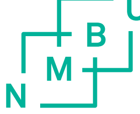

Norwegian University of Life Sciences
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Philosophiae Doctor (PhD)
Thesis 2023:33

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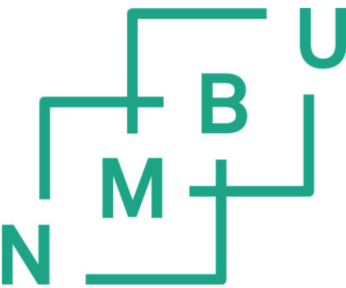

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I request the highest of fives – the self-five

Adapted from American sitcom
How I Met Your Mother
CBS Broadcasting Inc.

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1 Abbreviations and definitions

AI	Artificial insemination
AUC	Area under curve
cDNA	Complementary DNA
CYTO	Cytological endometritis
CFAI	Interval from calving to first artificial insemination
DE	Differentially expressed
EGA	Embryonic genome activation
GO	Gene ontology
HF	High fertility
IETS	International Embryo Technology Society
IFNT	Interferon-τ
LDA-LEfSe	Linear discriminant analysis effect size
LE	Leukocyte esterase
LF	Low fertility
MET	Maternal-to-embryonic transition
MOET	Multiple ovulation embryo transfer
NDHRS	Norwegian dairy herd recording system
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NGS	Next generation sequencing
NMBU	Norwegian University of Life Sciences
NR	Norwegian Red
NRR	Non-return rate
OPU-IVF	Ovum pickup *in vitro* fertilization
PAG	Pregnancy associated glycoproteins
PMN	Polymorphonuclear cells
PVD	Purulent vaginal discharge
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cells
RNA-seq	RNA sequencing
ROC	Receiver operating characteristics
rRNA	Ribosomal RNA
SCE	Subclinical endometritis

2 Summary

The productivity in the dairy cattle industry is highly dependent on fertility. In the last decades, an alarming decrease in the reproductive capacity has been detected in most dairy cattle breeds. Subfertility in dairy cattle is multifactorial, and the most common mechanism for failure of pregnancy is embryo loss which occurs within the first 42 days of gestation. Embryo survival depends both on the environment in the reproductive tract of the mother, and the quality of the embryo as such. Hence, embryo loss may be attributable to any negative impact along the developmental axis and determined by both maternal and paternal impact.

Norwegian red (NR) is notable for its good reproductive performance, which is probably a result of emphasis on fertility and health traits in the breeding program since the 1970's. With the implementation of genomic selection in the breeding strategy, interest in new phenotypes that can add information on fertility and health has been increasing. The strategy also has a new focus on embryo production, which requires a high level of knowledge concerning all aspects of the embryo.

The overall aim for this thesis was to gain knowledge about factors affecting embryo loss in NR. To do this, specific knowledge gaps related to both maternal and paternal aspect were targeted. In the cow, we provided information about cytological endometritis (CYTO) at artificial insemination (AI) in NR through a prospective cohort field trial of 1,648 cows in 116 herds, using the cytotape technique. Here, prevalence, effect on fertility and embryo loss, and risk factors for the condition was reported. This was complemented by a heritability estimate for the condition using data from the same population. Further, we investigated the uterine microbiota at AI in NR using a smaller study population from one herd. To investigate the paternal contribution to the early embryo, we performed a transcriptome study of embryos derived from bulls with high and low field fertility, respectively.

The prevalence of CYTO was 28% at AI, when the condition was defined by a 3% cut-off of the proportion of polymorphonuclear cells in cytological smears. The prevalence was higher than previously described at AI in other breeds. We reported a negative association between pregnancy incidence to first AI and CYTO. However, the overall pregnancy incidence to first AI was high, suggesting that the biological effect of this condition appears to be modest in comparison with results from other breeds. We speculate that an advantageous uterine immunology or perhaps certain traits in the microbiota could be the explanation for this outcome. The detected risk factors for CYTO were AI personnel, interval from calving to first AI, vaginal mucus condition, red blood cells in sample, barn type, and season. Late embryo loss and fetal loss was not

associated with CYTO at first AI, suggesting that decreased fertility in CYTO positive cows is a result of either early embryo loss or fertilization failure.

CYTO detected at AI was indicated to be heritable in NR by a trait definition of 5% polymorphonuclear cells. The heritability estimate was 0.04 and comparable to estimates of other diseases and disorders included in the total merit index for NR. Hence, CYTO can be considered as a new phenotype for fertility. With future development of a more feasible method to diagnose CYTO, it has the potential to be used for breeding purposes.

The uterine endometrial microbiota at AI was investigated at a superficial level with a cytobrush sample, and at a deep level with a biopsy sample. The microbiota from both sites was compared to the findings in vaginal swabs. In the biopsy samples, we found a high relative abundance of *Oscillospiraceae UCG-005*, *Bacteroidetes_vadinHA17*, *Ruminococcus*, *Bacteroides*, *Alysiella* and four different genera of the family Lachnospiraceae. These taxa were barely present in the other sample types, and not present in the negative extraction control. *Streptococcus* was highly abundant in both cytobrush and vaginal swabs, and *Escherichia-Shigella* was detected in mainly the vaginal swabs. The cytobrush samples and the vaginal swabs shared a similar taxonomic composition, suggesting that vaginal swabs may suffice to describe the surface-layer uterine microbiota at estrus. Interestingly, Fusobacteriota, which is associated with the development of metritis and purulent vaginal discharge, and a common finding in the uterine microbiota in other breeds, was not detected in any of the sample types. The biopsy samples were qualitatively different and more even than that of cytobrush and vaginal swab samples. We hypothesize that the deep layer endometrial microbiota might be associated with diseases and disorders in the uterus, even when superficial layer microbiota does not show such a correlation.

Embryos derived from bulls of high or low field fertility had a differing gene expression in genes linked to cholesterol metabolism, cytokine signaling, regulation of apoptosis, adhesion and attachment in placental development, formation of tight junctions, and regulation of redox activity. These are all processes or pathways that are known to be crucial for embryo development at the blastocyst stage and around attachment.

The collected results presented in this thesis are valuable as we continue to explore the underlying mechanisms of fertility in dairy cattle. For NR, the results are applicable for breeding and management recommendations, as well as in herd health investigations. This contributes to maintaining the current status of NR as a high fertility breed, and possible future improvements.

3 Norsk sammendrag

Produktivitet i melkeindustrien er i høy grad avhengig av dyrenes fruktbarhet. I løpet av de siste tiårene, har det blitt rapportert en markert nedgang i reproduktive resultater hos de fleste melkeraser. Lav fruktbarhet hos melkeku skyldes flere faktorer, og den vanligste årsaken til uteblitt drektighet er embryodød de første 42 dagene etter paring. Embryoets overlevelse er avhengig av både miljø i morens reproduksjonsorganer og kvaliteten av embryoet selv. Embryodød kan derfor skyldes enhver negativ innvirkning langs hele utviklingsaksen og påvirkes av både maternale og paternale aspekter.

Norsk Rødt Fe (NR) er en rase med svært god fruktbarhet. Dette er sannsynligvis et resultat av avlsprogrammets vektlegging av egenskaper koblet til fertilitet helt siden 1970-årene. Med implementering av genomisk seleksjon i avlsstrategien, har man sett en øket interesse for nye fenotyper som kan inkluderes i avlsarbeidet. I den nye strategien satses det også på embryoproduksjon. Dette krever et høyt nivå av kunnskap om alle aspekter av embryoutvikling.

Det overordnede målet for denne avhandlingen var å innhente kunnskap om faktorer som påvirker embryodød hos NR. For å oppnå dette, rettet vi fokus mot spesielle kunnskapshull relatert til både maternal og paternal innvirkning. Hos mordyret, presenterte vi ny informasjon om cytologisk endometritt (CYTO) ved kunstig sædoverføring (KS) hos NR. Data ble samlet inn i felt fra 1648 kyr i 116 besetninger med hjelp av *cytotape* og brukt til en prospektiv kohortstudie. Her rapporterte vi prevalens, effekt på fertilitet og embryo død, og risikofaktorer koblet til tilstanden. Dette ble videre komplettert med en beregning av estimert arvbarhet for CYTO med data fra den samme populasjonen. Videre undersøkte vi uterin mikrobiota ved KS hos NR i en mindre studiepopulasjon fra én besetning. For å undersøke det paternale bidraget i tidlig embryoutvikling, gjennomførte vi en transkriptomstudie av embryo med opphav fra okser med henholdsvis høy eller lav feltfruktbarhet

Prevalensen for CYTO var 28% ved KS når tilstanden ble definert ved en grense på 3% av polymorfonukleære celler i celleutstryk. Den rapporterte prevalensen var høyere enn det som tidligere har blitt beskrevet ved KS for andre raser. Vi fant en negativ assosiasjon mellom drektighet etter første KS, og CYTO. Til tross for dette, var den totale drektighetsinsidensen etter første KS høy, hvilket indikerer at den biologiske effekten av denne tilstanden hos NR tilsynelatende er moderat sammenlignet med andre raser. Basert på disse funnene, har vi diskutert om resultatene kan være koblede til en fordelaktig uterin immunologi, eller muligvis spesielle trekk i uterin mikrobiota hos NR. Risikofaktorer for CYTO var inseminør, intervall fra kalving til første KS, karakteristika ved vaginal utflod, røde blodlegemer i prøven, fjøstype og sesong. Sen embryodød og fosterdød var ikke assosiert med CYTO ved første KS,

hvilket indikerer at forstyrrelser i fertiliteten hos CYTO positive kyr er et resultat av enten tidlig embryodød eller uteblitt fertilisasjon.
CYTO ved AI er tilsynelatende arvelig hos NR når egenskapen blir definert ved en grense på 5% polymorfonukleære celler. Arvbarheten ble estimert til 0.04, hvilket kan sammenlignes med andre tilstander og sykdommer som blir inkludert i samlet avlsverdi for NR. CYTO kan bli betraktet som en ny fenotype for fertilitet. Under forutsetning av at det utvikles en mer gjennomførbar metode for å diagnostisere CYTO, har egenskapen potensiale til å bli brukt i avlsprogrammet for NR.
Den uterine mikrobiotaen ved KS ble undersøkt i overfladisk endometrium med en *cytobrush* prøve, samt i dypere endometrium med en biopsi. Mikrobiota fra både overfladisk og dyp prøve ble sammenlignet med funn fra vaginalsvaber. I biopsiprøvene ble de funnet en høy relativ tilstedeværelse av *Oscillospiraceae UCG-005*, *Bacteroidetes_vadinHA17*, *Ruminococcus*, *Bacteroides*, *Alysiella* og fire forskjellige genera av familien Lachnospiraceae. Disse taxa var nesten ikke til stede i de andre prøvetypene, og ble ikke funnet i negative ekstraksjonskontroller. *Streptococcus* forekom i høye relative mengder i både *cytobrush*-prøvene og vaginalsvaber, og *Escherichia-Shigella* ble detektert hovedsakelig i vaginalsvabere. *Cytobrush*-prøvene og vaginalsvabrene hadde lignende taksonomisk sammensetning, hvilket indikerer at det kan være tilstrekkelig å samle en vaginalsvaber for å bestemme overfladisk uterin mikrobiota ved brunst. Et interessant resultat fra studien viste også at Fusobacteriota, som er assosiert med utvikling av metritt og purulent vaginal utflod, og et vanlig funn i uterin mikrobiota hos andre raser, ikke ble funnet i noen av prøvetypene i vår studie. Biopsiprøvene var kvalitativt forskjellige og hadde en mer jevn fordeling av de forskjellige taxa, sammenlignet med *cytobrush*-prøvene og vaginalsvabrene. Vi spekulerer på om mikrobiota fra dyp endometrium kan være assosiert med sykdommer og tilstand i uterus selv i de tilfeller hvor man ikke ser en slik korrelasjon med mikrobiota fra overfladisk endometrium.
Embryo med opphav fra okser med høy eller lav feltfruktbarhet hadde forskjeller i genekspresjon i gener som er koblet til kolesterolmetabolisme, cytokinsignalisering, regulering av apoptose, embryoets innbinding til uterusveggen ved utvikling av placenta, utvikling av av *tight junctions*, og regulering av redoks-aktivitet. Disse biokjemiske prosessene er kjente for sin essensielle rolle i embryoutvikling rundt blastocyststadiet og rundt tiden da embryoet fester seg til endometriet.
De samlede resultatene presentert i denne avhandlingen vil være verdifulle for videre forskingen på mekanismer som påvirker fruktbarhet hos melkeku. For NR, er resultatene nyttige ved anbefalinger rundt avl og stell, i tillegg til besetningsutredninger. Dette bidrar til å opprettholde nåværende status hos NR som en rase med høy fruktbarhet, samt videre forbedring av fruktbarheten.

4 List of papers

Paper I
Diaz-Lundahl Sofia, Garmo Randi Therese, Gillund Per, Blystad Klem Thea, Waldmann Andres, and Krogenæs Anette (2021). Prevalence, risk factors, and effects on fertility of cytological endometritis at the time of insemination in Norwegian Red cows. Journal of dairy science 104: 6961-6974.
Doi: 10.3168/jds.2020-19211

Paper II
Diaz-Lundahl Sofia, Heringstad Bjørg., Garmo Randi Therese, Gillund Per and Krogenæs Anette (2022). Heritability of subclinical endometritis in Norwegian Red cows. Journal of dairy science 105:5946-53.
Doi: 10.3168/jds.2021-21752

Paper III
Diaz-Lundahl Sofia, Foyn Nørstebø Simen, Blystad Klem Thea, Duncan Gilfillan Gregor, Dalland Marianne, Gillund Per and Krogenæs Anette. The microbiota of uterine biopsies, cytobrush and vaginal swabs at artificial insemination in Norwegian Red cows.
Submitted to Theriogenology

Paper IV
Diaz-Lundahl Sofia, Meenakshi Sundaram Arvind Yegambaram, Gillund Per, Duncan Gilfillan Gregor, Olsaker Ingrid and Krogenæs Anette (2021). Gene expression in embryos from Norwegian Red bulls with high or low non return rate: an RNA-seq study of *in vivo*-produced single embryos. Frontiers in Genetics 12:780113.
Doi: 10.3389/fgene.2021.780113

Papers I, II and IV have been included in this thesis with the publishers' permission.

5 Introduction

5.1 Global concerns about decreased fertility and increasing embryo loss

In the last few decades, milk production in the global dairy cattle industry has increased significantly (Gross, 2022). During the same time, an alarming decrease in the reproductive capacity has been detected in the majority of dairy cattle breeds. This negative development was expressed as an emerging issue in several reviews in the early 2000's (Lucy, 2001; Silke et al., 2002; Pryce et al., 2004). The dairy cow needs to give birth in order for milk production to start (Lucy, 2001). Then, to maintain a high production throughout the cow's life, she needs to get pregnant at certain intervals. For instance, in the Norwegian dairy production it is common to aim for one calf per year (Geno SA, 2020d). Hence, the productivity in the dairy cattle industry is highly dependent on fertility. It has also been recognized that the entire production cycle of each animal needs to be optimized in order to lower the emission of greenhouse gases per unit of produced milk (Place and Mitloehner, 2010).

In the early 2000's, several studies pointed to a genetic antagonism between milk production and reproduction (Pryce et al., 2004; Lonergan et al., 2016). It has been reported that the two systems are competing for the same resources in the body. Physiological adaptations for a higher milk yield may explain part of the total reproductive decline. However, on an individual level, it is not always the case that the highest producing individual has the lowest fertility (Leblanc, 2010; Lonergan et al., 2016). More recent literature states that genetic gain in both milk production and reproduction is possible in a well-managed breeding program (Berry et al., 2016). As an example, some countries have managed to both halter and reverse the negative development in fertility while still maintaining a high milk yield (Lonergan et al., 2016).

Subfertility in dairy cattle is multifactorial. The mechanisms that have a negative impact on fertility includes post-partum anestrus, failure of ovulation, fertilization problems, and any issues along the developmental axis of the embryo and growing fetus. Some general causes for subfertility include heat stress, disease, nutritional issues, and genetics (Lonergan et al., 2019). Traditional fertility measurements in dairy cattle can be divided into fertility scores such as conception rate or non-return rate (NRR), and interval measurements such as days open or calving interval (Pryce et al., 2004). The measurements that indicated a decline in fertility in the dairy cattle

included decreased first-service conception rate and calving rate to first service, and increased services per conception, days from calving to first artificial insemination (AI), and days open (Lucy, 2001; Pryce et al., 2004).

In 2006, Diskin and colleagues presented data showing that the immense drop in dairy cattle fertility was mainly due to a proportionate increase in early embryo loss (Diskin et al., 2006), see Figure 1. Embryo loss is defined as the death of a conceptus before day 42 of gestation (Sreenan and Diskin, 1986; Zavy and Geisert, 1994). The total occurrence of embryo loss can be estimated by the difference between fertilization rate and calving rate, taking into account the amount of fetal death or abortions (Sreenan and Diskin, 1986). In literature, embryo loss is mainly divided into early and late embryo loss. The division between the two is generally appointed to day 24 of gestation (Diskin et al., 2006).

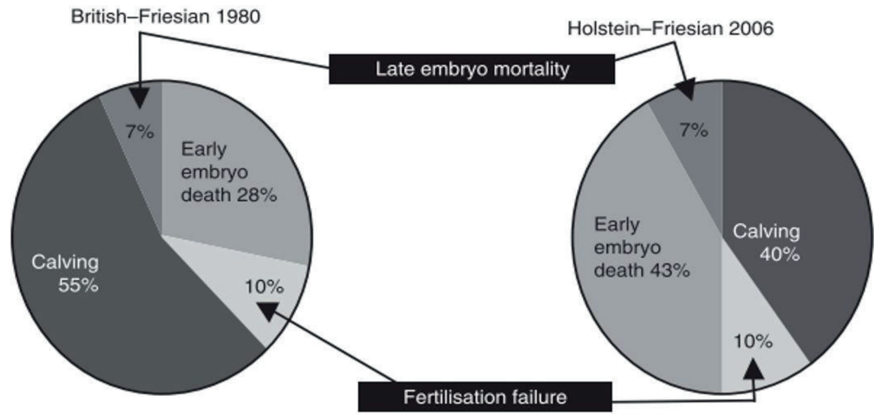

Figure 1. Reproductive outcomes in 1980 (British-Friesian cows) vs 2006 (Holstein-Friesian cows). Early embryo death is defined as the death of the embryo before day 28 of gestation in the figure. Figure reproduced with permission from Diskin et al. (2006), Copyright Elsevier.

