

Figure 8. Progesterone concentration at insemination (green line), at day 10 (red line), at day 21 (black line), and at day 30 (blue line) after AI by ovulation number for cows with pregnancy losses (solid line) and pregnant cows (dotted lines), day 1 after AI to calving.

Progesterone levels at the day of insemination and during the gestation were found to affect the pregnancy results. Cows with pregnancy losses had significantly higher basal P4 levels at the day of AI compared to pregnant cows (Figure 8). Cows with pregnancy losses between days one after AI and calving had significantly lower P4 concentrations at days 10, 21 and 30 after AI compared to subsequent calving cows.

Progesterone levels at AI decreased, and P4 levels at days 10, 21 and 30 after AI increased, with increasing ovulation number for both pregnant cows and cows with pregnancy losses. We also observed that P4 concentrations at AI at the first and second oestrous cycles were higher compared to at later oestrous cycles. Insemination of cows at later ovulations, where also a stronger oestrous expression was found, may increase the chance of successful pregnancies.

4.3 Genetic analysis of atypical progesterone profiles in Holstein-Friesian cows from experimental research herds (*Paper III)*

A total of 487 profiles (30.2%) were atypical P4 profiles and the proportion of the atypical profiles differed between the countries. In the Swedish and the Dutch data, prolonged luteal phase was the most common atypical P4 profile (19 and 17%, respectively) while in the Irish data delayed cyclicity (16%) and in the British data cessation of cyclicity (13%) were the most common atypical P4 profiles. Normal P4 profiles had a mean oestrous cycle length (IOI) of 23 days and 49% of the cycle lengths were between 18-24 days, 21% were shorter than 18 days and 31% were longer than 24 days. Cows with delayed cyclicity had a CLA of 71 days while cows with a prolonged luteal phase and cessation of cyclicity had a CLA of 25 days and 26 days, respectively.

First parity cows had a higher incidence of delayed cyclicity, prolonged luteal phase and a longer CLA compared to cows in later lactations while the incidence of cessation of cyclicity decreased with older cows. Cows with a long CLA had longer CFH, CFS and CI (Table 4).

Delayed cyclicity and CLA had moderate heritabilities (0.24 and 0.18 respectively) while heritabilities for cessation of cyclicity, prolonged luteal phase, IOI, ILI and LPL were low (0-0.08; Table 4).

Table 4. Estimated heritability (h^2), and SE in parentheses, and genetic correlations with the classical fertility traits and milk yield

Trait		Genetic correlations					
	h^2(SE)	CFH[1]	CFS[1]	PFS[1]	FLS[1]	CI[1]	ECM[1]
Delayed cyclicity	0.24 (0.05)	0.32 (0.26)	-0.14 (0.19)	1.00 (1.21)	0.32 (0.56)	NC[2]	0.57 (0.14)
Prolonged luteal phase	0.02 (0.04)	-0.94 (1.11)	0.42 (0.56)	NC[2]	0.30 (1.09)	NC[2]	-0.60 (0.54)
Cessation of cyclicity	0.00 (0.04)	-	-	-	-	-	-
lnCLA[3]	0.18 (0.04)	1.00 (0.17)	0.35 (0.12)	0.20 (0.43)	NC[2]	0.54(0.27)	0.45 (0.09)
lnIOI[3]	0.03 (0.04)	0.11 (0.29)	0.76 (0.24)	0.25 (0.42)	NC[2]	NC[2]	0.31 (0.18)
lnILI[3]	0.08 (0.14)	-0.17 (0.20)	-0.07 (0.15)	-0.36 (0.38)	NC[2]	-0.03(0.33)	0.25 (0.11)
lnLPL[3]	0.08 (0.05)	-0.38 (0.19)	0.25 (0.14)	0.38 (0.18)	NC[2]	0.03(0.30)	-0.02 (0.10)

[1] CFH=interval from calving to first observed heat; CFS=interval from calving to first service; PFS=pregnancy at first service; FLS=interval from first to last insemination; CI=calving interval; ECM=milk production at day 60 after calving.

[2] NC= Not converged

[3] Natural log (ln) of CLA=commencement of luteal activity; ILI=inter-luteal interval; LPL=luteal phase length; IOI=inter-ovulatory interval.

Delayed cyclicity and CLA was also found to have moderate genetic correlations with milk yield in early lactation (Table 4) which could imply a possible deterioration in these traits if not considered in breeding goals.

High milk production in early lactation was found genetically correlated to delayed cyclicity, a longer CLA, longer ILI and longer IOI. To reduce IOI, CFH and CFS, it is important that cows starts cycling earlier after calving.

4.4 Genome-wide Associations Study for Normal and Atypical Progesterone Profiles in Holstein-Friesian Dairy Cows (*Paper IV)*

The GWAS, with the Bovine 50K SNP genotypes, resulted in a total of 44 significant SNPs associated with the seven endocrine fertility traits. These SNPs were detected across the genome, except on chromosome 5, 7, 10, 13, 18, 20, 25-28. The strongest significant SNP, associated with cessation of cyclicity, was found on BTA17 at 47.02Mbp, and had a $-\log_{10}$(P-value) of 6.19. A promising region was found between 52-56Mbp on BTA17, with significant SNPs for delayed cyclicity, CLA and IOI.

Twenty four significant SNPs associated with the classical fertility traits were found on BTA 1, 2, 4, 5, 8, 10-12, 14-15, 19, 25 and 28. The greatest numbers of significant SNPs were found on BTA1 where significant SNPs associated with CFS, CFH, PFS, and FLS were found.

At BTA8, 17 and 23 significant associations were found for four of the studied traits; delayed cyclicity, cessation of cyclicity, CLA and IOI, which is the reason we focused on imputing these chromosomes to sequence level, for a further chromosome-wide association study (**CWAS**). On BTA8, significant SNPs were also found for the classical fertility traits CFH and CFS.

In Table 5 information about the number of SNPs and imputed variants on BTA8, 17 and 23 are presented. After imputation, with a imputation accuracy (**AR2**) ≥0.7, the total number of variants on BTA8 increased from 1,962 SNPs on the 50K to 23,051 variants on sequence level, on BTA17 from 1,338 SNPs on the 50K to 26,901 variants and on BTA23 from 914 SNPs on the 50K to 21,376 variants. This is an increase with 12, 20 and 23 times respectively for BTA8, 17 and 23.

Table 5. *Number of SNPs at the 50K SNP genotypes and the number of imputed sequences for all imputed variants (All), with an imputed accuracy (AR2)≥0.2 and with an AR2≥0.7, and the number of Indels on BTA8, BTA17 and BTA23*

	BTA8	BTA17	BTA23
Total number of SNPs[1]	1,962	1,338	914
Total number of imputed variants:			
All	1,798,450	1,299,024	1,119,123
AR2[2]=0	1,594,607	1,075,177	937,466
AR2≥ 0.2	69,207	79,895	70,055
AR2≥0.7	23,051	26,901	21,376
After quality control (AR2≥0.7)	22,506	26,268	20,821
Number of indels[3]	3,156	3,488	3,089

[1] SNPs at the Bovine 50K SNP genotypes

[2] AR2=imputation accuracy

[3] Indels from the sequence data with an AR2≥0.2

For delayed cyclicity two QTL regions were found on BTA8 (between 38.9-40.9 and 58.9-60.9 Mbp), one on BTA17 (between 52.8-55.2 Mbp) and two QTL regions on BTA23 (between 29.90-31.90 and 41.2-43.2 Mbp) (Figure 9). For cessation of cyclicity one significant variant on BTA8 (at 36.22 Mbp), one on BTA17 (at 47.01 Mbp) and one on BTA23 (at 7.91 Mbp) were found (Figure 9).