In the individual animal, late embryo loss has a larger economic impact than early embryo loss. This is due to the fact that maternal recognition of pregnancy happens around day 16 (Wiltbank et al., 2016). A cow that experiences late embryo loss will return to estrus in an irregular manner (Diskin et al., 2011), after day 24, meaning that she will have more days open. This is especially concerning in systems with seasonal calving where there is an increased risk for culling (Sreenan et al., 2001; Diskin et al., 2011). On the other hand, cows that experience early embryo loss (in the cases where the embryo dies before around day 16) will return to estrus within 24 days from the previous estrus. Nevertheless, considering the total amount of early and late embryo loss, it is clear that the overall economic impact is much larger for early loss. Hence,

early embryo loss stands out as the most important research area (Sreenan et al., 2001).

While early embryo mortality has increased, the data presented by Diskin et al. in 2006 also showed that late embryo loss seems to be unchanged (Diskin et al., 2006). The same trend goes for fertilization rate, which is described as the proportion of cleaved ova or embryos out of all recovered (unfertilized or fertilized) units. Fertilization rate is estimated to be 90 to 100% for heifers and probably a bit lower and more variable in high-producing dairy cows (Diskin et al., 2016). In herds where estrus detection and management around the AI timepoint or procedure are suboptimal, fertilization failure could be responsible for a larger part of the overall subfertility. Increasing herd sizes create more challenges when it comes to visual registration of heat. However, these matters have been automated and improved to some extent by the use of synchronization protocols, behavior registrations and activity measurements. Further advancements of these tools are likely to determine the time of ovulation with increasing accuracy (Crowe et al., 2018; Santos et al., 2022). Hence, embryo survival becomes an increasingly important determinant of the outcome and economy in large herds which are driven by advanced technology.

5.2 Embryo development in the bovine

The bovine embryo's life begins after fertilization in the oviduct (Lonergan et al., 2016). Here, it continues its repeated mitotic divisions without increasing in size (Valadão et al., 2018). The embryo spends about 4 days in the oviduct before entering the uterus at the 16-cell stage (Lonergan et al., 2016). Floating freely, it develops a compact ball made up of cells which starts to form tight junctions – the morula. From there, a blastocyst consisting of an inner and outer cell mass, and a fluid filled cavity, the blastocoel cavity, is formed. The inner cell mass is the origin for the fetal tissues and consists of primitive endoderm that will form the amniotic sac, and pluripotent cells that will differentiate and form the fetus. The outer cell mass, the trophectoderm, will form the fetal side of the placenta (Wiltbank et al., 2016). It is common to refer to the combination of all these structures as the conceptus (Peippo et al., 2011). The *zona pellucida* is an acellular protein coat surrounding the oocyte and the early conceptus (Bleil and Wassarman, 1980). At the end of the blastocyst stage, this coat opens, a process referred to as hatching, allowing the conceptus to change its size and shape. At hatching, the embryo becomes dependent on histotroph, which are maternal secretions from the uterine glands. To date, it is still not possible to imitate this maternal environment, making it impossible to cultivate embryos *in vitro* passed the hatching of the blastocyst (Lonergan et al., 2016). After hatching, the bovine conceptus, mainly the trophectoderm, performs a vast and exponential elongation, associated with a massive protein production (Bai et al., 2013; Lonergan et al., 2016). Just before elongation and with an increased intensity during the growth, the conceptus starts producing interferon-τ (IFNT), which is the signal for maternal recognition of pregnancy. With maternal recognition, luteolysis of the corpus luteum is blocked and the pregnancy is maintained (Rizos et al., 2017; de Souza Ribeiro et al., 2018). The critical period for this signaling is around day 16 of pregnancy. IFNT also serves the purpose to regulate the maternal immune system so it does not reject the conceptus as a result of paternal origin components (Wiltbank et al., 2016). Starting at around day 19-21, the conceptus attaches to the uterine endometrium (Bai et al., 2013). In the bovine, the term attachment is preferred rather than implantation, as the fetal membranes are attached to the endometrium, whereas in other species, such as the human, the embryo is inserted into the endometrium (Peippo et al., 2011). With attachment, the source of nutrition gradually changes from the histotroph to a yolk sac placenta and is finally provided through the circulatory system of the chorioallantoic placenta (Wiltbank et al., 2016). Figure 2 provides a timeline of the bovine embryo development.

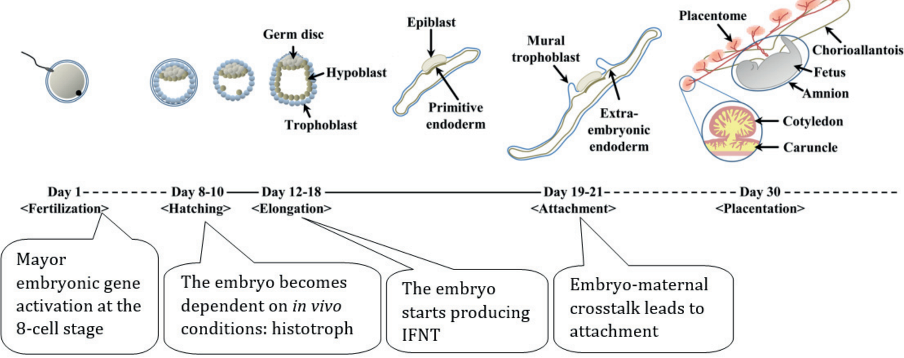

Figure 2. A timeline demonstrating important events in the bovine embryo development. Interferon-τ (IFNT). Figure adapted with permission from Bai et al. (2013), Copyright Japanese Society of Animal Reproduction.

5.3 Regulation of embryo development

Many of the cellular processes that promotes the development of the zygote to a functional organism are driven by cell signaling that activate different key pathways. Some central questions of developmental biology are, how and when the genetic code translates to signals or instructions, how these signals are emitted at a correct level, time, and place, and how the signals are interpreted and acted upon (Basson, 2012).

5.3.1 Maternal-to-embryonic transition and differentiation

From fertilization, the active RNAs and proteins within the embryo comes from the gametes of the mother (Graf et al., 2014b) and the father (Gross et al., 2019). The functions of these products include the activation of the embryo's own genome, and as development proceeds, these parental products are slowly degraded (Graf et al., 2014b). The maternal-to-embryonic transition (MET) is the timespan during which there is a shift in the transcriptional origin. During MET, the onset of the embryo's transcription, which occurs in waves, is a key event in preimplantation embryo development (Graf et al., 2014a; Wiltbank et al., 2016).

Transcripts with embryonic origin have been detected already from the one-cell stage (Memili and First, 1999). However, the exact onset is not well defined in the bovine, and the conclusion seems to depend on the methods used (Graf et al., 2014a). It has been suggested that the earliest activated genes are related to RNA processing, transport, and translation of mRNA. The largest proportion of embryonic gene activation in the bovine occurs at the 8-cell stage (on around day 3), an event referred to as the major embryonic genome activation (EGA). During this stage, the number of transcripts with embryonal origin seemingly begins to exceed the embryo's content of maternal transcripts (Graf et al., 2014b).

In the very beginning, the cells within the embryo are totipotent. From around the 16-cell stage, and after the major EGA, embryo development requires proper differentiation into distinct cell lineages in addition to the rapid cell division. Genes that regulate cell lineage differentiation have been detected mainly at the blastocyst stage in the bovine (Wiltbank et al., 2016).

5.3.2 Key pathways and cell signaling

The mammalian embryo's development is affected by two types of cell signals: intrinsic and extrinsic. These signals activate biological pathways through which the establishment, segregation and differentiation of pre-implantation cell lineages is made, leading to morphogenesis and expansion of the blastocyst, and its preparation for implantation (Eckert et al., 2015). The intrinsic signaling is mediated within the

embryo as a part of the autonomous developmental program. The extrinsic signals are the two-way communication between the embryo and the surrounding environment, i.e., the maternal genital tract or the *in vitro* environment. The effects from early cell signaling on the embryo, such as gene expression and gene expression regulation have short- and/or long-term consequences that can extend even into adult life (Eckert et al., 2015).

The identification of signals acting during embryo development, and the understanding of their properties and functions has seen many advances during recent years. For instance, we now know that there is repeated use of the same biological pathways in different places in the embryo and at different times. It is also known that there is a crosstalk between distinct pathways, allowing cells to have unique responses to the same signal, which is needed in differentiation (Sanz-Ezquerro et al., 2017).

The intrinsic signaling systems creates cell polarization and asymmetry by which distinct cell linages can be formed. The first intrinsic signaling pathway activated in the fertilized oocyte is the phosphoinositide signaling pathway, which is initiated by sperm-derived components. Among several functions, it initiates the activation of the embryonic genome. At compaction, cell-cell adhesion, cell polarization and membrane cytoskeleton reconstruction become important events. These events are mainly mediated by the Ca^{2+}-dependent E-cadherin- catenin complex and form the basis for subsequent blastocyst morphogenesis and trophectoderm formation (Eckert et al., 2015). Regulation of differentiation and blastocyst formation is also mediated by the signal from essential SRC family kinases. Through their action on growth factor receptors, they regulate proliferation, cell adhesion, growth, and survival of the embryo (Zhang et al., 2014). The trophectoderm and the inner cell mass of the embryo is further controlled by several transcription factors such as Cdx2, Oct4, Sox2 and Nanog. Cdx2 supports trophectoderm differentiation, while the others coordinate the viability of the inner cell mass (Eckert et al., 2015).

The extrinsic signaling affects the developmental programs in terms of differentiative potential of individual cell lineages, cellular proliferation, and metabolic activity. In the post-conception maternal tract, there are a variety of communication processes, which are often mediated through growth factors and cytokines (Eckert et al., 2015). These molecules and/or their receptors are present in the embryo itself and in the oviduct and uterus (Lonergan, 1994). Hence, the signaling does not only affect the embryo but also the uterine receptivity to implantation. The zona pellucida has a high permeability, allowing direct access of paracrine components to the cells within the embryo (Robertson et al., 2015). Insulin and amino acids are two examples of extrinsic signaling molecules that affect early embryogenesis in mammals. Effects of insulin on the embryo is mediated through the insulin receptor which activates several intracellular pathways, including the PI3K-akt signaling pathway (Cheng et al., 2010).

PI3K-Akt signaling has a central role in embryo survival, regulating differentiation and cell growth, proliferation, anti-apoptosis and calcium metabolism (Robertson et al., 2015). AMPK is another energy sensing pathway which has a crosstalk with insulin signaling. It is considered the key pathway responsible for the maintenance of cellular energy balance, and its downstream effects include regulation of fatty acid metabolism (Eckert et al., 2015).

Another extracellular signal active in the uterus include TGF-β family growth factor (Lonergan, 1994) that acts on the homeobox protein Nanog, with the function of maintaining pluripotency in human embryonic stem cells (Pera and Tam, 2010). The actions from the glycoproteins FGF2 and WNT also inhibits stem cell differentiation in human and bovine embryos (Pera and Tam, 2010; Ortega et al., 2017; Sidrat et al., 2020). FGF2 have the additional effect of stimulating the production of IFNT in the bovine (Cooke et al., 2009). Other key pathways for the developing mammalian embryo includes the hedgehog signaling pathway and NOTCH (Sanz-Ezquerro et al., 2017). The hedgehog signaling pathway controls cell proliferation and patterning of neural progenitors (Jiang and Hui, 2008), while a similar role has been suggested in the preimplantation bovine embryo for the NOTCH pathway (Li et al., 2021).

The details of cell signaling and the related key pathways in embryo development in general are not fully understood. The regulation of transcription factors cascades in the embryo will continue to be an important research focus in the future (Eckert et al., 2015).

5.4 Studying embryo developmental potential

The results of cell signaling within the embryo can control the expression of transcription factors, which again regulates cell specification and cell fate (Sanz-Ezquerro et al., 2017). Through their actions, many of the signals actively keep the embryo alive, so that the loss or inhibition of a specific signal is only revealed by increased apoptosis (Basson, 2012). Mammalian embryos also show regulative development, meaning that compensatory mechanisms can be triggered by damage or loss of cells to support a continued embryo development (Pera and Tam, 2010). Stress on the embryo can be defined as a weakened cell ability to produce normal and sufficient products upon differentiation, or a slower pace of stem cell accumulation. It is important to remember that stress response in the embryo is also normal in development and is elevated at certain mayor events such as genome activation, compaction, cavitation and at the differentiation of the first lineages (Puscheck et al., 2015). It has been suggested that the metabolic activity of poor-quality embryos is higher than in normally developing embryos (Leese, 2002; Baumann et al., 2007). This hypothesis was however revised in 2022 with the conclusion that developmentally successful embryos will have an optimal level of metabolic activity (not too active, not too quiet) referred to as *the Goldilocks zone* (Leese et al., 2022). Transcription factor activity is commonly changed as a response to stress. Some examples from literature are Oct1 and Oct4, Cdx2, Sox2 and Nanog. The affected pathways include PI3K, Akt, AMPK, SAPK and MEK1/2. Typically, stress lowers embryonic developmental and proliferative rates, but it can also induce a faster differentiation of stem cells. More research is needed to elucidate the details of stress in the embryo (Puscheck et al., 2015).

One of the first ways to investigate embryo developmental potential was through studying morphology and the rate of development such as blastocyst developmental rates. The morphological evaluation is commonly made based on detailed descriptions of stage and quality published by The International Embryo Technology Society (IETS) (https://www.iets.org/). The estimation of embryo stage and quality is still important in several aspects of research, for instance as selection criteria of embryos for comparisons based on differing conditions. Several new and alternative methods to estimate embryo quality are emerging (Rocha et al., 2016).

5.4.1 Sequencing of embryos

In recent years, advances have been made in technologies that increases the amount of information and accuracy in the investigation of embryos. The first large scale transcriptome profiling of bovine blastocysts was made using microarrays on *in vitro* produced (Sirard et al., 2004) and *in vivo* produced embryos (Kues et al., 2008). This provided an important basis of the current knowledge about the developing embryo.

However, the results from microarray studies were limited by the probe set of each microarray (Graf et al., 2014b). Later, next generation sequencing (NGS) became commercially available and more affordable. The first study using RNA-seq on bovine embryos was done in 2010 on pools of *in vitro* produced embryos (Huang and Khatib, 2010). The sequencing by NGS can be targeted to different cell products, following the line of the central dogma in biology, so that one can study both DNA and different RNAs. The complete genome can be extracted, subjected to fragment library construction, amplified, and sequenced, leading to massive data sets (Mardis, 2013). For RNA-sequencing (RNA-seq), RNA extraction is followed by a conversion to complementary DNA (cDNA) (Wang et al., 2009). It is now clear that the functionality of the genome in mammalian embryos is dependent on a variety of regulatory mechanisms including RNA modification, RNA editing, translation and stability of RNA, and non-coding RNAs. These mechanisms work together to produce the functional transcriptome (Niemann et al., 2007).

The data from NGS provides a solid basis when studying development in terms of onset of specific genes, and up/down regulations of certain genes and their related pathways at different settings. As an examples, Graf et al. (2014b) sequenced pools of oocytes and embryos from 4, 8, and 16-cell stages in addition to blastocysts. Through their work, they elucidated the details of MET and appointed major EGA in cattle to the 8-cell stage. Another study compared degenerated and normal blastocysts using RNA-seq and found differences in the expression of several genes that could be linked to crucial pathways in embryo development, such as steroid biosynthesis and cell communication (Huang et al., 2010). Sequencing data can also reveal similarities between embryos of different species. Jiang et al. (2014) compared NGS transcriptome data from human, mice, and cattle. They found that the bovine embryo is a better model for human embryonic development than the mouse embryo, implying that studies on the bovine embryo are highly relevant beyond the field of veterinary science. With NGS, one can also get a detailed picture of all that is affected by a certain phenotype of the mother or father animal, which is relevant for the methods used in the current thesis.

Most transcriptome studies of embryos used *in vitro* fertilized embryos. *In vitro* culture has the disadvantage of significantly affecting morphological and biochemical characteristics of mammalian embryos (Wright Jr and Ellington, 1995). Even though *in vivo* development of embryos is much less feasible to study, it is important to confirm results from *in vitro* produced embryos with an *in vivo* approach. Similarly, pooling of several embryos was used in the earliest RNA-seq studies (Huang and Khatib, 2010; Graf et al., 2014b), while recent advances make it possible to sequence nucleotides from single embryos (Chitwood et al., 2013) and even single blastomeres (Xue et al., 2013).

5.5 Approaching embryo death – previously known causes about when and why embryos die

While early embryo mortality is more common than loss at the later stages of gestation, there are also periods within the definition of early loss at which the embryo is more likely to die (Diskin et al., 2016; Ribeiro and Carvalho, 2018). It is challenging to precisely determine the exact timepoint for embryo loss, especially before the living embryo is detectable by ultrasound. The majority of embryo mortality occurs within 16 days from breeding, and probably within the first 8 days for cows with a high milk yield (Diskin et al., 2016). There are massive number of changes and growth in the embryo that occurs up until the blastocyst stage at day 8, which would explain that many embryos fail to pass this stage in suboptimal conditions.

Embryo survival depends both on the environment in the reproductive tract of the mother and the quality of the embryo as such (Hansen, 2002). Hence, embryo loss may be attributable to any negative impact along the developmental axis (Lonergan et al., 2016). The causes for embryo loss have been approached in different forms in literature. Diskin et al. (2011) categorized the cause into problems related to genetics, physiology, endocrinology, and environment. The genetic causes include chromosomal defects, individual genes, and genetic interactions, on both the male and female side (Diskin et al., 2011). As an example, Kadri et al. (2014) found a recessive mutation in Nordic Red cattle consisting of a deletion that leads to embryo death. They hypothesized that lethal mutations could account for a non-negligible part of embryo loss in dairy cattle.

Environmental causes refer to the ecosystem that the embryo experiences (Sirard, 2021). A previous review presented a specific list of bacterial, viral, protozoan, and fungal infections associated with embryo loss (Givens and Marley, 2008). The effect on the embryo depends on the agent's pathogenicity and virulence, and the immune competence and developmental maturity of the embryo when the infection occurs (BonDurant, 2007). Moreover, uterine infections and/or other factors induce inflammation in the uterus. An inflamed environment has negative effects on the embryo quality *in vitro* (Hill and Gilbert, 2008), and *in vivo* (Puscheck et al., 2015).

Endocrine conditions that affect the embryo survival include suboptimal levels of progesterone. Progesterone levels seems to affect the embryo and the surrounding environment in several ways. In the cycle immediately before AI, low levels of progesterone might lead to the ovulation of oocytes that are too advanced in their maturation, which in turn has a negative effect on the subsequent embryo development. It may also be correlated to the embryo's capacity to secrete IFNT.

Endometrial morphology and its ability to support embryo development is affected by progesterone levels in both the cycle preceding the AI and the days following AI (Diskin et al., 2011).