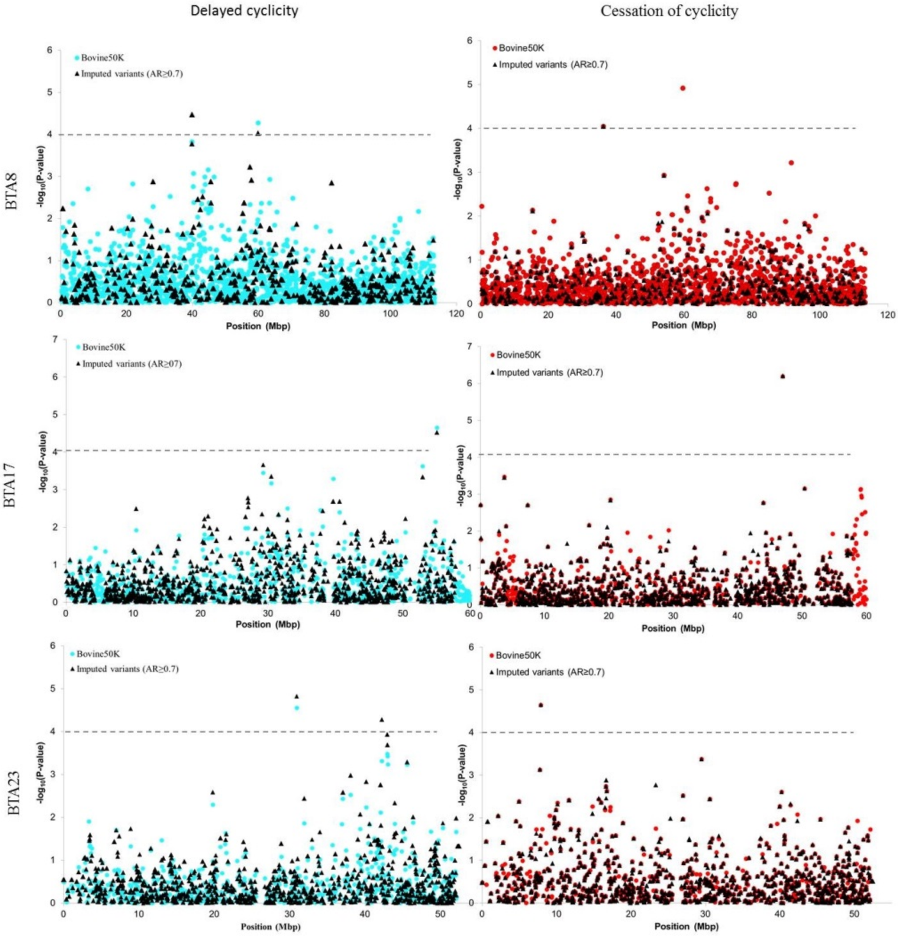

Figure 9. Chromosome-wide association study for delayed cyclicity (turquoise) and cessation of cyclicity (red) on BTA 8, 17 and 23. Bovine 50K SNP genotypes (turquoise and red circles) overlaid with imputed variants with an imputation accuracy of ≥0.7 (black triangles). The black dotted line is the genome-wide significance level of $–\log_{10}$(P-value) ≥ 4 based on the 50K SNP genotypes.

For CLA one QTL region was found on BTA8 (between 58.9-60.9Mbp), one on BTA17 (52.8-55.2 Mbp) and one on BTA23 (41.2-43.2 Mbp) (Figure 10). For IOI one QTL region was found on BTA17 (between 52.8-55.2 Mbp) (Figure 10).

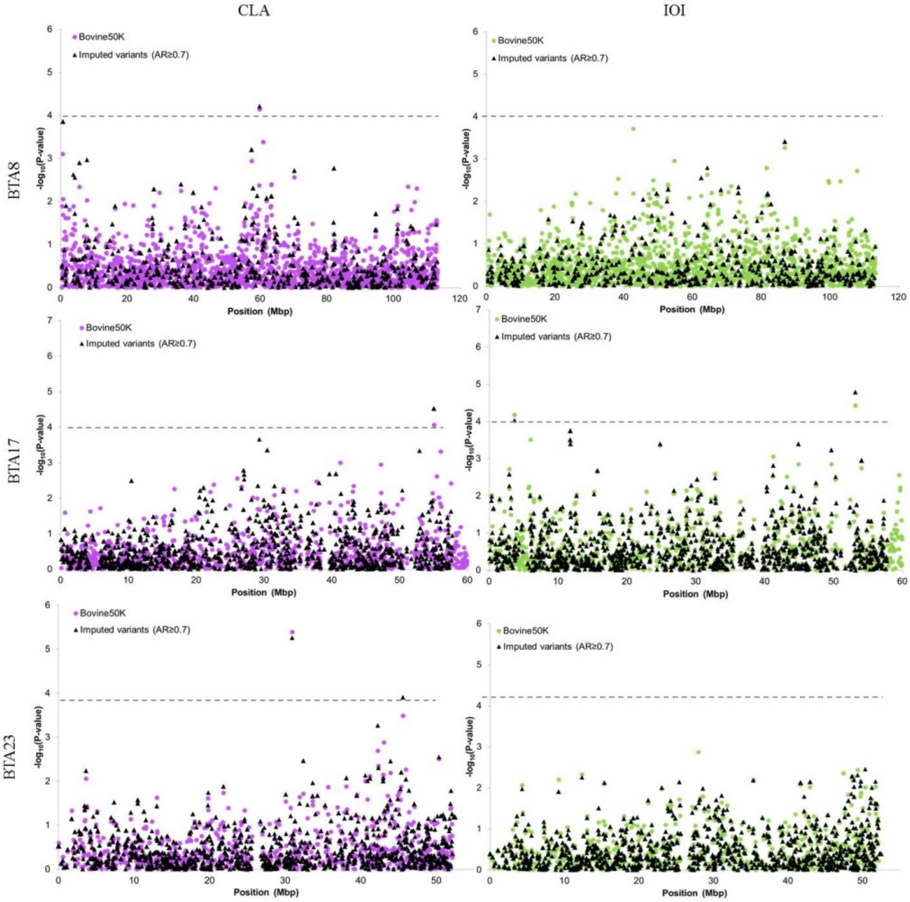

Figure 10. Chromosome-wide association study for commencement of luteal activity (CLA; purple) and inter-ovulatory interval (IOI; green) on BTA8, 17 and 23. Bovine 50K SNP genotypes (purple and green circles) overlaid with imputed variants with an imputation accuracy ≥0.7 (black triangles). The black dotted line is the genome-wide significance level of $-\log_{10}$(P-value) ≥ 4 based on the 50K SNP genotypes.

Figure 11 shows the overlapping regions for delayed cyclicity and CLA on BTA8 (Figure 11;a1-3) and BTA23 (Figure 11;c1-3), and for delayed cyclicity, CLA and IOI at BTA17 (Figure 11;b1-2). Many genes were found in the interesting QTL regions but only those genes that have been reported to affect i.e. the reproduction system, embryo phenotype, or hormones affecting fertility and endocrine morphology/physiology were considered in this study. Nine genes on BTA8, nine genes on BTA17 and five genes on BTA 23 were found. These genes are presented and described in Table 6. Due to high linkage disequilibrium (**LD**), it was not possible to specify genes or polymorphisms as causal factors for any of the regions.

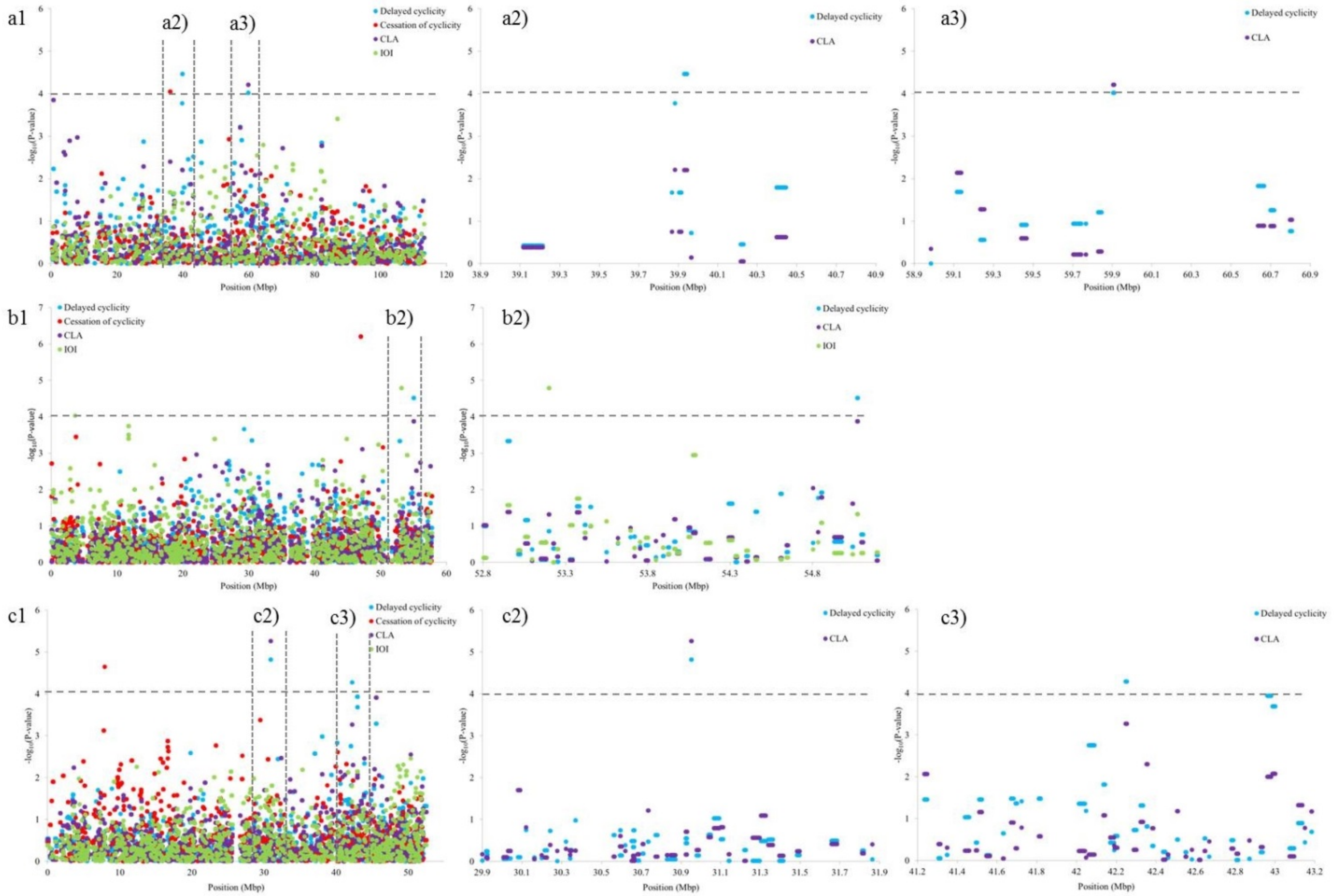

Figure 11. Fine-mapping (imputed sequences with and AR2≥0.7) of a1) BTA8, b1) BTA17 and c1) BTA23 showing an overlay of delayed cyclicity, cessation of cyclicity, commencement of luteal activity (CLA) and inter-ovulatory interval (IOI). Fine-mapping of delayed cyclicity and CLA between 38.9-40.9 Mbp (a2) and 58.9-60.9 Mbp (a3) on BTA8, of delayed cyclicity, CLA and IOI between 52.8-55.2 Mbp (b2) on BTA17, and of delayed cyclicity and CLA between 29.9-31.9 Mbp (c2) and 41.2- 43.2 Mbp (c3) on BTA23. The black dotted line is the genome-wide significance level of $-\log_{10}$(P-value) ≥ 4 based on 50K SNP genotype.

Table 6. Position of top variant on BTA8, 17 and 23, possible candidate genes, and function of the genes

BTA	Top SNP position (Mbp)	Candidate gene	Possible function
8	39.93	PDCD1LG2	Female fertility, endocrine gland phenotype
		PLGRKT	Female fertility, fertility measurements
		INSL6	Reproduction hormone activity
		JAK2	Reproductive system, embryo phenotype, abnormalities in the endocrine gland
	59.90	SIT1	Abnormal endocrine gland morphology/physiology
		CA9	Abnormal endocrine gland morphology/physiology
		HINT2	Abnormal endocrine gland morphology/physiology
		TLN1	Embryo phenotype
		GBA2	Reproductive system, abnormalities in the endocrine gland
17	53.20	SCARB1	Reproductive system, embryo phenotype, abnormalities in the endocrine gland
		KMT5A	Embryo phenotype
		CDK2AP1	Embryo phenotype
		TCTN2	Embryo phenotype
		CCDC62	Oestrogen receptor binding
		ABCB9	Endocrine gland phenotype
	55.08	CLIP1	Reproductive system phenotype
		KDM2B	Reproductive system phenotype, embryo phenotype
		ORAI1	Endocrine gland phenotype
23	30.92	HIST1H1C	Reproductive system, embryo phenotype, abnormalities in the endocrine gland
		HIST1H1D	Reproductive system, embryo phenotype, abnormalities in the endocrine gland
.	42.25	RANBP9	Reproductive system, abnormalities in the endocrine gland
		JAARID2	Embryo phenotype, abnormalities in the endocrine gland
		CD83	Reproductive system, abnormalities in the endocrine gland

5 General discussion

5.1 Introduction

The multifactorial nature of reproduction traits requires an integrated approach from environmental factors, such as e.g. management, oestrus detection, housing, feeding, and inseminations, to genetic factors, such as e.g. correlations to production traits and genes affecting fertility. In this thesis we have focused on oestrous expression traits and endocrine fertility traits based on milk progesterone (**P4**) concentrations. We have also looked at classical fertility traits derived from insemination and calving dates. We wanted to investigate traits that reflect the ability of the cows to return to cyclicity after calving, become pregnant, and keep the pregnancy. Figure 12 shows a schematic picture of the female fertility traits included in this thesis.

In the Nordic breeding evaluation the breeding goal for fertility aims for the cows to resume luteal activity early after calving, showing oestrus so they can be inseminated at the optimal time, become pregnant after the first insemination, and calve successfully (NAV, 2008b). Fertility has been included in the breeding evaluation in Sweden since 1974, and in the Nordic genetic evaluations female fertility traits based on insemination- and calving dates are used. The drawbacks are that the classical fertility traits are highly influenced by farm management decisions and have low heritability. The low heritability makes the selection slow and less effective. Endocrine fertility traits, such as P4 profiles, are expected to be more objective, i.e. less biased by farm management decisions, and more directly reflecting a cow’s reproductive physiology.