Physiological causes to embryo loss are related to nutrition and energy balance. After parturition, there are vast increases in the nutritional demand as a result of a sudden increase in milk yield. When the demand for energy exceeds the dietary intake, the cow experiences a negative energy balance (NEB) (Diskin et al., 2011). A positive energy balance in early lactation has been shown to have a positive association with conception rate to first service. Moreover, circulating levels of IGF-I, dry matter intake and body condition score affects conception rate (Patton et al., 2007). Further, the expression of genes related to oxidative stress regulation and fatty acid synthesis is disrupted in oocytes and blastocysts as a result of NEB (Eckert et al., 2015). NEB might also change the endometrial gene expression (Wathes et al., 2009) and increase the risk for inflammatory diseases in general and in the uterus, due to a less effective immune response (Diskin et al., 2016).

The various mentioned causes also impact one another. Hence, the categorization and approach to understanding embryo loss is not straight forward. Liver blood flow and metabolic clearance rate of steroids is related to the physiology of the animal post-partum, but it will also affect the levels of circulating hormones, including progesterone, and hence endocrine causes for embryo loss. The circulating levels of progesterone will also affect the uterine environment. Similarly, post-partum NEB is related to uterine inflammation, which can also be a result of uterine infections from the environment. The interactions between physiology, endocrinology, and uterine environment here, are just a few examples creating challenges to investigating the causes for embryo loss in a separate manner. Moreover, the patterns and causes of early embryo loss might be different in high-producing dairy cows compared to low-yielding cows and heifers (Diskin et al., 2011).

There are also other ways to address embryo loss. Lonergan et al. (2016) discussed the matter in terms of compromised follicle development or suboptimal reproductive tract environment. Risk factors disturbing the follicle and oocyte included heat stress, NEB and other nutritional disturbances, and lactation as such. For the reproductive tract environment, progesterone levels and metabolic stress from lactation were mentioned as factors affecting the embryo survival (Lonergan et al., 2016). Another review (Wiltbank et al., 2016) categorized embryo loss based on physiological events in the first week (period 1), during elongation (period 2), and during placentome development (period 3). This approach might be beneficial for the understanding of the issue, as it does not isolate the causes and has a higher focus on the challenges that the embryo might experience. In period one, they targeted oocyte quality disruption

by heat stress, uterine inflammatory disease, loss of body condition and suboptimal preovulatory progesterone levels as important factors. In the second period, emitted levels of IFNT and the resulting maternal recognition of pregnancy are pointed out as crucial, as well as possible deficiencies in the uterine histotroph (Wiltbank et al., 2016). Another study (Ribeiro and Carvalho, 2018) stressed that many different metabolic, endocrine and nutritional imbalances or deficiencies affects this stage of development. The third period extends into the placental development. Here, the nutritional source for the embryo changes from histotroph to chorioallantoic placental nourishment. Unsuccessful placental development is caused by uterine and non-uterine disease such as mastitis, metabolic and nutritional conditions, and hormonal disturbances such as, again, suboptimal circulating progesterone levels (Wiltbank et al., 2016).

5.6 Maternal contributions to the early embryo

As described in the section 5.5, most studies about embryo death have investigated causes in the female. In this section, I will elucidate some details of the maternal contribution, focusing on uterine inflammation and particularly subclinical endometritis.

The microenvironment of the embryo is largely established by the mother (Hansen, 2002). The oocyte develops within the follicle. At ovulation, it is released to the oviduct where the fertilization occurs. Further, the zygote is passed on to the uterine lumen. Maternal factors that affect the early embryo life includes compromised follicle development, incompetent oocytes which results in an intrinsic defect in the embryo, a suboptimal oviductal and uterine environment, asynchrony between the embryo and the mother, or a maternal error in responding to embryo signals (Hansen, 2002; Lonergan et al., 2016).

5.6.1 The uterus

The bovine uterus consists of three distinct regions: the uterine body and two uterine horns (Nabors and Linford, 2014), and all share a common lumen. The cervix is the anatomical restriction between the uterus and the vagina and outside environment. It consists of muscle cells and mucosa arranged in longitudinal and circular folds, creating a series of notches and ridges (Nabors and Linford, 2014). The cervical mucosa is further lined by columnar epithelium (Mullins and Saacke, 2003). In the follicular phase, changes in the structure of the connective tissue makes the cervix become softer (Breeveld-Dwarkasing et al., 2003). The uterus consists of three tissue layers. The endometrium is the first layer from the luminal side, followed by the muscular seams in the myometrium, and finally the peritoneum, which is a

continuation of the abdominal peritoneum (Nabors and Linford, 2014). The endometrium consists of an epithelial lining that also houses superficial and deep glandular epithelial cells and fibroblast-like stromal cells (Forde and Lonergan, 2012). Further, the endometrium can be divided into two distinct regions, caruncular and intercaruncular. The caruncular regions are evenly distributed throughout the endometrium (Schlafer et al., 2000). They have a raised mucosa and are highly vascularized. During pregnancy, these regions form the placentomes of the bovine placenta together with cotyledons from the fetal membranes (Nabors and Linford, 2014).

5.6.1.1 Uterine immunology and regulation

At parturition or release of the placenta, the endometrium is commonly damaged (Sheldon et al., 2019) and the caruncular regions are exposed to the lumen (Pascottini and LeBlanc, 2020). The damage and the influx of environmental microbes create an inflammation which is part of a normal port partum physiology (Pascottini and LeBlanc, 2020). Neutrophils are a commonly entering the uterine tissue in the inflammation process (Sheldon et al., 2019). Their presence is initiated by the activation of macrophages and immune receptors on uterine epithelial cells, which again induces secretion of chemokines and inflammatory cytokines. These mechanisms provoke release of neutrophils from the bone marrow to the blood, which allows a further migration to the uterus. Here, they recognize pathogens and induce the killing and clearing of bacteria before they undergo apoptosis (Pascottini and LeBlanc, 2020). Acute phase proteins and complement factors also play a role in this process (Sheldon et al., 2019). Even though much is known about the initiation of inflammation in the uterine tract, less information is available on how inflammation is limited and switched to a healing state (Pascottini and LeBlanc, 2020). Phagocyted or apoptotic neutrophils play a role through the initiation of anti-inflammatory or restorative cytokines, including IL-10 and TGF-β. Other mediators such as maresins, resolvins and protectins, synthezised from omega 3-fatty acids also partly contributes to bacterial clearance and the self-limitation of inflammation (Serhan, 2014). When bacterial killing or apoptosis of neutrophils fail, or when the activation of pro-inflammatory pathways is not limited, a chronic inflammation of the uterus may arise (Pascottini and LeBlanc, 2020).

In addition to a naturally occurring defense mechanism, tissue damage and an inflammatory response in the endometrium can be caused by certain pathogenic bacteria that enter the uterus at the events around parturition. The amount and pathogenicity of bacteria, and the effectivity of the immune system to limit the activity in these pathogens, will determine if uterine disease develops (Pascottini and LeBlanc, 2020). The individual animal's ability to deal with pathogenic microbes depends on both resistance (the capability to limit the pathogen burden) and tolerance (the ability

to limit disease severity caused by a certain pathogen). It has been suggested that tolerance to bacteria is more important than resistance in the development of uterine disease (Sheldon et al., 2019).

Systemic inflammation as a result of disease outside the reproductive tract, as well as the metabolic state of the animal, appears to have an effect on the modulation of bacteria and inflammatory response in the reproductive tract. Some examples include mastitis, lameness, respiratory disease, digestive problems (Ribeiro et al., 2016), metabolism related to hepatic function and adipose tissue mobilization (Bradford et al., 2015). The negative effect from non-uterine disease was observed in cows subjected to AI, but also those receiving an embryo through embryo transfer, suggesting that non-uterine disease not only affects the embryo as such, but the reproductive tract as well (Ribeiro et al., 2016). Fat mobilization and increased levels of non-esterified fatty acids (NEFA) result in the release of proinflammatory agents such as IL-6 and tumor necrosis factor, which in turn stimulates the production of acute phase proteins including haptoglobin. β-hydroxybutyrate release from the liver is also a consequence of elevated NEFA levels. All these products from a suboptimal metabolism dysregulates and impairs neutrophil function and hence the immune regulation in the uterus (Pascottini and LeBlanc, 2020).

After parturition, a remodeling and regeneration of the endometrium results in re-epithelialization of the caruncular regions, in addition to a weight reduction of the uterus from about 9 kilos at parturition to about 1 kg. These changes take about 30 days (Sheldon et al., 2019). However, inflammation appears to have a carry-over effect on the endometrium which can last for at least 4 months (Ribeiro et al., 2016). It is common to perform the subsequent insemination within this time. This long-term memory might be due to resident immune cells or possibly by a permanent effect through epigenetic mechanisms, affecting the proliferation and differentiation of the uterine epithelium after disease (Lucy et al., 2014). The theories about these long-term effects must be subjected to further research, with the potential to add new perspectives in the development of strategies to reduce embryo loss (Ribeiro et al., 2016).

5.6.1.2 Uterine inflammation and the embryo

Inflammation in the uterus affects the uterine environment, which is associated with direct harm to spermatozoa through oxidative stress, and harm to the embryo (Gilbert, 2011). In *in vitro* conditions, bovine sperm exposed to oxidative stress has reduced motility, which could indicate a lower probability for conception in the inflamed uterus. Moreover, such sperm also resulted in a lower cleavage rate of oocytes, and a reduced developmental competence in the oocytes that passed the first cleavage state (Hendricks and Hansen, 2010).

An *in vitro* study that exposed embryos to normal and inflamed aseptic endometrial fluid from the same cow showed that inflammation reduced embryo quality. The observed mechanism was a reduced absolute number of trophoblasts and an increased inner cell mass:trophoectoderm ratio, which led to the theory that the trophectoderm is inhibited by inflammation. Further, they suggested that the trophectoderm protects the embryo from inflammatory components such as cytokines (Hill and Gilbert, 2008).

Ribeiro et al. (2016) set up a series of *in vivo* studies, investigating the carry over effect from disease occurring from parturition to the day before breeding on different developmental stages of the embryo. The investigated disease included both uterine disease (retained placenta and metritis) and non-uterine disease (mastitis, lameness, digestive and respiratory problems). They concluded that diseased animals (with both uterine and nonuterine disease) had reduced zygote cleavage, reduced survival to morula stage, impaired conceptus elongation and reduced secretion of IFNT. The changes observed were also confirmed with concurrent alterations in the transcriptome of conceptus cells. Interestingly, uterine disease had a negative effect on fertilization based on cleavage of potential zygotes whereas non-uterine disease was not associated with this effect (Ribeiro et al., 2016).

5.6.2 Clinical uterine disease

In the present dairy cattle industry, up to 40% of the individuals develop uterine post-partum disease (Galvão et al., 2019; Sheldon et al., 2019) which is an increase compared to the situation 50 years ago (Sheldon et al., 2019). Furthermore, uterine disease is targeted as the leading cause of reproductive inefficiency in dairy cattle (Barlund et al., 2008).

Clinical uterine disease consists mainly of metritis and clinical endometritis. Metritis in the bovine is defined as an enlargement of the uterus and uterine discharge with a putrid odor that range from watery red-brown to a viscous pus. It most commonly appears before day 10 post-partum and can result in systemic signs of illness and fever, and even toxemia. Clinical endometritis is characterized by the presence of purulent or mucopurulent discharge from the vagina, that appears at day 21 or more post-partum (Sheldon et al., 2019). The severity can be evaluated by a grading system based on the amount of pus within the mucus. One example of such grading was developed by Williams et al. (2005). It has been shown that a large proportion of cows with uterine discharge did not have endometrial inflammation as investigated by uterine cytology samples. To deal with this discrepancy, the term purulent vaginal discharge (PVD) was suggested when vaginal discharge indicated endometritis, but no further evidence of its origin was available (Dubuc et al., 2010a). The incidence of clinical uterine disease varies between herd, breed, and country (Sheldon et al., 2019).

The reason for differences in reported numbers is also probably due to inconsistency in the definition of disease. Metritis has a reported incidence of approximately 20%, with a range from 8% to about 50% (Pérez-Báez et al., 2021). Clinical endometritis and cervicitis, both resulting in PVD, has a joint prevalence of about 32%, while about 13% suffers from endometritis alone (Deguillaume et al., 2012). Both metritis and clinical endometritis affect the profitability of the dairy cattle industry in several aspects, with negative impacts on both milk production and fertility (Piñeiro, 2016; Sheldon et al., 2019).

The risk factors for uterine disease are related to the three distinct groups (1) trauma to the genital tract (2) metabolic disorders and (3) hygiene issues. Trauma allows bacteria to reach the uterine stroma, and can occur as a result of dystocia, large offspring, stillbirth, twins, induction of parity, first parturition and retained placenta (Sheldon et al., 2019). Metabolic disorders compromise immune cell function including neutrophil activity (Hammon et al., 2006).

Most studies performed before around 2010 relied on culture-dependent studies. The knowledge about the uterine microbiota in clinical uterine disease have advanced with the use of NGS. Metritis is associated with dysbiosis and a reduced richness of the bacterial flora (Galvão et al., 2019). Pathogens that are most commonly associated with clinical uterine diseases include *E.coli, F. necrophorum*, *T. pyogenes*, and *Prevotella* and *Bacteroides* genera. *T. pyogenes* is thought to be the most critical pathogen leading to endometritis, while *F. necrophorum* is the main pathogen associated with metritis. However, there is probably a synergy between the mentioned (and additional) bacteria where some might initiate disease while others cause clinical signs or contribute to endometrial pathology (Galvão et al., 2019; Sheldon et al., 2019).

5.6.3 Subclinical endometritis

In 2004, Kasimanickam et al. (2004) investigated the occurrence of polymorphonuclear cells (PMN) in the bovine post-partum endometrium of clinically healthy cows, and its associations with the reproductive outcome. With this work, they established the term subclinical endometritis (SCE). Since then, a fair body of studies have investigated this condition in different continents and under different settings. Subclinical endometritis is now considered a global problem in dairy farming due to its effect on fertility with reported prevalence of 5-75% depending on applied diagnostic criteria and time of examination (Wagener et al., 2017). Per definition, the condition consists of three aspects: (1) the presence of PMN on the endometrial surface or lumen, (2) a reduction in reproductive performance, and (3) the absence of clinical symptoms of endometritis (Kasimanickam et al., 2004; Sheldon and Owens, 2018).

It has been suggested that the sole occurrence of PMN in the endometrium should be referred to as cytological endometritis (CYTO), as the definition of SCE also represents a negative effect on fertility (Dubuc et al., 2010a; Pascottini et al., 2016). There are studies showing that in some cases, PMN in the uterus did not have such negative effect (Plöntzke et al., 2010; Prunner et al., 2014). In those cases, as well as studies of new populations in which the outcome is unknown, the term CYTO is more appropriate. However, there are large inconsistencies in which of the two terms are being used in literature.

To diagnose SCE, endometrial cytology is the most common approach. Cells are collected from the uterine lumen and/or endometrial lining, then transferred to a microscopic glass slide and stained. Epithelial cells and PMN are counted (and, in some cases other white blood cells as well), and the percentage of PMN determines the SCE diagnosis, based on a set cut-off (Wagener et al., 2017). The first tool to obtain these cytological samples, was a cytobrush that was attached to the top of a metal rod. The brush was passed through the cervix protected by a plastic catheter and a plastic sleeve. Once inside the uterus, it was rolled against the endometrium using a slight pressure on the device with a finger from the rectal side. The cytobrush was then drawn back into the catheter and retracted from the reproductive canal (Kasimanickam et al., 2004). Later, the cytotape, consisting of a piece of paper tape glued onto the top on a steel inseminator, was invented. Cytotape sampling can be combined with AI in one sole passage of the cervix (Pascottini et al., 2015).

The cut-off of PMN to diagnose SCE differs between different populations and the timepoint at which the sampling is performed. This makes the comparison of results between studies a complex matter (Wagener et al., 2017). Sampling has mainly been performed 3-5 or 5-7 weeks after calving (Arias et al., 2018). A few studies performed sampling at AI, and suggested that this is a critical timepoint for uterine health status, in addition to allowing a standardization of the sampling timepoint (Pascottini et al., 2016; Pascottini et al., 2017a).

The causes for SCE are largely unexplained. A recent hypothesis states that SCE is mainly affected by uterine immune regulation (Pascottini et al., 2020). Known risk factors on the individual level include metabolic imbalances such as elevated concentrations of NEFA and β-hydroxybutyric acid (Galvão et al., 2010), heat stress, uterine disease such as metritis or systemic disease such as ketosis (Cheong et al., 2011). On the herd level, housing and bedding material was detected to increase the risk for SCE (Cheong et al., 2011). At AI, the established risk factors were season, parity, and days in milk (Pascottini et al., 2017b).

The microbiome of the bovine reproductive tract is considered to be underexplored in terms of specific taxonomy and functional aspects (Appiah et al., 2020; Adnane and Chapwanya, 2022). New technologies such as 16s rRNA seq enable culture-independent studies. While the pathogenic bacteria related to clinical endometritis and metritis have been described many times in literature, less in known about the association between subclinical endometritis and pathogenic bacteria. After the initiation of the current thesis, two studies have investigated microbiota of the superficial layers of the uterus in cows with subclinical endometritis, comparing it to healthy animals. Both studies concluded that SCE is not associated with changes in the uterine microbiota (Wang et al., 2018; Pascottini et al., 2020). However, these studies provide limited information as they only investigated the superficial endometrial microbiota, at set timepoints post-partum. Information about the microbiota at AI is still missing in general, as well as investigations of the deeper endometrial tissue.

Further, it is not known to which extent SCE affects embryo loss. Early embryo death is the most common mechanism for failed pregnancy, and SCE is common and claimed to be underestimated in herds with fertility issues (Pascottini and LeBlanc, 2020). SCE consists of an inflammation, and as previously described, an inflamed environment has negative effects on the oocyte, embryo and the reproductive tract. Moreover, both the endometrial transcriptome and the embryo gene expression is altered as a result of subclinical endometritis (Hoelker et al., 2012).

5.7 Paternal contributions to the early embryo

Historically, the largest component for the primary evaluation of the infertile human couple have been focused on the woman, and infertility has been considered principally a female problem (Moldenhauer et al., 2003). Earlier investigations on the father's contribution to fertility mainly focused on morphological assessments of the spermatozoa's ability to reach and fertilize the oocyte (Moldenhauer et al., 2003; Daigneault, 2020). A former commonly accepted theory stated that early mammalian embryo development was exclusively regulated by the mother. This was based on the fact that the female gamete is much larger than the male gamete and consequently had the capacity to house the necessary regulating factors such as transcripts and proteins (Immler, 2018). In the bovine, the cow has been the main target for studies of embryo mortality. This is probably a result of the negative association between high milk yield and reproductive outcome, detected along the period of increased embryo loss (Kropp et al., 2014).