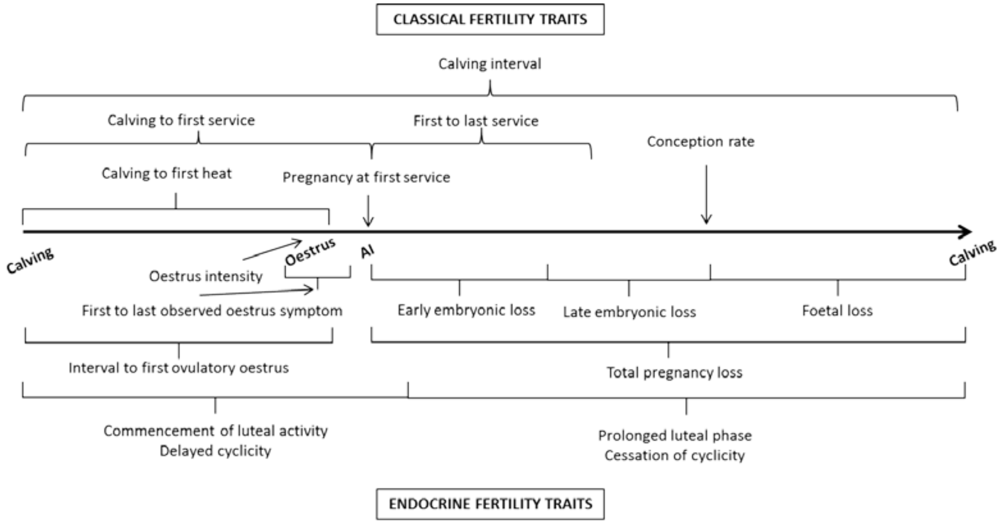

Figure 12. Schematic picture of the endocrine and classical fertility traits included in this study.

5.2 Oestrous expression and pregnancy losses

Poor oestrous expression and the inability to detect oestrus are major factors to reproductive failure. To ensure timely insemination and thereby maximize conception rates in the herd and profitability of the farm, it is important that the farmers capture the short period of when the cow is fertile and sexually receptive. In the absence of bulls, the farmers need to detect cows in oestrus by observing physical and behavioural symptoms that a cow exhibits only during oestrus or at a higher frequency during oestrus compared to other times during the oestrous cycle. It is difficult to document natural oestrous behavior of cows since oestrus intensity and duration have a large individual cow variation and is affected by social interaction among cows (Lucy, 2001; Roelofs, 2008). Both oestrus intensity and duration have decreased during the last decades. From frequently expressing more obvious symptoms such as standing oestrus and mounting behavior to expressing more non-specific oestrous behavior such as increased anxiety, chin-resting and licking on other cows (Roelofs et al., 2005; Dobson et al.,2008).

Failure to express oestrus, also called silent oestrus, is another important issue resulting in AI timing error. Silent ovulations are most common at first ovulation while diminishing in second and subsequent ovulations. With lack of visible oestrous symptoms at first ovulation AI could preferably be performed at later ovulations when oestrus is more expressed. In Paper II we found, in agreement with earlier studies, that the longer time after calving, the more intensive oestrus and cows with delayed cyclicity had stronger oestrus intensity and longer CFH compared to cows with a normal start of cyclicity (Paper III). An early insemination after calving increased the amount of pregnancy losses and cows with delayed cyclicity was found to be subjected to fewer early embryonic losses (Paper II). Cows with a shorter planned calving interval had higher amount of pregnancy losses and pregnancy losses was found to decrease with ovulation number. Another issue with too early inseminations could be the short time for the cows to recover from the previous pregnancy. From these results we can conclude that cows inseminated later in the lactation may reach better pregnancy results. However later ovulations, later inseminations and longer planned calving interval may be beneficial for embryonic survival these results can be suboptimal for other reasons. For example, a longer interval from calving to the start of luteal activity resulted in a longer interval to first insemination and a longer calving interval (Paper III) which may reduce milk production and the revenue at the farm.

5.2.1 Oestrous detection

The most common way to observe cow oestrous characteristics is by visual observations. The most common observed oestrous symptoms in this thesis, found by visual observations, were the external symptoms vulvar discharge and redness of the vulva. For a well performed visual observation it is important with frequent and continuous observations. In this study oestrus observations were only performed during daytime for approximately 20 minutes three times per day, which probably lead to a high frequency of missed oestrous symptoms. Visual oestrus observations require skilled observers that can distinct oestrous behavior from behavior/signals also expressed during the rest of the oestrous cycle. By using video recording the visual observations could be enhanced and the observer can analyse the data retrospectively and repeatedly, and also in a larger scale. The drawbacks with visual observations, with or without video recording, are that they are subjective, time consuming and labor intensive. With larger herds and cows in loose-housing systems more automated systems are required to support or replace visual oestrus detection observations. Automated oestrus detection could be seen as more objective, since they are less influenced by the farmer, compared to the visual observation. Even though automated oestrus detection is important the addition of visual observations is most likely necessary to reach sufficiently high detection rates and to detect the local oestrous symptoms that cannot be detected by any automated detection device. By using more than one oestrus detection method more oestrous symptoms may be found.

When the common Nordic breeding goal (NTM) was established in 2005, oestrus intensity (**OI**) was excluded from the daughter fertility index, as these recordings only are available from Sweden. The recordings are used as an indicator trait in the fertility index and EBVs for OI is still estimated for herd owners wanting to have this information. This trait is based on a five-pointed scoring system, from very weak to very strong OI. Oestrus intensity could be considered to be reintroduced into the common breeding goal due to its importance in e.g. optimizing the time for insemination. Oestrus intensity is a low heritability trait (Roxström et al., 2001) and based on subjective visual oestrus observations but even traits with low genetic variation are possible to improve through breeding (Berry 2015). Even though oestrus intensity and duration, estimated by automated recordings, have been reported with low heritabilties (0.02-0.08) (Løvendahl and Chagunda. 2009; Ismael et al., 2015), automated oestrus registrations are more objective. By adding oestrus intensity, based on these automated registrations, as an indicator trait to the reintroduced OI the accuracy for oestrus intensity could be improved. If the farmer more easily can find a cow in oestrus the timing of insemination will be improved which would have a great economic impact by e.g. higher conception rates and lower number of inseminations.

5.3 Progesterone in relation to pregnancy

To reach high pregnancy rates optimal P4 levels at the day of AI and during the gestation is of high importance. It is well established that deviated P4 levels are associated with pregnancy losses where the majority of losses occurs during the early embryonic period. More cows were subjected to early embryonic losses when the P4 level was somewhat higher (suprabasal) at AI, compared to cows with low basal P4 levels, which is in agreement with earlier studies. Cows with suprabasal P4 levels at the time of AI had greater risk of repeated AIs and longer calving intervals (e.g. Waldmann et al., 2001; Båge, 2003). We also found that cows with pregnancy losses had lower P4 concentration during the gestation compared to pregnant cows. Many studies have linked poor P4 secretion during the post-ovulatory period to poor embryo development and early embryonic loss (e.g. Mann and Lamming, 1999 and 2001). Since one of the main tasks for the luteal P4 in the body is to maintain an optimal uterine environment and to support the uterus to develop the embryo/foetus during the gestation a drop in P4 levels could indicate a coming pregnancy loss. To continuously measure P4 (with e.g. HerdNavigator^{TM}) around the planned AI and during the gestation period, pregnancy losses might be foreseen. Although, continuous P4 sampling during the gestation is both costly and challenging. How often the P4 have to be measured is not yet clear. In Paper II we found that cows with pregnancy losses at any time during the gestation had lower P4 values throughout the whole gestation period. For example cows that lost the foetus in the later part of the gestation had lower P4 levels already in the early gestation compared to the cows that remained pregnant throughout the same period. This could suggest that measuring P4 in the start of the gestation could be enough to foreseen a coming pregnancy loss. This assumes that there are pregnant cows with P4 registrations for comparison. Another reason for measuring P4 during the gestation could be to decrease the number of inseminations on pregnant cows. Pregnant cows sometimes show oestrous behavior, even standing oestrus regularly with an interval of around 21 days, and are inseminated because the farmer supposes that the cow is not pregnant. This could lead to pregnancy losses due to infections transmitted from the inseminations.