Scientific progress in the early 2000's indicated that the paternal contribution had been underestimated (Krawetz, 2005), and during the last decade, research on the father's contribution to the embryo has seen major advances (Immler, 2018). It is now clear that paternal effects extend far beyond the fertilization process itself. Recent evidence demonstrates that both parents contribute to embryo programming through genetic and epigenetic components, and via RNAs and proteins directly deposited within the zygote (Gross et al., 2019; Daigneault, 2020; Wu and Sirard, 2020), see Figure 3. As an example, information stored within both maternal and paternal gametes can influence embryonic and maternal gene expression, affecting blastocyst morphogenesis (Eckert et al., 2015). One recent study showed that bulls of differing field fertility gave rise to embryos of different quality (visible from day 7 of gestation), even when the fertilization rate was equal between the two groups (O'Callaghan et al., 2021). With the knowledge from the mentioned studies, one can define both male and female fertility as the capacity of fertilization and continued embryo and fetal development until birth. Consequently, the contribution from both parents can be responsible for embryo death.

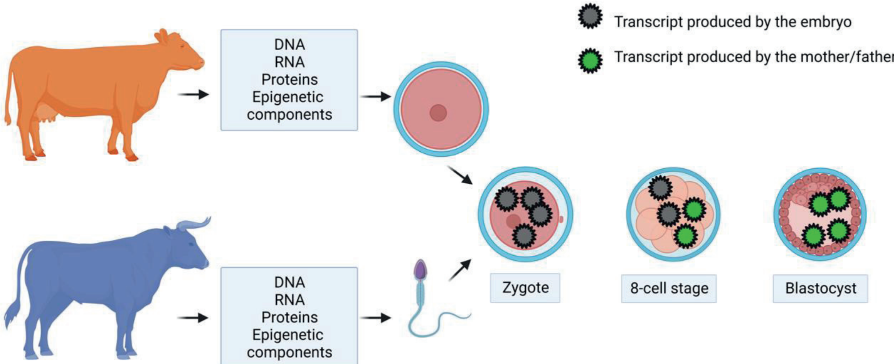

Figure 3. Maternal and paternal contributions to the embryo through the gametes, and transcript origin. Grey stars represent transcripts derived from the parents, whereas green stars represent transcripts produced by the embryo. Created with BioRender.com.

Separate investigation of male fertility is crucial, as the correlation of genetic progress in the fertility of the bull and the cow is low (Taylor et al., 2018). Moreover, with the employment of AI it is common to use semen from one elite bull for the insemination of a large number of females. This means that the success in the industry can be largely affected by targeting genes related to bull fertility in breeding strategies. Even though early embryo loss has seen a large increase during an intensive selection for high milk yield, a considerate proportion of pregnancies also failed due to early embryo loss even in the earlier statistics (Diskin et al., 2006). It is not known how much of this earlier proportion of embryo loss was due to paternal contribution. There might be a significant gain for the industry in elucidating paternal factors, which are not related to other challenges (such as metabolic and nutritional challenges) that the dairy cattle industry is facing.

As mentioned under section 5.4.1, the use of microarrays and NGS are common ways of investigating the outcome in the embryo as a result of different settings. These methods serve in the investigation of paternal contribution to embryo development, for instance by studying gene expression and other cellular components in embryos derived from fathers with different phenotype. One study investigated the transcriptome of *in vitro* fertilized blastocysts derived from the same father animal at either 10, 12 or 16 months of age. Using microarray data, they found several genes to be differentially expressed depending on the age of the father (Wu et al., 2020). Another study looked at gene expression in *in vitro* produced blastocysts as a result of heat stress to the bull. Even though they found a higher cell death rate and a lower blastocyst rate in the high temperature group, no differences in gene expression was targeted (Luceño et al., 2020). However, they used real time quantitative polymerase chain reaction (qPCR) targeting 25 genes known to be important for embryo

development, meaning that they could not conclude on the effect on the complete transcriptome. This underlines the benefit of NGS of the whole transcriptome. Also at the blastocyst stage, Kropp and colleagues demonstrated that *in vitro* produced embryos from bulls of different fertility had different gene expression (Kropp et al., 2017).

5.8 Norwegian Red

Norwegian red (NR) is the most common breed of dairy cows in Norway, constituting of more than 90% of the individuals and a total population size of about 180,000 (TINE Rådgiving, 2021). Even though it is a dairy breed, it can be considered as dual-purpose, since the main proportion of beef also derives from dairy cattle in the Norwegian production (Geno SA, 2020d). The NR breeding programs have emphasized fertility and health, such that female fertility has been included in the total merit index since the 1970's (Andersen-Ranberg et al., 2005).

NR cows are notable for their good reproductive performance, low prevalence of disease and high milk yield. The latest reported pregnancy incidence was 62.9%, with a calving rate to first AI of 56.3% (Garmo et al., 2008). Statistics showed a NRR on day 56 of 72.9% in 2019 (Geno SA, unpublished results). The occurrence of reproductive disorders is low. For instance, treatments against metritis or endometritis equal to 1.7/100 cow-years, while both cystic ovaries and retained placenta was treated 1.8/100 cow-years in 2021 (TINE Rådgiving, 2021). The average milk yield exceeds 8,000 kilos per year (Geno SA, 2020d), and the average herd size is 30.9 (TINE Rådgiving, 2021).

On a global level, functional traits such as reproduction and health traits are receiving increased focus because of economic and environmental reasons and animal welfare concerns (Andersen-Ranberg et al., 2005). Due to the characteristics of NR, frozen semen from the breed is exported and used for cross-in with other cattle breeds (Geno SA, 2020e).

5.8.1 Health recording system and breeding program

The Norwegian Dairy Herd Recording System (NDHRS) is a unique collection of data which is based on a collaboration of registration made by farmers, veterinarians, AI technicians, advisors, slaughterhouses, claw trimmers and laboratories. Individual health recordings follow the animal through its life even if it is sold to another farm (Geno SA, 2021b), and any diagnosis is set using pre-defined health codes (Heringstad and Østerås, 2013). The NDHRS is owned by the national dairy co-operative TINE, which again is owned by Norwegian dairy farmers. The data management is performed by Mimiro AS. With its complete national coverage since 1975 (Østerås et al., 2007), it is well-established, and includes about 98% of the Norwegian dairy herds, containing 92% of all dairy cows (TINE Rådgiving, 2021).

The organization that controls the NR breeding program, Geno SA, is owned by the Norwegian dairy farmers since 1935 (Geno SA, 2020e). Up until 2012, the selection of

breeding animals was solely based on progeny testing, where the bull's breeding value was of major importance due to more registered progeny per animal. During the last few years, the breeding strategy for NR has been gradually changed to genomic selection. The new strategy integrates information about both genotype and phenotype to calculate a genomic breeding value. Both bulls and heifers can be selected for breeding before they have progeny, as phenotype data comes from a pedigree matrix from other relatives. The new strategy results in a faster breeding progress (Geno SA, 2020c). Each year, about 100,000 heifers and bull calves are born, respectively. With the use of data from the NDHRS, around 8,000 bull calves and 12,000 heifers are chosen for genotyping by registering around 55,000 singe nucleotide peptides from a tissue sample. Based on a combination of phenotype information and genotype, each animal is appointed an estimated genomic breeding value. This breeding value is used to select 150 bull calves and 90 heifers which are purchased by Geno SA to be used as breeding animals. The chosen animals are further genotyped on a deeper level, revealing 777,000 single nucleotide peptides. Inbreeding is controlled by making sure that all breeding animals are used. Each second week the genomic breeding values are updated with the continuous incoming data (Geno SA, 2020a).

Figure 4. An interaction between one of the elite bulls at the breeding station (Geno SA), and the thesis author. Photo credit: Irma Caroline Oskam.

The bulls (See Figure 4 for an example of a NR elite bull) are now in semen production at an earlier age. However, the evaluation of fertility is still made by registrations from the NDHRS, by a breeding soundness evaluation at initiation, and by a microscopic and macroscopic semen quality control performed for each batch (Geno SA, 2020f). As for the chosen heifers, they account for the NR embryo production through multiple ovulation embryo transfer (MOET) or ovum pickup in vitro fertilization (OPU-IVF).

5.8.2 Fertility index and future perspectives in NR fertility

The breeding goal emphasizes fertility in several aspects. The daughter fertility index consists of the registrations from the NDHRS with number of inseminations as heifer and cow, respectively, and data about calving to first insemination. The sum of these registrations is weighted with 11.4% of the total merit index (Geno SA, 2021a). Heritable fertility disorders also form part of the calculations of the breeding value and includes calving ease (2.9%) and still birth (0.6%). Retained placenta, metritis, cystic ovaries, and silent heat are the four most common fertility-related diseases or disorders in NR (Heringstad, 2010). These are included in the total merit index as a composite trait "other disease", which has a relative weight of 0.3% (Geno SA, 2021c).

Even though NR has seen a great development in fertility through a targeted breeding, we still have a long way to go in understanding the details of the underlying genetics. Fertility traits have a generally low heritability (Pryce and Veerkamp, 2001). They are largely affected by environmental factors, and they are quantitative, meaning that each trait is regulated by several genes (Holmberg and Andersson-Eklund, 2006). With the implementation of genomic selection in dairy cattle breeding, interest in new phenotypes that can add information on fertility and health has been increasing. The new focus on embryo production requires a high level of knowledge concerning all aspects of the embryo, which is why research relating to this topic is highly important for the industry.

Fertility in NR had a negative development a few years back, being of major concern and one of the reasons for the initiation of the project *Reproductive sustainability in NR* (Project number 255097/E50). That project funded parts of the studies included in the current thesis. The NRR at 56 days was reduced from 74.2 in 2011 to 72.4 in 2014 (Geno SA, 2012; 2014). However, from 2014 the NRR has been non-changing and even with positive development from 2018 (Geno SA, unpublished results). NR still has a high fertility compared to many other breeds, and it is of high importance to maintain that status in the future.

5.9 Knowledge gaps

When this thesis was initiated, we lacked information about the prevalence and meaning of SCE in NR. This is a condition that we know have a negative impact on fertility on a global scale, and presumably it contributes to embryo loss as this is the most common mechanism for loss of pregnancy. We did not know whether NR cows had a good fertility due to a low SCE prevalence, or despite of a high prevalence. On a global scale, it was not known whether SCE had a heritable component. Such knowledge would make it possible to consider controlling this condition by breeding.

Further, we had little information about the uterine microbiota in dairy cattle, especially at AI and by biopsy, which might be of concern for SCE and related to survival of the embryo.

As for the paternal side, there is a general lack of information on a global scale about how the sire affects embryo development, especially *in vivo* produced embryos. The advancement of this knowledge and related actions might have a mayor effect on the negative impact that embryo loss constitutes in the dairy cattle industry today. On a national level, it is valuable to gain more insight about the embryo in general, due to the newly implemented embryo focus in the breeding strategy. New technology such as NGS has provided us with the tools to investigate several aspects of the paternal contribution and leads us closer to finding biomarkers that aid the prediction of bull fertility.

It is highly important to elucidate these maternal and paternal fertility matters, and their relations to the embryo, in the quest of maintaining and even improving the status for NR as a high-fertility breed.

6 Aims of the thesis

The overall aim for this thesis was to gain knowledge about factors affecting embryo loss in NR. To do this, we approached the matter using both the maternal and paternal aspect through the following objectives:

- Provide knowledge about the prevalence of CYTO in Norwegian red cows (Paper I)
- Estimate the effect of CYTO on the fertility of Norwegian red cattle, including the risk for embryo loss (Paper I)
- Gain information about the risk factors for CYTO in Norwegian Red (Paper I)
- Estimate the heritability of SCE in the NR population and discuss whether it could be relevant to consider this condition as a phenotype for breeding purposes (Paper II)
- Develop an understanding of the uterine microbiota at AI in Norwegian red (Paper III)
- Describe potential differences in the reproductive tract microbiota between SCE positive and healthy NR cows at AI (Paper III)
- Investigate the paternal contribution to embryo development at the blastocyst level by a global transcriptome study of *in vivo* produced embryos (Paper IV)

7 Summary of papers

7.1 Paper I

Prevalence, risk factors, and effects on fertility of cytological endometritis at the time of insemination in Norwegian Red cows

The present study aimed to assess the occurrence of CYTO at first AI post-partum in NR cows. Further, risk factors for CYTO manifestation and its effect on reproductive success and late embryo loss were evaluated. To pursuit this objective, we set up a prospective cohort field trial which included 1,648 cows located in 116 herds. Endometrial cytology samples were collected using a cytotape technique, and sampling was performed at mainly spontaneous estrus. A total of 300 representative epithelial cells and PMN were counted using a microscope at 400x magnification. Rectal palpation or analysis of pregnancy associated glycoproteins was the basis for the pregnancy diagnosis. The cut-off level for the definition of CYTO was set to 3.0% PMN. This decision was based on the construction of a receiver operator characteristics (ROC) curve, and the level at which the PMN occurrence affected pregnancy outcome with the highest summation of sensitivity (32.4%) and specificity (74.9%).

To acquire more information about the animals, vaginal mucus obtained by Metricheck™ as well as body condition score was recorded at the sampling day. Furthermore, milk samples for progesterone analysis were collected at AI and 21 days later. Data on each animal and herd was obtained by the Norwegian Dairy Herd Recording System. Three logistic models with herd included as random factor were constructed. The outcome for the three models was (1) the likelihood for CYTO based on the endometrial samples, (2) pregnancy to first AI, and (3) embryo loss. Cows with >3 ng/mL at AI were excluded from the material in the late embryonic loss model. Late embryonic loss was defined through high progesterone levels 21 d after the first AI, in cows that were not pregnant on rectal palpation or pregnancy associated glycoproteins (PAG) analysis ≤42 d after the first AI. This loss was also appointed to cows with no recording on pregnancy status who were presented for a new AI ≥ 42 d after first AI.

The proportion of CYTO was 28.0% (461/1648). The overall pregnancy incidence to first AI was 59.8% (866/1449) and the average interval in days to first AI was 71.7 d (SE ± 0.7). Pregnancy to first AI was lower in CYTO positive cows (odds ratio= 1.51, CI = 1.17-1.94). Other factors affecting pregnancy to first AI were AI personnel, test day milk yield, barn type, and obstetrical conditions or fertility treatments before first AI.

The likelihood for CYTO at first AI was associated with the following risk factors: AI personnel, calving to first AI interval, vaginal mucus characteristics, amount of red blood cells in sample, season, and barn type. The proportion of late embryo loss and abortion was 8.6% (82/948) and 2.8% (24/866), respectively. Late embryo loss was associated with treatment against fertility disorders prior to first AI, but not associated with CYTO.

7.2 Paper II

Heritability of subclinical endometritis in Norwegian Red cows

The current study aimed to (1) estimate the heritability of SCE in the NR population and (2) discuss future perspectives of the condition as a fertility phenotype for breeding.

Based on the material from paper I, 1,642 cows had sufficient sample quality and available animal history to be included in the present study. Different trait definitions were examined, and SCE was defined as binary traits, based on the following cut-off levels of PMN: Cyto0 = PMN >0, Cyto3 = PMN >3%, Cyto5 = PMN >5%, Cyto10 = PMN >10%, and Cyto20 =PMN >20%. The mean ranged from 0.07 (Cyto20) to 0.59 (Cyto0), and the mean proportion of PMN was 5%. PMN was also analyzed as a continuous variable using percent PMN. Information on the animals and herds was obtained from the Norwegian Dairy Herd Recording System. The pedigree of cows with data included 24,066 animals. To estimate the variance components, we used a linear animal model that included the known risk factors for CYTO presented in paper I as fixed effects. The only trait definition that had an estimated genetic variance larger than the standard error was Cyto5, with an estimated heritability of 0.04. For all other definitions, the genetic variance was not significantly different from zero. Hence, the current study indicates that SCE is heritable in NR when defined with a 5% cut-off level of PMN.

The heritability estimate was comparable to other diseases and disorders included in the total merit index for NR. Hence, SCE could be considered as a new phenotype with the potential to be used for breeding purposes. The currently most validated method to diagnose SCE is performed through a highly time-consuming cell count routine. To implement SCE in the breeding strategy, a more feasible method to diagnose SCE needs to be developed.

7.3 Paper III

The microbiota of uterine biopsies, cytobrush and vaginal swabs at artificial insemination in Norwegian Red cows

In this study we investigated the uterine microbiota in NR at AI by biopsy (deep endometrium) and cytobrush (superficial endometrium) samples and compared the findings to the vaginal microflora. The second objective was to describe potential differences at these distinct depths of the endometrium, in healthy vs SCE positive NR cows. Thirty-two lactating and clinically healthy Norwegian red cows were subjected to sampling. At the sampling timepoint, they were in their second heat or more after calving and presented for first AI. The sampling consisted of two distinct passages through the cervix. First, we acquired a vaginal swab and a cytobrush sample, in addition to a cytotape to investigate the animal's uterine health status with respect to SCE. Secondly, we obtained a biopsy sample from the uterine endometrium. Bacterial DNA from the 16S rRNA gene was extracted using the QIAamp Cador mini Pathogen kit. As a negative control for the extraction process, DNA was extracted from DEPC water, and the resulting material was sequenced in the same manner as the rest of the samples. Further, the bacterial DNA was sequenced with Illumina sequencing of the V3-V4 region by using the 341F/785R primer pair. Data processing and statistical analysis was performed in the QIIME2 pipeline and included the estimation of alpha and beta diversity and taxonomic composition. To investigate which taxa that best could explain the differences between the groups, we applied Linear discriminant analysis effect size (LDA-LEfSe) using the online Galaxy tool (https://huttenhower.sph.harvard.edu/galaxy/). The bacterial count in the different sample types was estimated using qPCR quantification.

The final number of animals that had results from sequencing was 24, and out of these only three animals were positive for SCE. As expected, the bacterial load was much higher in the vaginal swabs than in the uterine samples. The microbiota of endometrial biopsies was qualitatively different, richer, and more even than that of cytobrush and vaginal swab samples. The three biopsy samples belonging to SCE positive animals were clustered together in the beta diversity analysis, but that cluster did not differ visually from the other biopsy samples.

In total, 319 bacterial genera were identified. The dominant represented phyla were Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota. According to the differential abundance analysis, Bacilli was enriched in the cytobrush and vaginal swab samples compared to the biopsies, while Firmicutes was also enriched in the vaginal swabs compared to biopsy. The cytobrush samples and the vaginal swabs shared a similar taxonomic composition. In the biopsy samples, we found a high abundance of *Oscillospiraceae UCG-005*, *Bacteroidetes_vadinHA17*, *Ruminococcus*, *Bacteroides*, *Alysiella* and four different genera of the family Lachnospiraceae. These taxa were

barely present in the other sample types, and not present in the negative extraction control. We could not detect any difference in the taxonomic composition associated with SCE status.