5.3.1 Pregnancy results in relation to milk yield

During several decades the world-wide breeding objectives in dairy cattle have mainly focused on increasing milk production, with exceptions for the Nordic countries that also have included other traits such as functional traits e.g. female fertility in the breeding goal. Milk yield traits have an unfavourable genetic correlation with fertility which means that fertility decreases with increasing milk yield. An inclusion of the fertility traits in the breeding goal have led to a more balanced breeding, without affecting the genetic gain for milk notable. This is desirable since milk has the major impact on the revenue at the farm. Although there are contradictory reports on the effect of milk yield on conception and pregnancy rates. In a review by Diskin et al. (2012) high-producing dairy cows were reported to have greater embryonic loss compare to low-producing dairy cows while Santos et al. (2004), reviewing several investigations, reported a small or no indication that milk production could be a risk factor for increased pregnancy losses in dairy cows. In this thesis we did not study the associations between classical fertility traits and milk yield but we found unfavorable genetic correlations between milk yield during early lactation and delayed cyclicity and CLA of (r_g=0.57 and 0.45 respectively). Milk yield was also found to have a favorable genetic correlation with prolonged luteal phase (-0.60) in this study, which is in agreement with Tenghe et al. (2015) who reported favorable correlations between milk yield and proportion of sample in luteal activity, and luteal activity days 25-60 postpartum (r_g=-0.17 and -0.34). Tenghe et al. (2015) also reported unfavorable genetic correlations between milk yield and CFS and CI (r_g=0.14 and 0.56 respectively) and milk yield and CLA (r_g=0.18). Early embryonic loss was found to increase with increasing milk production while oestrous expression traits were not found to be affected by milk yield (Papers I and II). Since there is a continuous breeding for higher milk yield, and delayed cyclicity and CLA are genetically correlated with milk, deterioration in these traits will occur if not considered in breeding goal.

Increased milk yield is generally associated with increased feed intake and increased metabolism of P4 in the liver (Sangsritavong et al., 2002; Wiltbank et al., 2014). As discussed earlier P4 levels at the day of insemination and during the gestation is important for the pregnancy results. High yielding dairy cows have a higher P4 metabolism which results in low levels of circulating P4 in the blood. This could lead to a slower rise in P4 during early dioestrus which reduce the development of the early embryo and increase the risk of pregnancy losses (Robinson et al., 2006). Mattos et al. (2000) reported that increased concentrations of plasma P4 both before and after insemination was associated with higher pregnancy rates. Earlier studies showed that feeding cows' supplemental dietary fat increased the plasma P4 (Garnsworthy et al., 2008a and 2008b). An increased availability of fatty acid precursors allows an increase in P4 secretion, which can improve ovarian and uterine functions and influence the oestrous cycle. Garnsworthy et al., 2008b also concluded that negative energy balance (**NEB)** and losses in body condition score (**BCS**) are associated with a lower quality of the oocyte, reduced embryo development, lower conception rates and lower serum P4 concentration. NEB in early lactation has also been reported to delay ovarian activity (Berglund et al., 1989) and recovery of postpartum reproductive function (Butler, 2013). This could be another underlying reason for delayed cyclicity and should be further investigated. In other words, the extent and duration of the NEB is an important indicator deciding when the cow will return to normal ovarian cyclicity after calving. Because high producing cows lose more energy, primarily to milk, most of them experience a period of NEB. Reducing the extent and duration of NEB and body condition loss could therefore be beneficial for fertility (Garnsworthy et al., 2008b).

5.4 Progesterone profiles

In this thesis P4 profiles were programmed in SAS, by plotting the P4 concentration against postpartum to first service. These plots were used as a base for the definition of the P4 profiles. The definitions were validated by manually observing a random number of plots and then by comparing them to the computed P4 profiles. This work was labor intensive and it took a lot of time trying to make them as accurate as possible and also as comparable between the four countries as possible. The distribution of P4 profiles differed between the countries. For Sweden and the Netherlands prolonged luteal phase was the most common atypical P4 profile, while delayed cyclicity for Ireland and cessation of cyclicity for UK. These differences may be attributed to several contributing factors such as different management and production systems as well as frequency of P4 sampling. How frequent the P4 is sampled and analysed is important for the accuracy of the derived profiles, a more frequent sampling improves the accuracy. By using more continuous and frequent sampling and also a more standardized sampling procedure (e.g. HerdNavigator^{TM}) the results may be more accurate and as well as more comparable between the countries.

When trying to find a method to define traits based on P4 levels, as the P4 profiles, it is important to be aware of a between-cow variation in the basal P4 level (Sorg et al., 2016). A predefined threshold level of P4 for luteal activity was used for all cows in all countries. The programmed P4 profiles for some cows did not seem to correspond with the manually observed profiles. For example, some oestrous cycles were defined as prolonged luteal phases but when manually looking at these oestrous cycles we could not exclude they were normal cycles. One reason could be that these cows had higher basal P4 levels compared to other cows. These cows may reach the predefined threshold level for luteal activity earlier and pass the threshold for end of luteal activity later which may increase the number of cycles defined as a prolonged luteal phase. An automated in-line registration based on individual cows may be a solution where the individual cows P4 levels are considered e.g. by considering previous profiles of the cow, as recently suggested in a study by Blavy et al. (2018).

For dairy farmers decreasing costs of production is often as valuable, or even more valuable, than increasing income. This can be confirmed by the weight that functional traits today are given in the breeding goal together with production traits. For several reasons it is important that cows resume cycling early after calving. A later start of cycling may result in longer intervals to first service and longer calving intervals which will affect the economy at the farm. Delayed cyclicity had a moderate heritability (0.24) and a moderate genetic correlation to CFH (r_g=0.35). Calving to first observed oestrus was based on insemination and calving dates which are highly influenced by the farmers' decisions and the management. Interval from calving to first oestrus has been investigated in other studies, where e.g. the interval to oestrus has been measured by automated registrations, such as P4 and activity sensors. Løvendahl and Chagunda (2009) reported moderate heritability estimates for interval to first oestrus, measured by either an activity device or by P4 concentrations (0.24 and 0.27, respectively). Ismael et al. (2015) reported a moderate heritability estimate of 0.16 for calving to first high activity derived from an electronic activity tag. Furthermore, Ismael et al. (2015) also reported a strong genetic correlation between calving to first high activity and CFS (0.96). The relationship between delayed cyclicity and the interval from calving to first oestrus detected by automated registrations of e.g. P4 or high activity (we can call this trait autoCFH) would be interesting to further investigate. Hypothetically if we could estimate at least a moderate genetic correlation between delayed cyclicity and autoCFH and together with the strong genetic correlation between autoCFH and CFS, indirect selection for cows with a shorter autoCFH could reduce the number of cows with delayed cyclicity and increase the chance for a more rapid return to cycling. Information about both CFS and autoCFH would give higher selection accuracy compared with the accuracy obtained from CFS information only.