7.4 Paper IV

Gene expression in embryos from Norwegian Red bulls with high or low non return rate: an RNA-seq study of *in vivo*-produced single embryos

In the present study, we investigated the differences in the global transcriptome in *in vivo* produced embryos, derived from sires with either high or low field fertility. The fertility was measured as the NRR on day 56 after first AI of the inseminated cows. In total, 12 bulls were selected from a database of 470 NR sires, born between 2010 and 2014, all with at least 500 registered first inseminations. The 12 selected bulls represented the highest and lowest fertility among all registrations, with a NRR from 49.3 in the low fertility group to 80.5 in the high fertility group, and an average of 72.5 (s.e. = 3.5). Semen from either high fertility (n = 6) or low fertility (n = 6) bulls was used in the artificial insemination of superovulated heifers (n = 14) that were in the age span of 12-15 months. Uterine flushing to retrieve embryos was performed on day seven after insemination. Embryos of IETS stage 5 (early blastocyst), 6 (blastocyst) or 7 (expanded blastocyst) and first grade quality were selected for further processing. In total, RNA extracted from 24 embryos was sequenced using Illumina sequencing, followed by differential expression analysis and gene set enrichment analysis.

Comparing the embryo gene expression between the two groups, 62 genes were found to be differentially expressed (adj.p-value<0.05). Out of these, several genes were linked to pathways that could explain the different developmental capacity. Transcripts highly expressed in the embryos from low fertility bulls were related to terpenoid backbone synthesis and sterol metabolism, while transcripts highly expressed in the high fertility embryos were linked to anti-apoptosis and the regulation of cytokine signaling. Both fertility groups had enrichments in the leukocyte transendothelial migration and insulin signaling pathways. In addition to this, some highly expressed transcripts in both groups can be considered as new candidates in the regulation of embryo development.

8 Methodological considerations

The details of material and methods for each of the studies that make up the foundation of this thesis, is presented within the papers and will not be described in detail here. Table 1 present an overview of the study populations and methods used in all papers. This section contains additional considerations and reflections.

Table 1. A summary over the study populations and methods used in papers I-IV. Norwegian Dairy Herd Recording System (NDHRS). Norwegian University of Life Sciences (NMBU)

	Study population	**Sampling method for principal sample**	**Data acquisition**	**Statistical analyses**
Paper I	Cows presented for first AI after parturition (n= 1,648) from 116 herds in Norway.	Cytotape collected at AI	Cytology NDHRS	Logistic models Receiver operating characteristics curves
Paper II	Cows presented for first AI after parturition (n= 1,642) from 116 herds in Norway.	Cytotape collected at AI	Cytology NDHRS	Linear animal model
Paper III	Cows from NMBU Center for livestock production (n= 32)	Cytobrush, uterine biopsy and uterine swab collected at AI	Illumina 16s rRNA-sequencing	QIIME2 bioinformatic pipeline. Linear discriminant analysis effect size
Paper IV	Breeding bulls from Geno SA archive (n= 12) and heifers from NMBU Center for livestock production (n= 14)	Blastocysts from superovulated heifers, collected at day 7 after first AI	Illumina RNA-sequencing	DESeq2 differential expression analysis. Gene set enrichment analysis

8.1 Animal use and welfare

The ethical approval for animal use in all studies was provided by the Norwegian Food Safety Authorities with approval ID 17/152686–1 (study I and II) and 11732 (study III and IV). In study I and II, animals were sampled with cytotape at AI. In study III we collected cytotape, cytobrush and biopsy samples from the endometrium as well as vaginal swabs. Embryo production was a way to investigate paternal contribution in study IV. In that study, heifers were subjected to super ovulation and insemination followed by embryo flushing.

8.1.1 Cytotape

For paper I and II, cows were sampled from the uterus at AI with a cytotape as developed by Pascottini et al. (2015). The calculation of sample size was based on a presumption of the prevalence and effect of CYTO, which was again based on earlier publications (Kaufmann et al., 2009; Pascottini et al., 2017a; Wagener et al., 2017). The prevalence and the difference in pregnancy to first AI were both expected to be 10%. The desired power and confidence interval was 90% and 95%, respectively.

The cytotape technique has previously been investigated regarding its effect on reproductive outcome (Pascottini et al., 2017a). In paper I, we could confirm that the sampling did not affect reproductive outcome. Regarding the subjective experience of the animal, the cytotape sampling is only marginally different from a normal AI. The possibility to combine the sampling with AI in the same passage of the cervix, was one of the reasons why we chose to sample at this timepoint and not earlier after parturition.

8.1.2 Uterine biopsy

For the collection of endometrial biopsies (paper III) we used a biopsy forceps designed for uterine sampling. Naturally, the procedure could result in a negative effect on the reproductive outcome, and possibly induce pain to the animal. It was not an option to use organs from slaughterhouses, as the microbiota could have significant changes compared to the organs of living animals. The animals included in our study had a lower NRR56 than the mean for NR, which indicates that the biopsy sampling did have a negative effect. One review argued that biopsy sampling did not affect the pregnancy outcome (Ramirez-Garzon et al., 2021). However, in that review, none of the investigates studies obtained the biopsy the same day as the AI took place, which was the case in our study.

As for the animal's experience with this procedure, we did not observe any signs of pain or discomfort upon sampling that could not be considered normal at rectal palpation. We could have considered epidural anesthesia before sampling, which itself is associated with stress in the bovine (Petyim et al., 2007). One previous study that also collected biopsies from the uterus did not

mention any anesthesia, and neither signs of pain in the animals (Knudsen et al., 2016). A much older study and one of the first to collect endometrial biopsies, specifically mentioned that the animals did not seem to be exposed to pain at the clipping of the biopsy (Skjerven, 1956). However, in the human, endometrial biopsy sampling without local anesthesia has been reported to induce different levels of discomfort (Trolice et al., 2000).

To obtain the different uterine samples for paper III, it was necessary to pass the cervix twice, in addition to the AI. This is likely to induce discomfort or soreness in the animal, both trough the reproductive canal and the rectal palpation (Alam and Dobson, 1986). A large part of discomfort from rectal palpation can be due to stress in animals that are not used to handling. As a refinement in our study, we used animals from the NMBU Center for livestock production, which are highly accustomed to human contact. In 2020 and after the initiation of our study III, a new multi-purpose sampling device was presented by Helfrich et al. Using this device, it is possible to sample uterine secretion to diagnose SCE, in addition to a cytobrush and biopsy sample by one sole passage through the cervix (Helfrich et al., 2020).

8.1.3 *In vivo* embryo production

In paper IV, we performed an *in vivo* embryo production by inducing a super-ovulation in heifers. The objective with super-ovulation is to obtain as many *in vivo* produced embryos as possible (Bó and Mapletoft, 2014). To reach this, the animal must be treated with several intramuscular injections with follitropin and lutinizing hormone. In our protocol, this treatment was given as two daily injections for four consecutive days. In addition, the heifers were treated with cloprostenol twice with a 12-day interval for heat synchronization. After the superovulation, they also received two injections with cloprostenol to induce luteolysis. A high number of injections may be stressful to the animal. Furthermore, the stress may interfere with the efficiency of the embryo production (Mapletoft et al., 2002). To this point, such treatment regimens have been necessary due to FSH having a short half-life in the circulation. However, new protocols with recombinant and long half-life FHS that require a single injection are emerging (Sanderson and Martinez, 2020).

Another important animal welfare concern is whether the animal feels pain or stress as a result of the hormonal changes and the subsequent reactions in the ovaries and other organs. This consideration needs further research in cattle, especially since it is known that prey species tend to minimize or mask signs of pain (Ashley et al., 2005). Furthermore, common side effect from similar treatments in the human include abdominal distention, abdominal pain and discomfort, and nausea (Felleskatalogen, 2023).

The heifers used in this study were 12-15 months old. In NR, it is common to start the first AI from an age of 14-16 months and a weight of around 400 kg (Animalia, 2019). For that reason, some of the heifers were small and both rectal palpation and passage of the cervix was slightly challenging. As in study III, we used animals from the NMBU Center for livestock production that are well accustomed to handling.

Following the super ovulation treatment, the heifers were inseminated, and an embryo collection was performed on day 7 of gestation through flushing. For this procedure we administered epidural anesthesia. However, embryo flushing requires rectal palpation and the insertion of a foley catheter, and can be a lengthy procedure, which was the case in some of these young heifers.

8.2 Terminology: CYTO vs SCE

As indicated in the introduction (5.6.5), there is an inconsistency and overlap in the use of the terms CYTO and SCE. CYTO is the increased proportion of PMN in the endometrium, while SCE is defined by the two aspects (1) absence of clinical symptoms and (2) reduced reproductive performance, in addition to the CYTO diagnosis (Dubuc et al., 2010a).

In paper I we performed the first investigation of CYTO and its risk factors in NR. With no earlier indications of how CYTO affected the reproductive outcome, we reasoned that CYTO would be the more appropriate term. Also, we did not want to exclude animals with vaginal discharge which we would have to do had we chosen the term SCE. In previous investigations of the prevalence of CYTO and risk factors, some have used the term CYTO (Pascottini et al., 2016; Pascottini et al., 2017a), while others have used the term SCE (Cheong et al., 2011; Prunner et al., 2014).
Later, when it was known that CYTO did have a negative effect on reproductive outcome in our study population, we used the term SCE in our paper II. For paper III, it was more challenging to decide which term was more appropriate. We did know that CYTO without clinical symptoms in NR would mean SCE in general, but this was not certain for each of the 32 individual animals included in our study of the uterine microbiota. Here, we reasoned that SCE would be the better option as it is more commonly used, and it was also used in the two previous studies of the microbiota in this condition (Wang et al., 2018; Pascottini et al., 2020). However, using the term CYTO would probably be more consistent with our earlier use of these terms.

8.3 The prevalence of CYTO, effect on reproductive outcome and risk factors

In paper I, a study population of 1,648 NR cows presented for first AI after parturition, was used to determine the prevalence of CYTO, its risk factors and effect on fertility.

Vaginal mucus was collected using a Metricheck™, which has been described to better detect clinical endometritis than other methods such as vaginoscopy and gloved hand technique (Pleticha et al., 2009). The mucus was evaluated using a common scoring system based on 4 levels from translucent mucus (0) to discharge containing ≥50% purulent material (3) (Williams et al., 2005). Animals with score 2 or 3 were defined as having clinical endometritis, and we decided to include all animals in the study independent of their vaginal mucus score. Earlier studies of SCE have generally excluded animals with purulent vaginal discharge, due to interference between the definition of the conditions (Kasimanickam et al., 2004; Kaufmann et al., 2009; Plöntzke et al., 2010). However, these studies used manual vaginal examination or vaginoscopy, and might have underestimated the number of cows with a clinical condition. In some studies that investigated CYTO (Dubuc et al., 2010a; Pascottini et al., 2017a), the higher prevalence of positive animals might be explained by inclusion of individuals with clinical endometritis. In NR, treatments for endometritis and metritis are low (1.7/100 cow-years) (TINE Rådgiving, 2021). Therefore, we expected a low number of clinically affected individuals, and that inclusion of these would not skew our data. Only 10 cows (0.7%) had a vaginal discharge score indicating clinical endometritis in our study, hence it only had a negligible effect on the evaluation of CYTO.

Sampling was performed at AI with the benefit of placing the cytotape on the inseminator. Additional reasons for choosing to sample at AI were that (1) it is a standardized timepoint in the production cycle and (2) it is highly relevant to investigate the situation at AI as it is shortly after this timepoint that the embryo comes in contact with the reproductive organs that are affected by CYTO. However, the investigation of CYTO at earlier timepoints is also relevant, as inflammation in the endometrium can have a carryover effect of at least four months (Ribeiro et al., 2016).

8.3.1 Diagnosing cytological endometritis

The cytobrush was the first instrument to collect endometrial cells for the diagnosis of SCE (Kasimanickam et al., 2004). The cytotape is used in the same manner as the cytobrush. Both techniques yielded a similar result with respect to total cellularity and PMN percentage, whereas the cytotape sampling resulted in less blood and more intact cells (Pascottini et al., 2015). One of the reasons for choosing cytotape over cytobrush, was the feasibility in combining sampling with AI, which made it possible to assign AI personnel in two different counties of Norway to perform the sampling. Uterine lavage and ultrasound are two other techniques for the collection of cells (Wagener et al., 2017), which were considered to be less feasible.

Staining and cell-counting of the cytology slides was performed at the university and was a highly time-consuming component of this thesis. This also reflects the main obstacle of diagnosing SCE in field (Wagener et al., 2017). Different attempts to diagnose SCE in alternative and more feasible ways have been published. This includes measurements of total proteins, optical density, pH, and leukocyte esterase (LE) on endometrial fluids or cells collected from the endometrium mixed with a neutral fluid (Cheong et al., 2012; Couto et al., 2013; Van Schyndel et al., 2018). The most promising method was seen using a LE test from test stripes designed for urine samples (Cheong et al., 2012; Van Schyndel et al., 2018). The test was evaluated both on its association with results from cytology, and its effect on reproductive performance. Reported significant associations in both comparisons was presented, but the authors also pointed to the poor sensitivity and specificity for the test (Cheong et al., 2012). Couto et al. (2013) could not confirm the association between LE test results and reproductive performance. More recently, (Van Schyndel et al., 2018) reported a moderate diagnostic accuracy compared to cytology. They concluded that the agreement was reasonable enough to use leukocyte-esterase as a cow-side diagnostic test for SE in the clinical context on farm. The LE test constitutes a future possibility, provided that refinements of the test are successfully performed.

Another new promising approach to diagnose CYTO is by measuring the level of pro- and anti-inflammatory cytokines (IL1B, IL8, IL17A) in uterine secretions. The findings of these cytokines in CYTO positive animals correlated to the related gene expression in endometrial biopsies. To collect uterine secretions for this purpose, a novel sampling tool was presented (Helfrich et al., 2020).

The stained cytology samples were investigated under a microscope at 400x magnification where a total of 300 representative epithelial cells and PMN were counted in several fields, see Figure 5. Three veterinarians performed the counting. Determining the proportion of PMN through microscopic evaluation of endometrial smears is reliable and reproducible (Wagener et al., 2017). However, to ensure a good inter-observer reliability in our study, we performed reliability calculations on a subset of the observations (n = 475). The outcome showed a Kendall's W of 0.72, which was assessed as high (Martin and Bateson, 2007).

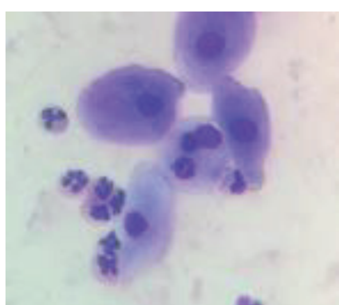

Figure 5. To diagnose CYTO, a cytological smear was prepared by rolling the cytotape sample from each animal on a glass slide. Further, 300 PMN and epithelial cells were counted under a microscope, and the proportion of PMN was calculated. To the left: cytological smears after staining (Photo credit: Sofia Diaz-Lundahl). To the right: microscopic image of PMN and epithelial cells from the endometrium (Photo credit: Randi Therese Garmo)

8.3.2 The cut-off level for PMN

To calculate the prevalence, effect on reproductive outcome and risk factors for CYTO, we built three logistic models. The first model assessed CYTO based on endometrial samples, the second model assessed pregnancy outcome based on risk factors including CYTO, and the third model considered embryo loss.

The cut-off level for the definition of CYTO was set to 3.0% PMN. This decision was based on the construction of a ROC curve (Dohoo, 2009), and the level at which the PMN occurrence affected pregnancy outcome with the highest summation of sensitivity (32.4%) and specificity (74.9%). At this level, the area under curve (AUC) equaled to 0.54, and the positive and negative predicative value was 46.6% and 62.2%, respectively. This indicated that a 3% cut-off had a modest ability to predict a CYTO-positive cow to become nonpregnant, but it was also close to what Pascottini et al. found in Holstein-Friesian at AI (AUC = 0.62) for their chosen cut-off (1% PMN) (Pascottini et al., 2017a). Furthermore, a modest AUC was seen in several studies that investigated CYTO (Kasimanickam et al., 2004; Dubuc et al., 2010a; Madoz et al., 2013), and could be explained by the fact that reproductive success is affected by more factors than just the inflammatory status of the uterus (Kasimanickam et al., 2004). As the ROC curve did not add much predicative ability to the model in our study, we also investigated whether it could be an option to define the probably best cut-off by a lowess smoother curve on a *logit* scale, which did not result in a very smooth curve. We also considered defining the cut-off by PMN present or not, which would result in a 0.3% threshold as we counted 300 cells. However, ROC is a common way to set the cut-off value for CYTO or SCE, and with advice from the paper reviewers, we landed on using this method and a 3% cut-off. The

modest AUC in our study could be explained by the fact that both CYTO positive and CYTO negative animals had a high overall pregnancy to first AI.

8.3.3 Estimating embryo loss

Milk samples for measuring the progesterone levels were collected at AI and 21 days later. The results were applied to estimate (1) whether the cow was in true estrus at sampling and (2) whether the cow was likely pregnant on day 21 after the first AI. A level of <3 ng/mL was used to indicate estrus. The likelihood for pregnancy at day 21 was used as an indicator of embryo loss.

Early embryo loss is more common (Diskin et al., 2006) and has a larger economic impact on the dairy cattle industry than late embryo loss (Sreenan et al., 2001). Therefore, it would be highly interesting to obtain a direct measurement of the correlation between SCE and early embryo loss. However, diagnosing embryo loss that occurs before 24 days of gestation is challenging, and requires a distinction between animals that failed to get pregnant and animals that lost an early embryo. There are no feasible methods to implement that investigation in a large study population such as the one in our study I. Therefore, we decided to estimate late embryo loss by drawing the line at day 21 and the assumption that the cows that carried a living embryo at that point, would not have a milk progesterone level indicative of a new estrus. With an estimate of late embryo loss and its correlation to SCE, we were able to apply an exclusion method in the interpretation of our results, to hypothesize on whether SCE likely affects early embryo loss or not.

Cows with >3 ng/mL at AI were excluded from the material in the late embryonic loss model. Late embryonic loss was defined as having happened in cows with progesterone levels >3.0 ng/mL 21 d after the first AI, who were (1) not pregnant on rectal palpation or PAG analysis ≤42 d after the first AI, or (2) presented for a new AI ≥ 42 d after first AI, and with no recording on pregnancy status. Using this definition assumes that all cows have a 21day estrus cycle, which is common in NR (Refsdal et al., 2014). This means that those with longer or shorter cyclase's could have been erroneously appointed as still pregnant on day 21, which would lead to an under-estimation of late embryo loss.