5.4.1 Genome-wide associations

For the observed endocrine traits we found many association signals spread over the genome showing that these traits are polygenic. This means that the traits are controlled by more than one gene (usually by many different genes). The complexity of the fertility traits was manifested with the absence of very strong associations for either of the traits suggesting that many genes with small affects are involved which makes it difficult to identify causal genes. For example, delayed cyclicity were found to be associated with significant SNPs and variants on all imputed chromosome, but also on a chromosome that was not imputed and only analysed with the 50K SNP genotypes, BTA16. Further exploration of the genetic basis for the complex and multifactorial endocrine fertility traits would require a much larger GWAS study in terms of many genotyped animals with P4 records. With the increased use of automated in-line recording, e.g. HerdNavigator^{TM}, and the routine genotyping of the cows a much larger GWAS should be feasible in the future.

With the increased use of automatic registrations, use of sensor technology and more cows that are routinely genotyped the possibility to include low heritable and complex traits in genomic selection (**GS**) is increasing. The animal breeding industry in the Nordic countries has applied genomic selection, based on the individual’s marker information, for approximately 10 years. In the traditional breeding evaluation, based on progeny testing, the bulls do not get a breeding value until their daughter get phenotypes in their first lactation. In GS, young bull calves can be assayed as soon as they are born. Genomic estimated breeding values (**GEBV**) can then be predicted and a selection decision can be made if the bull calf will be used for supply semen to the industry as soon as he is able to. This will result in a reduction of the generation interval with more than 2 year and double the rate of gain (Garcia-Ruiz et al., 2016). One prerequisite for GS is a large reference population with phenotyped and genotyped animals. With lower prices on genomic tests more dams are added to the reference population which has positive effects of the reliability of the genomic prediction (Thomasen et al., 2014).

Another way to increase the accuracy of genomic selection, except increasing the size of the reference population, is to increase the marker density and the LD between the traits and the marker regions (Hayes et al., 2009). Increasing the marker density would be expected to give a more precise detection of a causal mutation since the distance between the SNP and causative gene will decrease. Whole genome sequence (**WGS**) data have the greatest amount of genotypic information. The availability of sequence data should contain the causal mutations underlying the traits investigated and with GWAS these mutations is expected to be found (Meuwissen and Goddard, 2010). After imputing our data from 50K SNP genotypes to sequence data (with an imputation accuracy of ≥0.7) the density of genetic markers increased at least 10 times and the distance between genetic markers decreased (from 10Mbp with the 50K SNP to a few kilo bp with the sequence data). This resulted in a substantial increase of significant associations with the phenotypes. Despite the absence of strong association signals, several marker variants were identified within a region with nearby genes involved with different reproduction functions. Although we used the sequence data, we still could not with certainty pinpoint any causal factor underlying the fertility QTLs. Several reasons may explain this. First, most of the total genetic variants identified in the sequence data were filtered out, before the imputation as well as during the imputation, due to low accuracies or low quality scores (e.g. low MAF). Secondly, there were many variants with the same P-value as a result of the high linkage equilibrium (**LD**) among these variants. A strong or even perfect LD could confirm that our imputation of these variants were correct but our ability to make any firm conclusion on which variant to choose for further analysis was limited.

For validation and to identify causal variants a number of further studies have to be performed. Re-sequencing the animals in the regions of interest and maybe include a larger number of cows in the analysis could remove the imputation errors and also provide a statistically more independent data set.

Another option could be to include other breeds in the study to help us detect the variants and causative mutations. For example combining the HF data set with the Nordic Red breed could lower the number of probably causative mutations. If the associations persist across breed the genetic markers are likely to be very close to the QTL because of the limited extent of LD across breeds (Goddard and Hayes, 2009).

6 Conclusions

Oestrous expression and detection are important factors to reach optimal time for insemination. During the last decades oestrus intensity and duration have decreased and the dairy production has moved to larger herds which make it more difficult and labor intensive to use visual oestrus observations. From expressing more obvious oestrous symptoms, such as standing and mounting, to more non-specific oestrous behavior, such as e.g. anxiety and cheek resting, it is harder to detect the true oestrus. To optimize oestrus detection it is therefore recommended to use automated oestrus detection together with visual observations. To minimize labour it is also important to develop tools for automatic recording also including the local oestrous symptoms. By reintroduce oestrus intensity as a breeding goal in the Nordic breeding evaluation a stronger oestrous expression and easier oestrus detection may be achieved. This could increase the chance for the farmer to find the right time to inseminate the cows. Records from activity measures could then be added as an indicator trait to improve the selection accuracy.

Oestrous expression was found to be stronger at later ovulations and silent ovulations were found more common at the first ovulation This suggest that insemination later after calving increases the chance of finding an optimal time for insemination which would result in better pregnancy results. This might not always be applicable and economically relevant. It is therefore important to further investigate the physiological reason for the association between progesterone concentrations and ovulation number and to find ways to reduce the reproductive losses in general and specifically during the first cycles after calving.

A major challenge for future research will be to increase pregnancies per AI by both genetic and dietary improvements. More focus on oestrous expression traits would benefit early embryo survival. A more precise knowledge about the timing and possible background in combination with more powerful breeding tools such as genetic and genomic selection may create further improvements. Introduction of diets that alter e.g. circulating P4, and support special important events during the reproductive cycle is another research area which still needs to be investigated.

Endocrine fertility traits are more objective traits compared to classical fertility traits since they more accurately reflect the cows' biology. Delayed cyclicity and CLA had moderate heritability. These two traits were also found to have moderate genetic correlations with milk yield in early lactation which could imply a possible deterioration in these traits if not considered in the breeding evaluation.

A large number of potential chromosomal regions of interest for fertility traits were detected. The fine-mapping of QTL regions on BTA8, 17 and 23, using imputed sequence variants, identified several significant associations with delayed cyclicity and CLA that can contribute to an index of markers for genetic improvement of fertility. Several potential candidate genes reported to affect reproduction were also identified in the QTL regions. However, due to high linkage disequilibrium, it was not possible to specify genes or polymorphism as causal factors for any of the regions.

With genomic selection we will have an earlier selection of animals based on their genotype information. Based on large reference populations with genotyped and phenotyped animals a huge amount of data would be provided for genomic prediction and the selection candidates without phenotypes. With large reference populations and with sensor techniques, automation of registration and new equipment to do the measuring of new traits, such as CLA or OI, we could beneficially include these in the genomic prediction.

7 Future research

The sensor technology around oestrus detection is moving fast due to the need of detecting cows in oestrus and to optimize the time of insemination. With such a development and an increased interest in using in-line recordings, data for breeding cows with traits that can be used in larger herds and are more easily managed are rising. In the near future, focus is suggested to be on the following objectives.

- Use of automatic oestrus detection, such as activity sensors or in-line P4 measurements, together with visual observations to be able to optimize oestrus detection to capture more oestrous symptoms, and to develop sensors also for local oestrous symptoms.
- Reintroduce oestrus intensity as a breeding goal in the Nordic breeding evaluation and add automated oestrus registrations as an indicator trait to oestrus intensity. Further investigating the possibility to use oestrus intensity in genomic selection.
- Investigate how progesterone levels at specific times during early pregnancy could be used for selecting cows with a better ability to retain pregnancies. Further investigate how frequent progesterone sampling that is needed to predict pregnancy losses. The next step could be to estimate genetic parameters for pregnancy losses and investigate how these could be used in the genetic evaluation and genomic selection.
- Investigate diets that alter circulating progesterone concentrations and how the diet could support special important events during the reproductive cycle e.g. resumption of ovarian activity, fertilisation, embryo/foetal development and finally calving.
- Validate the genome wide association study by including more genotyped and phenotyped animals and/or include animals from another breed to reduce the LD structure and to lower the number of possible causative mutations.

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Popular science summary

Dairy cow fertility is of great importance for the economy in the dairy industry. The cows have to show oestrus, become pregnant and keep the pregnancy, together with calve within a certain interval and produce milk to be economically sustainable. In the Nordic countries fertility has been included in the genetic evaluation since the 70s. Traditional breeding includes fertility traits measures derived from insemination- and calving dates, which in general are highly influenced by on-farm decisions. Unfortunately the low heritability of these traits makes the genetic improvement slow. In Sweden artificial insemination (**AI**) is used and without oestrus synchronization and timed inseminations expression of oestrus symptoms is important for finding a cow in oestrus and for a correct timing of insemination. Weaker oestrous symptoms together with larger herds and fewer working hours per cow may result in reduced possibilities to find cows in oestrus.

The aim of the first study was to investigate the presence and importance of oestrous symptoms in the two main breeds in Sweden, the Swedish Red (**SR**) and Swedish Holstein (**SH**) dairy cows, and to analyse the relationship between oestrous expression and conception rate. This study included data from approximately 2000 oestruses studied during 16 years from a semi-commercial herd of the Swedish University of Agricultural Sciences, in Sweden. Oestrus observations were performed during 20 minutes three times per day where ten different oestrous symptoms were observed and registered. These oestrous symptoms were summarized in five classes based on the intensity and accuracy of the symptoms, from the weakest to the strongest oestrous symptom. Progesterone (**P4**) in milk was regularly analysed at regular times in all cows. Since the concentration of P4 changes depending on the stage in the oestrous cycle and the gestation it was used as an objective marker of the reproductive process. During oestrus the P4 concentrations are low which is why, in this study all oestruses were confirmed with a low P4 value. The P4 concentrations were also used to define normal and atypical oestrous cycles and as a marker for embryonic and foetal mortality. Of all inseminated cows, 24% of the cows with weak oestrous symptoms and 54% of the cows with strong oestrous symptoms became pregnant. This confirms our hypothesis that cows with strong oestrous symptoms have higher chance to become pregnant. Oestrus intensity (**OI**) increased with increasing ovulation number after calving and cows with longer oestrus duration expressed a stronger oestrus. Oestrus duration was defined as the time from first to last observed oestrous symptom. The general oestrus duration was estimated to 54h and the duration increased with increasing parity. The first and most frequently observed oestrous symptoms in a coming oestrus were red and swollen vulva, vaginal discharge and discharge color. The most obvious oestrous symptoms are standing to be mounted, mounting another cow and lowering of the back, but were seldom found in this study. One reason for this could be that the trend has gone toward fewer and less intensive oestruses. Another reason could be the low number of oestrus observations performed per day. To improve oestrus detection we need more frequent oestrus observations and we need to include all oestrous symptoms, even those that are weaker but expressed more often. By using oestrus detection that includes both automatic registrations, such as e.g. HerdNavigator™ or activity sensors, and visual observations more oestrous symptoms could be captured, which may result in better timing of insemination. To optimize the chance to find a cow in oestrus, and to find the optimal timing of insemination, oestrus intensity should be reintroduced as a breeding goal in the genetic evaluation. Automatic oestrus detection could be added as an indicator trait to oestrus intensity, which today is based on visual observations.

The aim of the second study was to investigate the extent and pattern of pregnancy losses. The data used for this study was the same as in the first part. Most pregnancy losses occurred during early pregnancy and embryonic loss was found to be 43% (from AI to day 60 in gestation), while foetal losses, which occurred from day 61 to calving, was 13%. The SR cows had lower total pregnancy losses compared to SH cows which support the first study where we found that SR cows had better pregnancy results compared to SH cows. Stronger oestrus intensity was found to decrease the amount of early embryonic loss from AI to day 24 after AI but no effect was found after day 24. One explanation for this may be that weak oestrus intensity results in an incorrect timing of insemination. Silent oestruses, which is an ovulation without any visible oestrous symptoms, is common in the first cycle. Oestrus intensity was found to increase with increasing ovulation number while early embryonic loss and total pregnancy loss was found to decrease at later ovulations. Even though later inseminations could benefit the early embryonic survival it would be suboptimal in other aspects. Later inseminations may result in longer calving to first insemination interval and longer calving interval which lower the milk production and affect the economy. Cows with pregnancy losses had higher P4 value at the day of AI and lower P4 value during the gestation compared to pregnant cows. This confirms that P4 levels play an important role for the pregnancy results.

In the third study Holstein-Friesian cows from four different countries were included. The aim was to provide information useful for a genetic evaluation of fertility by utilising P4 based fertility traits. The P4 pattern during the oestrous cycle is important for the pregnancy results. Delayed start of cyclicity and the interval from calving to start of luteal activity (**CLA**) were found to have higher heritabilities compared to the classical fertility traits that are used in e.g. the Nordic genetic evaluation. They were also found to be negatively affected by milk yield in early lactation, which may imply deterioration in these traits if not considered in breeding goals.

The aim of the fourth and last study was to provide information useful for genetic evaluation by using genomic information and to try to identify genetic markers associated with normal and atypical P4 profiles. With a genomic association study based on 50,000 genomic markers we found 44 markers associated with the seven observed endocrine fertility traits. Chromosome 8, 17 and 23, were further analysed, by using imputed sequences, which are millions of genetic markers based on a Holstein-Friesian reference population, to try to identify genes associated with the traits delayed cyclicity, cessation of cyclicity, CLA and oestrous cycle length. Five regions with several possible candidate genes related to reproductive functions were identified.

Genomic selection has been used in the Nordic countries for several years and has become an important tool in the breeding evaluation. In traditional breeding progeny testing is used which means that the bulls do not get a breeding value until their daughter get phenotypes in their first lactation. In genomic selection information about the traits can already be obtained when the animals are born. The animals can be selected in an earlier stage which will decrease the generation interval and improve the genetic gain. Genomic selection is more beneficial for traits with low heritability, such as the fertility traits. Fertility traits e.g. CLA and oestrus intensity, could be used as indicator traits in genomic selection provided there are large enough reference populations with both genotyped and phenotyped animals. In these large reference populations it would also be possible to use automated registrations of e.g. oestrus detection and P4 sampling.

Populärvetenskaplig sammanfattning

Mjölkkornas fruktsamhet spelar en avgörande roll för mjölkföretagens ekonomi. Korna måste visa brunst, bli dräktiga och bibehålla dräktigheten samt kalva med givna intervall och producera mjölk för att vara ekonomiskt försvarbara. I de nordiska länderna har fruktsamhet varit inkluderat i avelsvärderingen sedan 70-talet. Den traditionella avelsvärderingen inkluderar fruktsamhetsmått som är baserade på inseminerings- och kalvningsdatum, vilket betyder att de i hög utsträckning är beroende av besättningsägarens skötselstrategier. Tyvärr har den låga arvbarheten hos de klassiska fruktsamhetsegenskaperna bidragit till ett långsamt genetiskt framsteg. Då vi i Sverige använder artificiell insemination (**AI**) utan brunstsynkronisering med hormoner är det viktigt att korna visar brunsttecken som är tydliga för djurägaren dels för att hitta brunstiga kor och dels för att hitta rätt tidpunkt för inseminering. Svagare brunster i kombination med större kobesättningar och färre skötseltimmar per ko kan leda till sämre förutsättningar att hitta brunstiga kor.