8.4 Heritability estimation for subclinical endometritis

In our study II, we used the dataset from study I to estimate the heritability SCE in NR. The linear animal model used for estimation of variance components included the known risk factors for CYTO presented in paper I as fixed effects. The random effects were the additive genetic effect of animal and residual. An alternative approach that we considered, and that could have been appropriate for the binary traits, was to use a threshold model. However, linear models are robust and often the model of choice for routine genetic evaluations, and we therefore found it appropriate to use this approach for this initial genetic analysis of a new trait, to explore the potential.

Although the current study was based on the largest published data set on SCE with 1,642 usable registrations, the number of records was still low in the context of estimating heritability of disease in dairy cattle. For comparison, two recent heritability estimates of clinical endometritis included 14,810 cows (May et al., 2021) and 114,060 cows (Shabalina et al., 2020), respectively. Both studies used linear animal models as in the current study. Nevertheless, clinical endometritis is commonly defined through vaginal discharge which is much more feasible to collect at high throughput, than samples to diagnose SCE. Although the relatively low number of animals was an obvious limitation of our study II, it was valuable, as it represented the first published heritability estimate of SCE.

With the current dataset, it was not possible to decide whether the trait for SCE was genetically the same across parities. With more data, I would suggest making a distinction between first and subsequent lactations, as animals highly affected by SCE might have been culled after the first lactation. By doing that, the accuracy of our calculations might have been stronger. Furthermore, it is common to group animals based on lactation in heritability estimates of clinical uterine disease (Zwald et al., 2004; Shabalina et al., 2020; May et al., 2021).

8.5 The uterine microbiota at AI: Study design

In paper III, we investigated the uterine microbiota at AI by collecting an endometrial biopsy (deep endometrium) and cytobrush (superficial endometrium). We also investigated relations from these different depths of the endometrium with the vaginal microbiota as collected with a vaginal swab. The study population consisted of 32 healthy lactating NR cows from the NMBU Center for livestock production. The management conditions, housing and feeding is well controlled in the chosen farm. Furthermore, this population was chosen for feasibility, as sampling was performed at natural heat, meaning that only a few cows could be sampled on the same day. In NR, treatment for heat synchronization was only performed 1.9 times per 100 cow-years in 2021 (TINE Rådgiving, 2021), so by obtaining samples at natural heat, I believe we got a closer reflection of general conditions in NR.

The sample collection from the endometrium was performed in two phases, with the disadvantage that we had to pass the cervix at two occasions. Initially, we collected a cytotape (for SCE diagnosis) and the cytobrush sample using a combined tool, see Figure 6, and after that we obtained a biopsy. This design was chosen in order to obtain the status with respect to SCE before we proceeded with the biopsy sampling. In our original study design, we aimed to obtain an equal number of cows with and without SCE, respectively. By this, we wanted to realize a sub-goal of the study on whether the microbiota was different in cows positive for this condition. Hence, the repeated passages of the cervix would be justified, because biopsy sampling would not be necessary, had we already obtained the desired number of SCE positive or negative animals. However, the occurrence of SCE appeared to be low in the chosen population, and we ended up with only three SCE positive animals. For that reason, we were only able to land on a description considering microbiota in SCE positive cows.

Figure 6. A combined tool based on an insemination gun was used to collect a cytotape and cytobrush sample from the uterine endometrium. The insemination gun was protected with a disposable plastic tube. Figure reproduced with permission from Pascottini et al. (2015), Copyright Elsevier.

Contamination is an important issue in studies of microbiota, and in particular when the expected bacterial load is low, such as in the uterus (Sheldon et al., 2019). When the microbial biomass is low,

the proportion of contaminating microbiota from the environment or the laboratory increases might grow as big as the sample microbiota (Karstens et al., 2019). In our study, we did not use a negative control at sampling, which is advisable (Kim et al., 2017). The guidelines for best practice are constantly evolving, and our study design was planned in 2016-2017, while the sample collection was performed in 2017-2018. More recently, Pascottini et al. (2020) used a swab that was moved around in the open air in the barn as a control in their study of the uterine microbiota. A positive control is also recommended (Kim et al., 2017), and in our study we used the vaginal swabs as a positive control. As the instruments to collect endometrial samples passes the vagina, it was also a good way to control for contamination.

8.5.1 DNA-extraction

For the DNA-extraction, we used a negative control (DEPC water), which was processed and sequenced in the same manner as the rest of the samples. In each round of extractions, we included 4-5 samples at a time (a mix of the three different sample types), and one negative extraction control. The negative extraction control was the last in the line of samples to be extracted in each round.

Contamination from commercial DNA-extraction kits is a well described issue. DNA extraction kit contaminants depends on different kits or lots, and also the laboratory in which the samples are processed (Salter et al., 2014). To control the sequencing results, negative controls are crucial (Kim et al., 2017). It is also recommended to process all samples side by side in the laboratory (Kim et al., 2017). However, we avoided this as any error in the process might risk all our samples. Well-to-well contamination has recently been described. The recommendation to reduce such contamination is to randomize samples across plates and process samples of similar biomasses together (Minich et al., 2019). In our study, this would mean to process biopsies and negative controls alongside, which we did not. Even though it seems impossible to avoid contamination in the laboratory, we did try to control this issue by taking notes of the extraction rounds and including this information in our metadata and statistical analyses. That way, we could visualize potential differences in the beta diversity related to extraction round.

8.6 The paternal contribution to the early embryo: study design

In the study of the paternal contribution to the embryo (paper IV), we used semen from high fertility bulls (HF bulls) or low fertility bulls (LF bulls) to inseminate super ovulated heifers, for the *in vivo* embryo production. The design was based on the assumption that semen from the low fertility bulls would result in embryos with a lower developmental capacity, see Figure 7.

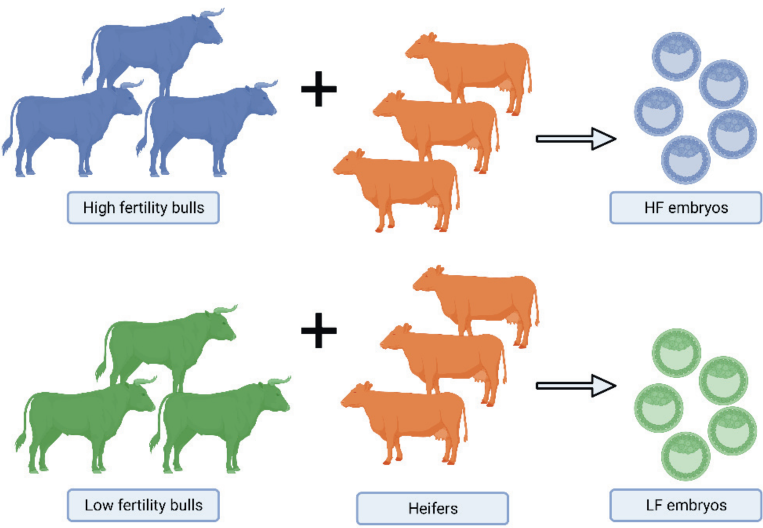

Figure 7: In paper IV, semen from high fertility (n = 6) or low fertility bulls (n = 6) was used to inseminate a homogenous group of superovulated heifers. The embryos from high fertility bulls (HF embryos) were assumed to have a higher developmental potential than the embryos from the low fertility bulls (LF embryos). Figure created with BioRender.com.

The bulls were chosen from a database of 470 NR sires, all with at least 500 registered first AI. Fertility was recorded as the NRR at 56 days after first AI of the inseminated cows. The semen had passed standard testing requirements performed by Geno SA, and the reason for the difference in fertility was not known. One limitation in the chosen design, was that it was not certain whether all low fertility bulls had the same negative effect on embryo. It is also not certain whether the mechanisms for low fertility that the chosen bulls exhibit, is common in the rest of the NR population. The study included 14 bulls, and in the end, we could sequence embryos from 3 low fertility bulls and 4 high fertility bulls. Studies using RNA-seq often have a small study population due to the costs of sequencing. When we initiated our study IV, the current recommendations were to use at least three biological replicates (Conesa et al., 2016), and this requirement was met in our study.

Our study used *in vivo*-produced embryos, whereas it is a more common approach to use *in vitro*-fertilized embryos. As the biochemical characteristics of mammalian embryos is affected by *in vitro* culture (Wright Jr and Ellington, 1995), it is important to use *in vivo* approaches parallel to this research. However, in order to obtain a sufficient number of embryos, it is common to treat the donor animals to have a superovulation (Mundim et al., 2009). This was also done in our study. In the interpretation of our results, it is important to remember that superovulation can alter the embryos gene expression (Mundim et al., 2009). This alteration applies to both LF and HF embryos in our study and should not affect the differences between the groups. Super ovulation can also have different effects in different animals (Mapletoft and Bó, 2014), which became clear in our study where the embryo yield per animal was between 0 and 19 embryos.

We decided to use one heifer per bull. An alternative approach would be to use one heifer for one bull and perform repeated superovulations. The latter would have resulted in embryos with the same maternal genome, which could have the benefit of better distinguishing the paternal effects from the maternal effects. However, such a design would also have its related disadvantages. First, superovulation and embryo collection likely have a negative effect on the animal welfare. In my opinion, using one heifer for the repeated use of these methods, would have reduced the total animal welfare in our study. Second, these methods themselves including the insemination might affect the conditions in the uterus, hence affecting the environment for the subsequent embryo group. Naturally, the outcome in the embryo gene expression could have been equally or more affected by these conditions, even if I could not find an answer to these questions in earlier literature. Third, the age of the heifer could also influence the embryo outcome, as age has been shown to alter both embryo gene expression (Takeo et al., 2013) and endometrial gene expression (Tanikawa et al., 2017). By using the same heifer on several occasions and considering that superovulation can be repeated after about two months (Geno SA, 2020b), the maternal contribution could be different in the early compared to the late collected embryos. As a fourth reason for choosing the current design over a repeated use of the same heifer, our study design was more feasible and could be performed within one month since the heifers were treated to have a synchronized heat. Another option would be to perform a cross-over where the heifers would be subjected to superovulation at two different timepoints and inseminated with semen from a low-fertility bull or a high-fertility bull at the two occasions, respectively. However, this design would still have the limitation of different mother animals, in addition to several of the drawbacks described from the one-heifer approach.

To reduce differences in the maternal contribution to the embryos, we used heifers from the NMBU Center for livestock production that were housed together with the same feeding regime and were within a pre-defined age span. We also secured that their genetics would be as similar as possible by choosing heifers derived from the same genetic line with 28 years of targeted breeding for high fertility and a low occurrence of clinical mastitis (Heringstad and Larsgard, 2010).

In our study, we performed a morphological evaluation of the embryos according to the guidelines by IETS. Even though this is a common method, it has the disadvantage of being biased by the evaluator subjectivity. Other alternative techniques to decide stage and quality are emerging, such as the combination of digital images with artificial intelligence (Rocha et al., 2016). In our material, we could see that the embryos from the different stages did not have vast differences in the gene expression, which justifies our use of three different stages of blastocysts. We also chose to sequence only embryos of the highest quality. One could argue that by doing this, we might have lost some interesting information, especially from the low-quality embryos deriving from low fertility bulls. We did consider this approach. However, with our low number of biological replicates, it was necessary to keep the individual variation within the groups to a minimum.

8.6.1 RNA extraction

In our study IV, we used the embryo gene expression as a way of investigating how the different fathers affected the embryo. By investigating the embryos transcriptome as an alternative to the genome, one can get a full image of the genes that are actually being expressed in the moment before freezing.

One mayor challenge in the extraction of RNA from the single embryos, was the lysing of the zona pellucida. Previous knowledge from our faculty showed that this process required tough handling, which had to be balanced against the risk to damage the embryo. We landed on a protocol that used cycles of freezing and thawing to open the zona pellucida. Repeated freezing and thawing is also known to degrade RNA (Kellman et al., 2021), which was probably the case in our study as well to some extent. Degradation of RNA is an issue that has been debated and discussed in the last years, particularly since the broader use of NGS began. It has been concluded that if material is not degraded in a manner that would bias a study´s conclusion (i.e., samples and controls suffer similar degradation), valid conclusions can still be drawn even from highly degraded samples (Gallego Romero et al., 2014). In such cases, presentation of quality measurements is important to demonstrate the similarity in input material. The cDNA tape station profiles acquired from the sequencing center demonstrated that the majority of RNA had not been damaged and the lengths of the RNA was as expected. Furthermore, the PCA plot showed that the outcome in i.e., the gene expression, was uniform enough to make comparisons between the two embryo groups.

8.7 Next generation sequencing and bioinformatics

In Paper III and IV we used NGS and subsequent bioinformatic analyses. In paper III, DNA in different sample types from the reproductive tract was extracted and bacterial 16S rRNA was targeted for the production of amplicons. In paper IV, RNA was extracted from embryos and the cDNA corresponding to the total mRNA was sequenced.

In studies using NGS, it is common to obtain large datasets consisting of millions of reads. To compare groups, the statistical analysis of these reads requires a correction for multiple hypothesis testing in order to not risk a high false positive rate. Some methods such as the Bonferroni correction might be too conservative and risk too many false negatives (Kim et al., 2017). The Benjamini and Hochberg correction was used in the statistical analyses for both paper III and paper IV. This is a commonly accepted method (Kim et al., 2017) and integrated in the QIIME2 pipeline and DESeq2. In both studies, an adjusted p-value (also referred to as the q-value) of 0.05 was considered to be significant.

8.7.1 16S rRNA

In paper III we used sequencing of 16S rRNA. It is possible to target different hypervariable regions or combinations of such in the sequencing of 16S rRNA gene. No region of 16S rRNA can differentiate between all bacteria. Some regions are more likely to better distinguish a certain set of bacteria than other regions, and vice versa (Chakravorty et al., 2007). Hence, there might be some discrepancies when our results from the V3-V4 region are compared with studies that used other combinations of 16S regions.

Amplicon data from the uterine microbiota was investigated using the QIIME2 pipeline (Bolyen et al., 2019) which is a common way to analyze data from 16s rRNA sequencing (Qian et al., 2020). The microbiota was investigated in terms of ecologic diversity and taxonomy. Ecologic diversity is commonly expressed as alpha and beta diversity (Qian et al., 2020). The alpha diversity refers to the variety of species within each sample and can be described as the richness (the total number of species) or the evenness (how well distributed the abundance is among the species) (Wilsey and Potvin, 2000; Gilbert and Lynch, 2019). There are also metrics that represent a combination of the two, such as Shannon or Simpson index (Qian et al., 2020). We chose to present three common metrics in the current study: Chao1 (richness), Pileous Evenness and the Shannon index. The Simpson index puts more emphasis on more common species, which would probably be a more appropriate option. However, the Shannon index was presented in earlier studies of the microbiota of SCE (Wang et al., 2018; Pascottini et al., 2020), which facilitated the comparison between studies. The beta diversity refers to the differences in microbiota between samples (Gilbert and Lynch, 2019; Qian et al., 2020). We applied the two commonly used metrics Bray Curtis dissimilarity and Weighted unifrac. The Bray Curtis dissimilarity quantifies the compositional dissimilarity and gives

more weight to common species. Weighted unifrac takes phylogeny into consideration and have a reduced emphasis on low abundant taxa (Qian et al., 2020).

The mayor challenge in the statistical analyses of our 16S rRNA amplicon data was the low microbial biomass in the biopsy samples. Firstly, the large proportion of host DNA compared to the bacterial DNA was a challenge. Host DNA probably interfered with the quality controls of sample content because the DNA concentration appeared much higher than what was obtained from the bacterial DNA. Also, some sequences appearing in the raw fastq files might come from host DNA. In order to eliminate host DNA from the sequencing data, the classifier that was trained on SILVA reference sequences was also adapted to the V3-V4 region of bacterial 16S rRNA, within the QIIME2 pipeline. Furthermore, all features that did not reach a classification of at least phylum level were removed. Secondly, the outcome from low biomass samples has a higher risk of being disturbed by contaminating taxa. The handling of sequences appearing in negative controls is still an ongoing discussion (Karstens et al., 2019). Some authors eliminate all taxa appearing in negative controls from the dataset as they might be a result of contamination. Such an approach might eliminate taxa with a biological relevance and might therefor be too strict (Glassing et al., 2016; Karstens et al., 2019). Other methods consist of passing the dataset through programs such as Decontam or SourceTracker, or removing low abundance taxa or taxa that are common contaminants. A more recently published method is to use a mock microbial community of known composition as a positive control. This can support the interpretation of possibly contaminating taxa and the findings in a negative extraction control (Karstens et al., 2019). This approach would have been a good option in our paper III, had the method been published before we planned our study design.

8.7.2 RNA-seq

In paper IV, we used RNA-seq to investigate the embryo gene expression. The distribution of gene expression in the individual blastocysts was visualized by principal component analysis plots where we could confirm that the embryos of different IETS stages 5, 6 and 7 where sufficiently similar to make a comparison between the high and low fertility bulls. Two embryos were shown as outliers and were removed from further analyses. The decision to remove them was supported by the fact that they had an inferior cDNA quality according to the TapeStation profiles.

The kit applied for production of cDNA from RNA (https://www.takarabio.com/a/114896) uses a poly-T primer, which only allows amplification of mRNA, thus excluding other RNA molecules from being subjected to sequencing. In the cDNA production, we used 18 cycles of amplification, which was the maximal number recommended by the kit used for cDNA production. The number of amplification cycles necessary was tested using embryos from other animals than those in the experiment. Our results from this testing showed that fewer cycles did not work on all test samples. Hence, to make the results comparable between samples we decided to use 18 cycles. In addition, all embryos from both LF and HF sires were treated in the same way, so we reason that the comparison is valid.

Differential expression analysis was performed using DESeq2 (Love et al., 2014), which generates a list of differentially expressed (DE) genes (a DE-list). A gene set enrichment analysis of the DE genes was performed using g:profiler (https://biit.cs.ut.ee/gprofiler/gost) (Reimand et al., 2007), which also integrated data from Gene Ontology (http://geneontology.org/), the KEGG Pathway database (https://www.genome.jp/kegg/pathway.html) and Reactome (https://reactome.org/). We also used Pathviews (https://pathview.uncc.edu/) (Luo et al., 2017) for functional analyses and ENSEMBL (https://www.ensembl.org/index.html) to study the function of individual genes. A large variety of tools for gene set enrichment analysis have been developed since 2002. In 2009, a review of 68 different tools was published (Huang et al., 2009), and this is still an active field of development (Sherman et al., 2022). In gene set enrichment analysis, there is a high risk of sampling bias, which can arise through (1) technology bias, (2) detection bias, and (3) biological bias. To reduce sampling bias in the interpretation of the functional enrichment analysis, it is recommended to generate an appropriate background expression list with genes that be considered to be significantly regulated in all samples. The list will depend on the studied tissue (Timmons et al., 2015). In our study, a custom gene list that were detected in the DE analysis was used as background for statistical domain scope in g:profiler.