Syftet med den första studien var att studera och beskriva brunsttecknens förekomst och betydelse hos två av våra mjölkkoraser, Svensk Röd och Vit boskap (**SR**) och Svensk Holstein (**SH**), och att analysera sambandet mellan brunstvisningsförmåga och dräktighetsresultat. Studien bygger på brunstdata från ca 2000 brunster registrerade under 16 år i en av Sveriges Lantbruksuniversitets tidigare försöksbesättningar. Brunstobservationer utfördes under 20 minuter tre gånger per dag och ett tiotal olika brunsttecken registrerades. Brunststyrkan sammanfattandes i en skala med fem klasser baserat på de enskilda tecknens styrka och säkerhet, från svaga till starka brunster. Hormonet progesteron (**P4**) analyserades kontinuerligt i mjölken hos alla kor. Nivån av P4 växlar beroende på fas i brunstcykel och dräktighet och är därmed en objektiv markör för fortplantningsprocessen. Under brunsten är P4-nivåerna låga och därför bekräftades alla brunster i denna studie med låga P4-nivåer. Progesteronanalyserna användes även för att definiera normala och atypiska brunstprofiler samt användes som en markör för embryo- och fosterdöd.

Av de kor som hade seminerats och som enbart visade svaga brunsttecken blev 24% dräktiga och av de kor som visade starka brunsttecken blev 54% dräktiga. Detta bekräftar vår hypotes att kor med starka brunsttecken har större möjlighet till ett bra fruktsamhetsresultat. Brunststyrkan ökade med ägglossningsnummer efter kalvning och kor med längre brunst visade även starkare brunst. Brunstens längd, som definierades som tiden från första till sista registrerade brunsttecken, var i genomsnitt 54 timmar och var längre för kor i senare laktationer än kor i tidigare laktationer. De första brunsttecknen som observerades och som även upprepades mest var vaginalflytningar och röd och svullen vulva vilket kan förväntas inför en kommande brunst. De klassiskt säkraste tecknen på brunst är upphopp på annan ko, stå för upphopp och svankning av länden. Dessa brunsttecken visades sällan i denna studie vilket delvis kan bero på att brunststyrka och brunstlängd har försämrats med tiden. Det kan även bero på att korna enbart studerades tre gånger per dygn vilket kan ha bidragit till en stor andel missade brunsttecken. För att förbättra brunstpassningen är det viktigt att ha tätare registreringar och inkludera brunsttecken som upprepas ofta och under en längre period som t.ex. flytningar och röd och svullen vulva. För att optimera brunstpassningen är automatiska brunstregistreringar, som t.ex. HerdNavigator^{TM} eller aktivitetsmätare, tillsammans med visulla registreringar att föredra, då man på detta sätt kan observera fler brunsttecken. Mer fokus borde ligga på brunsttecken då tydliga dvs. starka och långa brunster är förutsättningen för att hitta rätt tidpunkt för inseminering. För att öka chansen att hitta brunstiga kor och hitta rätt tidpunkt för inseminering bör brunststyrka återinföras som ett avelsmål i avelsvärderingen. För att ytterligare stärka egenskapen kan automatiska brunstregistreringar läggas till som en indikatoregenskap till brunststyrkan, som idag registreras med visuella observationer.

I den andra studien var syftet att studera förekomsten av dräktighetsförluster och när under dräktigeheten de sker. Här användes samma material som i första studien. Den största andelen dräktighetsförluster sker i tidig dräktighet och andelen embryoförluster (fram till dag 60 i dräktigheten) beräknades i vår studie till ca 43%. Fosterförlusterna (från dag 61 till kalvning) var ca 13%. Man kunde se att SR-korna hade lägre andel dräktighetsförluster än SH-kor vilket ligger i linje med vår tidigare studie där SR-kor hade ett bättre dräktighetsresultat än SH-kor. Andelen tidiga embryoförluster (mellan dag 1-24 i dräktigheten) minskade med ökande brunststyrka medan man inte kunde se någon påverkan efter dag 24. Detta kan delvis sannolikt förklaras av att en svag brunststyrka har lett till att inseminering skett vid fel tidpunkt och att en befruktning troligen inte skett, alternativt att embryot vid ett senare tillfälle inte varit kapabelt att leva vidare. I första brunstcykeln sker många tysta brunster vilket betyder att korna har ägglossning men inte visar några yttre brunsttecken. Insemination i senare brunstcykler har visats ge bättre dräktighetsresultat genom att starkare brunster ger lägre andel tidiga embryoförluster. Det är dock inte optimalt att rekommendera lantbrukare att vänta med inseminering till senare då det leder till ett längre kalvningsintervall, vilket resulterar i lägre mjölkproduktion och försämrad ekonomi. Kor som förlorar sitt embryo/foster under dräktigheten har högre P4-nivåer vid inseminering och lägre P4-nivåer under dräktigheten jämfört med dräktiga kor. Detta bekräftar att P4-nivåerna har en viktig roll för att uppnå bra dräktighetsresultat.

I den tredje studien studerades Holstein-Friesian kor från fyra olika länder. Syftet var att hitta användbar information om de progesteronbaserade fruktsamhetsegenskaperna för att kunna förbättra avelsvärderingen för fruktsamhet. Progesteronkurvans form under brunstcykeln har visat sig ha stor betydelse för dräktighetsresultatet. Onormala brunstcykler, så kallade atypiska progesteronprofiler, kan leda till senare inseminering, fler insemineringar samt längre kalvningsintervall, vilket i slutändan påverkar mjölkproduktionen och lantbrukarens ekonomi negativt. Intervallet från kalvning till start på luteal aktivitet (**CLA**) och försenad äggstocksaktivitet har visat sig ha högre arvbarhet än de klassiska fruktsamhetsmåtten som idag används i avelsarbetet. Måtten har även visat sig vara negativt påverkade av mjölkmängd, vilket kan tyda på en försämring av dessa egenskaper om de inte tas hänsyn till i avelsvärderingen.

Syftet med den fjärde och sista studien var att studera genetiska markörer och med hjälp av dem identifiera gener som kan ha samband med normala och atypiska P4-profiler. Materialet i denna studie var samma som i den tredje studien. Med en genomisk associationsstudie, baserat på 50,000 genetiska markörer, identifierades 44 markörer som kan ha samband med de sju endokrina fruktsamhetsmåtten. Kromosom 8, 17 och 23 analyserades vidare genom att använda miljoner av genetiska markörer baserade på en referenspopulation som bestod av Holstein-Friesan kor och tjurar. För försenad äggstocksaktivitet, avbruten äggstocksaktivitet, CLA och brunstcykelns längd identifierades fem områden med gener som kan kopplas ihop med olika fruktsamhetsfunktioner.

Genomisk selektion har använts i de nordiska länderna sedan flera år tillbaka och har blivit ett viktigt redskap i avelsvärderingen. I traditionellt avelsarbete använder man så kallad avkommeprövning vilket innebär att man måste vänta till att tjurarnas döttrar presenterat egenskaper från sin första laktation. I en genomisk avelsvärdering utnyttjar man information redan när djuren föds. Detta har resulterat i kortare generationsintervall och snabbare genetiskt framsteg. Genomisk selektion är framför allt fördelaktigt för egenskaper med låga arvbarheter, som t.ex. fruktsamhet. Olika fruktsamhetsmått, som t.ex. CLA och brunststyrka, skulle kunna användas som indikatoregenskaper i genomisk selektion om det i framtiden finns tillräckligt stora referensgrupper med DNA-testade djur, som också har tillräckligt många progesteronregistreringar. I sådana referensgrupper skulle det även vara möjligt att använda sig utav automatiska registreringsmetoder för att registrera brunsttecken och analysera P4.

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