Working with non-model species, it is a common method to compare the findings to human orthologues because it can provide new information, as the human genome is more thoroughly studied and has focused on other qualities than production-related parameters. Even though gene ontology annotation mirrors human annotation, more information regarding pathways associated with genes and their function are available for human compared to cattle. To enrich results interpretation, DE genes and the background list for statistical domain were converted to human ENSEMBL orthologs from bovine ENSEMBL IDs, by g:profiler, and run through gene ontology analysis in g:profiler and pathway analysis in Pathviews. Using this approach, we found that each pathway or gene ontology (GO) term had more of the genes from the DE-list associated in general. We also found stronger evidence codes for the genes to be linked to the pathway or GO-term.

9 Results and Discussion

Why embryos die is a vast research question and does not have one simple answer. Several attempts have been made to categorize the causes in order to point out the probably most important matters. The already known or suggested causes also interact with one another, making it challenging to define their relative importance. In early embryo loss, science faces the issue of distinguishing between fertilization failure and early embryo loss. With these challenges, the question of why embryos die will probably have to be elucidated by constructing the puzzle piece by piece.

In the planning of this thesis, we attempted to pinpoint relevant areas of focus within the concept of embryo development. We decided to focus on both maternal and paternal aspects, as it is important to underline that both parents have an impact on the embryo development and hence embryo death. On the maternal side, we focused on the uterine environment at AI, and SCE which has been shown to be a common issue on a global level. On the paternal side, we targeted the lack of knowledge on how the sire contributes to early embryo development. This thesis extends into the different scientific disciplines of epidemiology, genetics, transcriptomics, and bacteriology, all with the common link: factors that affect the development, life, and survival of the bovine embryo. This section provides a discussion of the results obtained in study I-IV, reflection of the contribution that our findings represent to the field, and future perspectives.

9.1 Maternal impact

In paper I and II we investigated SCE in NR with relation to prevalence and significance, as well as heritability within the breed. The study population was highly comparable with the total population of NR according to management system, herd size, season for AI and milk yield (TINE Rådgiving, 2019). In paper III, we studied the uterine microbiota at AI in NR in a smaller study population.

9.1.1 CYTO prevalence and risk factors

In paper I, we found that the proportion of animals with a CYTO diagnosis was 28% (461/1,648), using a 3% cutoff-level of PMN. Simulating cut-off at 0.3%, 1.0%, and 5.0% PMN, the proportion of CYTO-positive cows would be 58.4%, 42.7%, and 20.9%, respectively. Interestingly, the prevalence was higher than what has been reported at AI in Holstein (Dubuc et al., 2010a) and Holstein-Friesian (Kaufmann et al., 2009; Pascottini et al., 2017a). To the contrary, we expected a lower prevalence and speculated that a low CYTO prevalence could be one possible explanation for the high fertility in NR.

CYTO was reported as a risk factor for pregnancy success after first AI. The odds ratio was 1.51 for a CYTO-positive nonpregnant cow compared with a CYTO-negative pregnant cow. Hence, CYTO was reported as a fertility issue in NR and can therefore be defined as SCE. However, pregnancy to first AI was still high. Cows positive for CYTO had a successful pregnancy to first AI in 53.4% (217/406) of the cases, whereas the corresponding number for CYTO-negative cows was 62.2% (649/1,043). With these results we could establish that NR cows have a good fertility despite of a high prevalence of SCE, and that a positive SCE diagnosis only has a modest effect on pregnancy to AI. This is probably a reflection of the breeding program for NR which has emphasized fertility and health for decades. In our study, we hypothesized that NR has preserved a genetically advantageous regulation of uterine immunology. Uterine disease can develop as a result of metabolic stress and its effect on immunity (Sheldon et al., 2019). Systemic inflammation and negative energy balance plays an important role in the control of uterine immune function and the development of CYTO in Holstein cows (Dubuc et al., 2010b; Cheong et al., 2011; Pascottini and LeBlanc, 2020). In contrast to NR, Holstein is a breed with a tradition of a strong genetic selection for milk yield and could therefore experience more metabolic stress. A recent review (Sheldon et al., 2019) suggests that tolerance is more important than immunity, making failure of pathogen tolerance the cause for disease development, which leads to persistent inflammation. High pathogen tolerance could be one explanation of the relatively beneficial outcome of CYTO in NR.

The detected risk factors for CYTO were AI personnel, interval from calving to first AI (CFAI), vaginal mucus condition, red blood cells (RBC) in sample, barn type, and season. The finding that AI personnel influenced our results is an indication that training and calibration is crucial before sampling with cytotape. In our study, the AI personnel consisted of 11 experienced and competent

AI technicians and 3 veterinarians that were trained in a one-day practical course. A shorter CFAI interval was a risk factor for CYTO, which support the use of lower cut-offs to diagnose CYTO at AI than what has been used in most studies that sampled at earlier time points. Likewise, the variable RBC in the endometrial samples was associated with CYTO, which could have been due to a sensitive mucosa in CYTO positive animals. Neither CFAI nor RBC had an effect on pregnancy outcome.

Barn type was also associated with CYTO, where tie stall herds had less CYTO-positive cows. However, the pregnancy success to first AI was higher for cows in free stall herds, hence not largely affected by the higher incidence of CYTO. In correlation with this, one earlier study reported a higher reproductive success in Norwegian Red managed in free stalls (Simensen et al., 2010). There is a vast difference in how the parameter vaginal mucus condition has been treated in earlier studies of SCE, as described in 8.3, making comparison of the risk for this factor a challenge.

The risk for CYTO was lower in summer and spring compared to the winter season. However, the total pregnancy success was not affected by season. The seasonal variation in CYTO could indicate that the occurrence might be different in different regions of Norway, as Norway extends over three climate zones (warm-temperate, cold-temperate and polar climate). Our 116 herd were situated in Hedmark and Trøndelag county which has a central location in Norway in the north-south direction.

Late embryo loss and fetal loss was detected in 8.6% (82/948) and 2.4% (24/866) of the cows, respectively. There was no increased risk for late embryo loss or fetal loss related to CYTO. Late embryo loss was defined as death between day 21 and day 42, while loss from day 28 is a more common definition. This suggests that late embryo death in NR happens in less than 8.6% of the cows, and is comparable to the 7% reported by Diskin et al. (2006). Late embryo loss was only associated with treatment against fertility disorders before first AI, with an odds ratio of 4.37. Medical treatment as such may alter the metabolism of the uterus, or the disease leading to treatment may alter it. Our results suggest that late embryo loss and fetal loss is mainly influenced by other factors than CYTO, yet to be investigated.

In addition to achieving our objectives, our sampling for study I provided an estimation of the occurrence of clinical endometritis in NR, based on the appearance of vaginal discharge (Sheldon and Owens, 2018), sampled with Metricheck™. Only 0.7% (10/1,442) of the animals were affected, which can be compared to the 12% prevalence found by Dubuc et al. (2010a) at AI in Holstein cows, using the same sampling device. We could not find any other recent study of the prevalence of clinical endometritis in NR, and for comparison we used data from the NDHRS, where the number of treatments against metritis and endometritis was 1.3/100 cow-years in 2019 (TINE Rådgiving, 2019).

It is interesting that while the prevalence of clinical endometritis was very low, the prevalence of CYTO was high. This indicates that clinical endometritis and CYTO are conditions with different mechanisms and causes that requires a separate investigation of pathogenesis and genetics. This is supported by differences in uterine microbiota detected between cows positive for clinical endometritis and SCE, respectively (Pascottini et al., 2020). Furthermore, a genome-association study (May et al., 2021) identified genomic loci associated with different stages of endometritis and metritis. No overlap in candidate genes or single nucleotide polymorphism was found for the different stages, which indicates that even the clinical stages of uterine disease should perhaps be investigated separately.

9.1.2 Subclincial endometritis and heritability

Based on our results from paper I, we hypothesized that there is a genetic and heritable component in SCE. If such component was found, SCE could have a potential as a future fertility phenotype to be used for breeding purposes. This reflection led us to initiate the second study of this thesis.

In paper II, we investigated different trait definitions for SCE by estimating variance components and corresponding heritability. The only trait definition that had an estimated genetic variance larger than the standard error was Cyto5. Hence, SCE appear to be heritable using a 5% cut-off level of PMN. The estimated heritability was 0.04 with standard error 0.035. This is comparable to previous findings for clinical endometritis in Holstein with estimates of 0.03 (May et al., 2021) and 0.04-0.07 depending on lactation (Shabalina et al., 2020). Furthermore, one previous study estimated heritability of metritis in NR to 0.03 (Heringstad, 2010). The results from the present study should be interpreted with caution, as the standard errors of the estimates were relatively high.

In paper II, we also reflected upon whether SCE could be implemented as a fertility trait in the NR breeding program. On a global level, the low heritability values of fertility traits have been used as an argument against the use of genetics to improve fertility. However, as breeding towards a high milk yield contributed to a decline in fertility, then genetics can also be used to improve fertility (Cassandro, 2014). A recent review (Sheldon et al., 2019) suggests that one important factor to control uterine health in dairy cattle is through enhancing the animal tolerance to uterine pathogens. It has also been stated that the development of new fertility traits and the use of genetic selection is increasingly important (de Souza Ribeiro et al., 2018). Furthermore, genetic strategies are preferred over the use of antibiotics or other medicine and is better for the animal welfare. In NR, the four most common fertility related diseases or disorders, including metritis, are already included in the total merit index as a composite trait “other disease”. Our conclusion from the current study was, that SCE does appear to have a potential as a new fertility trait.

A 5% cut-off on PMN was defined as the correct trait definition for SCE, because it was the only definition by which SCE was confirmed as heritable. In paper I, a 3% cut-off resulted in the best

summation of specificity and sensitivity to predict pregnancy to first AI. However, a 5% PMN level also had a negative effect on pregnancy to first AI, and the difference in summation of sensitivity and specificity was marginal compared to a 3% cut-off. A 5% PMN level has been proposed as a general cut-off for the purpose of a common standard, when SCE is diagnosed at 21-62 days after calving (Madoz et al., 2013).

9.1.3 The uterine microbiota at AI

Although paper II demonstrated that SCE has a heritable component in NR, the majority of the variation was not explained by this factor. A beneficious immune regulation of the uterus in NR is one potential answer to why the negative effect of SCE was only modest in the breed. Another option could be that NR has a beneficious composition of uterine microbiota, in healthy or SCE positive individuals, or both. In paper III we investigated the microbiota of the uterus at AI, which represents a first step towards understanding the environment that the fertilized embryo experiences in NR. In addition to this main objective, we included cows positive and negative for SCE with the aim to guide the understanding of this condition in NR.

To investigate the uterine microbiota at AI, we used animals from the Center for livestock production at NMBU. Surprisingly, only 3 out of the sampled animals (n=32) were positive for SCE, which is a much lower prevalence than found in study I of 28%. Due to the low number of SCE positive animals, we could not make any statistically valid conclusions on whether the condition is related to certain traits of the uterine microbiota. However, the consideration with respect to SCE provided important knowledge about the uterine status in our study population in general. The low prevalence in the investigated herd underlines the importance of herd-related risk factors in SCE (Cheong et al., 2011; Wagener et al., 2017).

The investigation of the uterine microbiota at a superficial endometrial level (by cytobrush) and a deeper endometrial level (by biopsy) was based on sequencing results from 24 cows. The alpha diversity estimate showed a higher evenness in biopsy samples compared to cytobrush and vaginal swabs. Our results correlated with the findings in an earlier study that compared endometrial biopsies with uterine flush samples (Knudsen et al., 2016). The beta diversity was visualized in PCoA plots, and showed that the biopsy samples clustered together while the other two sample types were scattered in all directions. The PERMANOVA calculations confirmed that the biopsy differed in beta diversity compared to the other sample types. The microbiota from cytobrush samples and vaginal swabs had no significant difference in either alpha or beta diversity.

The most highly represented phyla in the present study were Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota. These results concord with earlier studies of the bovine reproductive tract using NGS in healthy cows or cows with SCE (Knudsen et al., 2016; Bicalho et al., 2017b; Wang et al., 2018). There were more inter-individual differences in the taxonomic composition of the cytobrush and vaginal samples, than in the biopsy samples. *Streptococcus* was

highly abundant in both cytobrush and vaginal swabs, and *Escherichia-Shigella* was detected in mainly the vaginal swabs. This correlates with earlier microbiome studies of the bovine vagina (Rodrigues et al., 2015). No difference in taxonomic composition was found between cytobrush samples and vaginal swabs according to the LDA-LEfSe analysis. The similarities in alpha and beta diversity and taxonomic composition between these sample types suggest that that the uterus and vagina share a common microbiota when the cow is presented for AI at natural heat. Hence, one may question the necessity of taking a cytobrush sample instead of a vaginal swab in future investigations of the uterine microbiota at AI.

The taxonomic composition was different in the biopsies compared to the other two sample types. Bacilli was enriched in the cytobrush and vaginal swab samples compared to the biopsies, while Firmicutes was also enriched in the vaginal swabs compared to biopsy. In the biopsy samples, we found a high abundance of *Oscillospiraceae UCG-005*, *Bacteroidetes_vadinHA17*, *Ruminococcus*, *Bacteroides*, *Alysiella* and four different genera of the family Lachnospiraceae. These taxa were barely present in the other sample types, and not present in the negative extraction controls.

The bacterial composition of the negative extraction controls was dominated by *Massilia, Burkholderia*, *Polaromonas* (which were also highly abundant in other sample types, especially biopsy) and *Flavobacterium* (mainly in negative controls). It is uncertain whether these taxa represent a kit contamination, if they are also present in the reproductive tract, or a combination of the two. Earlier literature shows that when the starting microbial mass is low, the proportion of contaminant bacterial DNA increases (Karstens et al., 2019). By qPCR, we could confirm the higher bacterial load in the cytobrush samples and vaginal swabs, compared to the biopsy samples. This concords with results from the human female genital tract (Chen et al., 2017). Hence, our results from the biopsies must be interpreted with caution. We reasoned that the taxa that distinguish the biopsies from the other sample types, are the ones appearing in the biopsies without detection in the negative extraction controls.

One interesting observation from our study, was that Fusobacteriota was not detected in any of the sample types. Fusobacteriota is associated with the development of metritis (Jeon et al., 2015; Bicalho et al., 2017b; Galvão et al., 2019) and purulent vaginal discharge (Bicalho et al., 2017a). In contradiction with our results, three earlier studies of the bovine uterus in Holstein all found high abundances of this taxa (Knudsen et al., 2016; Bicalho et al., 2017b; Wang et al., 2018). The reason for the differences in the abundance of Fusobacteriota might be partly due to that sampling in the mentioned studies was performed earlier after parturition. However, our finding might point to one possible explanation to why NR has a uniquely low occurrence of endometritis and metritis, and also beneficial uterine mechanisms with respect to SCE.

As mentioned in 8.5, we obtained only a small number of SCE positive cows in study III. No difference was detected between SCE positive and negative animals for either alpha or beta diversity, or taxonomic composition. The three biopsies from SCE positive animals formed a cluster

in the beta diversity PCoA visualization which indicates that there are similarities between them. However, this cluster did not seem to separate from the remaining biopsy samples. Two earlier studies that investigated the microbiota in SCE positive and healthy cows, at 30 and 10/21/35 days post-partum, respectively, also concluded on no differences between the groups (Wang et al., 2018; Pascottini et al., 2020). These two studies used uterine flush samples or cytobrush samples, but neither used biopsies.

In addition to a description of the uterine microbiota at AI in NR, our study underlines the importance of investigating the deeper layers of the endometrium that can be reached with a biopsy sample. This might reveal information that is interesting in investigations of disorders and diseases of the uterus, but also with respect to the environment that the embryo experiences. There is some evidence suggesting a transmission of pathogens from the gut to the uterus via the hematogenous route (Jeon et al., 2017). Possibly, deeper layers have a higher load of bacteria descended from this route while the superficial endometrium and vagina are more likely to be affected by extrinsic and ascending pathways.

9.2 Paternal impact

In paper IV, we investigated the paternal impact on embryo development by comparing the transcriptome in embryos from two different groups of bulls, selected based on their high or low fertility, respectively.

Worth noticing, there was a tendency of a higher blastocyst recovery rate from heifers inseminated with the HF bulls compared to the LF bulls. While 66.6% (20/30) of the recovered embryos derived from HF bulls (referred to as HF embryos) had developed to the blastocyst stage, the corresponding proportion for embryos derived from LF bulls (referred to as LF embryos) was 39.5% (17/43). Embryo cleavage and blastocyst rate *in vivo* has previously been reported to correlate with field fertility in the bull (Zhang et al., 1997; Ward et al., 2001; O'Callaghan et al., 2021). These results support the idea of an important paternal contribution to the early embryo developmental stages.

Embryos produced from the two groups of bulls differed in the expression of 62 genes. Such a low number of DE genes is common in transcription studies of embryos. Two previous studies of the paternal contribution to the embryo found 65 DE genes, both at the blastocyst stage (Kropp et al., 2017) and the 2-4 cell stage (Gross et al., 2019). Our collected understanding of this, is that gross changes in the embryo can be caused by a change in only a slight number of genes. This might be explained by the repeated use of the same biological pathways in different places in the embryo and at different timepoints, in addition to crosstalk between distinct pathways (Sanz-Ezquerro et al., 2017). Moreover, the LF embryos showed a higher metabolism than the HF embryos, detected by a higher number of genes enriched in the LF embryos. This concords with the earlier suggested *quiet embryo hypothesis* (Leese, 2002; Baumann et al., 2007). The differences in metabolism in general points to that our assumption about different developmental potential in the two groups is, at least to some extent, accurate.

The DE genes were investigated through pathway analyses and associated GO-terms. There was consensus between the different databases we used, and the human orthologues were related to almost identical pathways. In the LF embryos, the enriched genes were associated with 29 GO-terms. The highest association was found in processes related to metabolism of sterol, steroid, isoprenoids, and cholesterol, which includes the terpenoid backbone biosynthesis and steroid biosynthesis pathways. Both LF and HF embryos had enrichments associated with the insulin signaling pathway and the leukocyte transendothelial migration pathway.

The terpenoid backbone biosynthesis initiates the production of sterol isoprenoids, such as cholesterol, and nonsterol isoprenoids (Buhaescu and Izzedine, 2007; Miziorko, 2011), and is connected upstream to the steroid biosynthesis (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021). The genes enriched in the LF embryos associated with these pathways, including *HMGCR* and *HMGCS1*, underlie vast mechanisms of regulation. This includes an end-product feedback system that allows any absence of sterol isoprenoids to activate the transcription

of the *HMGCR* gene (Buhaescu and Izzedine, 2007; Niemann et al., 2007). HMGCR is a rate limiting enzyme and is of major importance for the entire downstream process (Goldstein and Brown, 1990). The transcription factors (sterol regulatory binding proteins) controlling the activation of the *HMGCR* gene have also been shown to increase mRNA expression for several enzymes along the entire pathway of cholesterol production (Sakakura et al., 2001). Hence, in our study, the high expression of enzymes associated to these pathways in the LF embryos, could be a result of a dysfunction, or exaggerated degradation or demands of its products. Cholesterol, being one of those products (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021), is essential for the developing embryo, as it forms part of the cell membrane, and acts in cell signaling crucial for developmental patterning, in collaboration with the hedgehog gene family (Porter et al., 1996; Roux et al., 2000). Furthermore, the essential role of *HMGCR* from the blastocyst stage on has been confirmed through a knockout-study (Ohashi et al., 2003). Interestingly, one earlier study that compared the transcriptome of blastocysts with a hypothetical difference in developmental potential (*in vitro* vs *in vivo* produced embryos) (Driver et al., 2012), also found enrichments in genes related to the biosynthesis of cholesterol. In their study, 5 genes were identical to our findings, including *HMGCR* and *HMGCS1*.

The insulin signaling pathway was enriched in the HF embryos by the genes *SOCS1* and *BAD*. *SOCS1* encodes an enzyme which inhibits the action of the insulin receptor (Mooney et al., 2001), which again suppresses the full insulin signaling pathway. It also has several roles in the negative feedback mechanism of cytokine signaling (Krebs and Hilton, 2001). Cytokines controlled by this pathway, such as interferon-γ, has several important roles in embryo development, but excess production is detrimental (Robertson et al., 2015). This implies that a well-functioning regulatory mechanism is beneficial for the embryo. *BAD* encodes an enzyme that is inhibited by the activation of the insulin signaling pathway, so a higher expression could be a consequence of the elevated activity in *SOCS1*. *BAD* is an antagonist of apoptosis. The high expression in the HF embryos in our study is interesting as a higher apoptotic cell ratio indicates a lower developmental competence in the embryo (Maddox-Hyttel et al., 2003).

Two of the most highly expressed genes in the LF embryos (*CLDN9* and *NCF1*) were represented in the leukocyte transendothelial migration pathway, which was also represented by one gene in the HF embryos (*CLDN10*). This pathway has been reported to have similarities to the human implantation process (Genbacev et al., 2003; Dominguez et al., 2005). *CLDN9* and *CLDN10* encodes claudins which forms parts of tight junctions, which again are crucial for morphogenesis. It has been hypothesized that the combined expression of claudin, or the "claudin signature", is critical for embryonic tissues (Gupta and Ryan, 2010). NCF1, also known as p47phox, acts in the production of reactive oxygen species (Babior, 2004). Redox activity controls programmed cell death (Pierce et al., 1991), and excessive oxidative stress is embryo toxic (Dennery, 2007). The higher expression of *NCF1* in the LF embryos in our study might point to a lower competence in the regulation of redox activity. This hypothesis is supported by the findings in one earlier study of the sire's contribution to embryo development (Kropp et al., 2017).

Some of the of the most highly expressed genes in the two groups were not represented in any pathways. These included *GIMAP4*, *HLX* and *POUF51* in the HF embryos and *BPI* in the LF embryos. *GIMAP4* is involved in cytokine secretion (Heinonen et al., 2015) and calcium signaling (Schnell et al., 2006). Calcium signaling have several functions in the pre- and peri implantation period (Armant, 2015). *HLX* is a regulator of cytokines and related to the development of the placenta (Rajaraman et al., 2010). *POUF51* encodes the transcription factor Oct4, which coordinates the viability of the inner cell mass (Eckert et al., 2015). For some of the highly expressed genes, such as *BPI* in the LF embryos, we could not find a function associated with current knowledge about the regulation of embryo development. As previously mentioned, the details of cell signaling and the related key pathways in embryo development in general are not fully understood (Eckert et al., 2015). Hence, these genes can be considered as new candidates in the regulation of embryo development.

In summary, the DE genes from LF and HF embryos found in our study were related to several pathways and processes known to be crucial for the developing embryo. We interpreted the meaning of the enriched pathways, GO-terms and individual transcripts in relation to embryo development. Our collected understanding of the results implies that the mechanism for the low fertility in the LF bulls might be explained by an unfortunate impact on (1) the metabolism of cholesterol and its precursors, (2) cytokine signaling, (3) regulation of apoptosis, (4) adhesion and attachment in placental development, (5) formation of tight junctions, and (6) regulation of redox activity. This further suggests that the sire's genetic contribution affects important processes, linked to pre-and peri implantation regulation in the developing embryo.

Our study IV is the first to compare the complete gene expression of *in vivo* produced embryos from sires with high and low field fertility, measured as high or low NRR, respectively. Our results only have few evident similarities with the outcome in a similar study that investigated bull field fertility and embryo transcriptomic profiles in *in vitro* produced blastocysts (Kropp et al., 2017). This underlines the importance of studying *in vivo* produced embryos even though it is a challenging approach.

9.3 Final remarks on embryo loss, and future perspectives

The overall aim for this thesis was to gain knowledge about factors affecting embryo loss in Norwegian Red. This thesis and the papers included contribute to this by being some of the equally important pieces of puzzle that we need to increase the understanding of embryo development and embryo loss.

The link between SCE and embryo loss is still not well understood. Possibilities to diagnose pregnancy within 21 days of gestation would be very helpful in order to set up studies that directly measure the effect of these conditions on the early embryo. With results from this thesis, we now know that SCE is common in NR. As CYTO positive animals had a higher risk of becoming non-pregnant, and the condition does not seem to affect late embryo loss or fetal loss, then it should have its effect on either fertilization rate or early embryo loss, or a combination of both. Presence of PMN in the uterus has been shown to lower fertilization rates (Carvalho et al., 2013). Equally, altered embryo quality was also reported in cows positive for SCE (Hill and Gilbert, 2008). Future investigations of the relative importance of fertilization failure vs embryo loss as a result of SCE are needed.

It is possible that there is a paternal aspect to SCE. Emerging evidence suggests that the father's well-being affects the reproductive tract environment in the female through both spermatozoa and seminal plasma. The well-being in this context was described by nutrition, age, and endocrine disrupting chemicals (Watkins et al., 2020). It has also been suggested that the transcriptome of the uterine endometrium can be regulated by semen, with the biological function of modulating vascular and immune related responses to the embryo which affects fertility (Watkins et al., 2020; Fernandez-Fuertes et al., 2022). One recent study demonstrated that *in vitro* produced conceptuses from HF bulls affected the endometrium in cows by stimulating pathways involved in regulating the immune response, compared to conceptuses from LF bulls (O'Callaghan et al., 2021). Further studies on this aspect of paternal contribution to embryo loss are indeed appropriate in the coming years.

SCE is related to NEB in the cow, which is also reported to alter the endometrial gene expression (Wathes et al., 2009). It would be highly interesting to investigate parameters that measure levels of negative energy balance in NR by a large-scale data collection, perhaps with the use of milk robot data (Churakov et al., 2021). Further, high pathogen tolerance or other immunological mechanisms could provide explanations of the relatively favorable outcome of CYTO in NR. Animals with SCE have an increased expression of genes encoding inflammatory mediators (Fischer et al., 2010). One previous study induced an aseptic inflammation in the endometrium of cows and used the cell-free uterine lavage to culture embryos *in vitro*. They reported an impaired development compared to the control group, which was cultured in a non-inflammation uterine fluid. By this, they hypothesized that the soluble mediators of inflammation exerts the harmful effect on the embryo,

rather than the infiltrating cells (Hill and Gilbert, 2008). Here, a study of the endometrial gene expression in SCE positive and negative NR cows, respectively, could also be a relevant continuation of the studies included in this thesis, even though such investigations has been performed in other breeds (Fischer et al., 2010; Ghasemi et al., 2012; Raliou et al., 2019). In such study, it would be interesting to include an investigation of the embryo gene expression to elucidate how SCE related changes in the endometrium affects the outcome in the embryo.

In paper II we provided a heritability estimate for SCE, and our finding suggested a heritable component in SCE. Our dataset was small in the context of heritability estimates, and the standard errors were relatively high. The currently most validated method to diagnose SCE is performed through a highly time-consuming cell count routine. In order to establish larger datasets on SCE, a more feasible diagnostic method is needed. Further investigations on the genetics behind the heritable trait would be interesting and could result in practically applicable knowledge.

Increased knowledge about the uterine microbiota and its effects on the endometrium, and indirectly or directly on the embryo, will supply important information about the embryo's living conditions that could affect embryo survival. Our study III provided new information about the microbiota of superficial and deep levels of the endometrium in NR, which had detectable differences. To further localize bacteria encountered in biopsies, it would be interesting to perform fluorescence in situ hybridization on chosen bacteria. This information could expand our knowledge about their role in persistent inflammation. The microbiota from endometrial biopsies might still have a correlation with SCE even if it was not detectable on our low number of SCE positive individuals. This matter requires further investigation.

Based on the following results and conclusions from study I, II and III: (1) a beneficious uterine immunology in NR, (2) a probable heritability detected (which might be different in different breeds), and (3) a non-detectable presence of Fusobacteriota, it might be that the different causes for SCE have a different weight in different breeds. Hence, breeds with large differences in the matter of metabolic stress or uterine microbiota, might need different approaches to reduce SCE or its effects on fertility.

Genomic technology such as NGS will provide an improved understanding of the underlying biological processes involved in embryo development and could identify genes responsible for improved embryo survival (Diskin et al., 2016). With the use of such technology, our study IV described transcriptomic differences in the embryo based on paternal fertility. The results points to the father's contribution to embryo development and underlines the importance of further studies that focuses on the male. It is necessary to stress this matter, as even recent articles tend to leave out the mentioning of paternal factors when describing embryo development and death. With our study, we added important information to the current understanding of the paternal influence in the developing embryo. Although the field of male fertility has received clear attention and progress during the last decade, further research is needed to clarify this complex matter.

It is not certain which mechanism or component in the spermatozoa that affected the embryos in our study IV. The differences in LF and HF embryo gene expression might be caused by either bull DNA, or regulation by transcription factors, proteins, or epigenetic factors deposited in the oocyte at fertilization. Epigenetics is an upcoming field in both maternal and paternal contribution to the embryo. Methylation marks, histones modifications, small RNAs, and chromatin state variations carried by spermatozoa may remain active in the early zygote and influence the embryonic period (Wu and Sirard, 2020). It has been reported that DNA-methylation patterns in semen differ between high and low fertility bulls (Kropp et al., 2017). Therefore, epigenetic investigations of the spermatozoa from the bulls used in our study, where the embryo outcome is available, would be highly interesting.

10 Conclusion

The appropriate cut-off level for PMN in the NR population was 3%, based on the highest summation of specificity and sensitivity from a ROC curve. When CYTO was defined by this cut-off, the prevalence was 28% at AI for NR, which is higher than previously reported at AI in other breeds.

The overall pregnancy incidence to first AI in NR was high and we reported a negative association with CYTO. However, the biological effect of this condition appears to be modest in comparison with results from other breeds. It is plausible that the inclusion of fertility in NR breeding programs for almost 50 years has resulted in improved production traits, with genetically advantageous uterine immunology possibly being one of the mechanisms behind this observation.

The detected risk factors for CYTO were AI personnel, CFAI, vaginal mucus condition, RBC in sample, barn type, and season. Late embryo loss and fetal loss was not associated with CYTO at first AI, suggesting that decreased fertility in SCE positive cows is a result of either early embryo loss or fertilization failure.

SCE detected at AI was indicated to be heritable in NR by a trait definition of 5% PMN. The heritability estimate was comparable to other diseases and disorders included in the total merit index for NR. Hence, SCE could be considered as a new phenotype with the potential to be used for breeding purposes. However, to implement this, a more feasible method to diagnose SCE needs to be developed.

We provided a description of the uterine microbiota at AI in NR, by superficial layer (cytobrush) and deep layer (biopsy) samples of the endometrium. The microbiota in the biopsies was qualitatively different and more even than that of cytobrush and vaginal swab samples. Hence, the microbiota from the deeper layers of the uterus might have a correlation with SCE even if the superficial bacterial population does not show such a correlation. The cytobrush samples and the vaginal swabs shared a similar taxonomic composition, suggesting that vaginal swabs may suffice to describe the surface-layer uterine microbiota at estrus. We also presented a description of the microbiota in the healthy and SCE positive NR cows at AI.

Embryos derived from bulls of high or low field fertility had a differing gene expression in genes linked to several processes or pathways that are known to be crucial for embryo development at the blastocyst stage and around attachment. This included cholesterol metabolism, cytokine signaling, regulation of apoptosis, adhesion and attachment in placental development, formation of tight junctions, and regulation of redox activity.

The new knowledge about CYTO and SCE in NR contributes to the understanding of fertility and embryo loss within the breed. The provided insights concerning the microbiota of the uterus represents an important first step in the investigation of its role in persistent inflammation and the early embryo's living conditions. On the paternal side, our results demonstrate the mechanisms by which the bull can affect embryo survival. This underlines the importance of expanding the knowledge of the paternal contribution to embryo development. The collected results presented in this thesis are valuable as we continue to explore the underlying mechanisms of fertility in dairy cattle. For NR, the results are applicable for breeding and management recommendations, as well as in herd health investigations. This contributes to maintaining the current status of NR as a high fertility breed, and possible future improvements.

11 References

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Paper I

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Prevalence, risk factors, and effects on fertility of cytological endometritis at the time of insemination in Norwegian Red cows

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ABSTRACT

The present study aimed to assess the occurrence of cytological endometritis (CYTO), a nonsymptomatic inflammation of the endometrium, at first artificial insemination (AI) postpartum in Norwegian Red cows. Further, risk factors for CYTO manifestation and its effect on reproductive success and late embryo loss were evaluated. In total 1,648 cows located in 116 herds were included in the study. On mainly spontaneous estrus, endometrial cytology samples were collected using a cytotape technique, and a total of 300 representative epithelial cells and polymorphonuclear neutrophils (PMN) were counted at 400× magnification. Vaginal mucus obtained by Metricheck (Simcro) and body condition score were recorded. Milk samples for progesterone analysis were collected at AI and 21 d later. Pregnancy was diagnosed by rectal palpation or analysis of pregnancy-associated glycoproteins. Based on the constructions of a receiver operator characteristics curve, the cut-off level for PMN defined as CYTO was set to 3.0%, representing the level at which the PMN occurrence affected pregnancy outcome, with the highest summation of sensitivity (32.4%) and specificity (74.9%). Three logistic models with herd included as random factor were constructed. The outcome for the first model was the likelihood for CYTO based on the endometrial samples, in the second model pregnancy to first AI, and in the third model embryo loss. The proportion of CYTO was 28.0% (461/1,648). The average interval in days to first AI was 71.7 d (standard error ± 0.7) and the overall pregnancy incidence to first AI was 59.8% (866/1,449). The likelihood for CYTO at first AI was associated with AI personnel, calving to first AI interval, vaginal mucus characteristics, amount of red blood cells in sample, season, and barn type. Pregnancy to first AI was lower in CYTO-positive cows (odds ratio = 1.51, confidence interval = 1.17–1.94). Other factors affecting pregnancy to first AI were AI personnel, test day milk yield, barn type, and obstetrical conditions or fertility treatments before first AI. The proportion of late embryo loss and abortion was 8.6% (82/948) and 2.8% (24/866), respectively. Late embryo loss was associated with treatment against fertility disorders before first AI, but not associated with CYTO. Overall, our results suggest that even if Norwegian Red cows show a fairly high prevalence of CYTO in the endometrium at first AI, it does not seem to have a major effect on the reproductive performance. The Norwegian Red breeding program has emphasized fertility and health for decades, and a genetically advantageous uterine immunology might be one of the preserved mechanisms.
Key words: Norwegian Red, embryo loss, cytotape, cytological endometritis, polymorphonuclear neutrophils

INTRODUCTION

Embryo loss, defined as loss of conceptus during the first 42 d of pregnancy (Zavy and Geisert, 1994), is one of the major contributing factors affecting reproductive efficiency in dairy cows (Diskin and Morris, 2008). Embryo survival is negatively affected by suboptimal uterine environment (Hill and Gilbert, 2008). Hence, postpartum uterine disease has a great impact on reproductive performance in the dairy industry, causing large economic losses (Sheldon and Dobson, 2004). As a result, recent years has witnessed an increase in studies investigating the prevalence and effect of subclinical endometritis in dairy cows (Kasimanickam et al., 2004; Madoz et al., 2013; Wagener et al., 2017).

Subclinical endometritis is defined by 3 aspects: (1) the presence of PMN on the endometrial surface or lu-

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men, (2) a reduction in reproductive performance, and (3) the absence of clinical symptoms of endometritis (Kasimanickam et al., 2004; Sheldon and Owens, 2017). The diagnosis is usually based on cytology samples collected from the endometrial surface or lumen, where cytobrush or uterine lavage are the most common collection techniques (Pascottini et al., 2016; Sheldon and Owens, 2017; Wagener et al., 2017). Cytological endometritis (**CYTO**) refers to these cytological findings alone (Dubuc et al., 2010a; Pascottini et al., 2016).

It is challenging to compare studies on the prevalence of subclinical endometritis or CYTO because they are based on dissimilar sample time points relative to parturition, as well as different cut-off levels to define the condition (Pascottini et al., 2015; Wagener et al., 2017; Arias et al., 2018). While most published studies on CYTO or subclinical endometritis are based on sampling 3 to 5 wk or 5 to 7 wk after calving (Arias et al., 2018), Pascottini et al. (2016, 2017a) performed sampling at the time of AI, with the argument that this is the most critical point for uterine health status. Further, sampling at the time of AI allows for greater standardization regarding what cut-off value of PMN to use for the diagnosis of CYTO, as well as a more standardized collection procedure (Pascottini et al., 2017a). To prevent unnecessary penetration of the cervix, cytology samples were collected with a piece of tape (cytotape) attached to the sanitary sheath covering the insemination gun (Pascottini et al., 2015, 2017a,b). A consecutive study showed that this collection method did not affect the pregnancy rate (Pascottini et al., 2017a), although there was a risk of the insemination procedure itself introducing a PMN response in the endometrium (Pascottini et al., 2017b). Earlier identified factors for the increased risk of CYTO at first AI in multiparous cows include parity, DIM, and season of the year (Pascottini et al., 2017b).

It has been reported in several studies that CYTO has a negative effect on pregnancy outcome in dairy cattle (Kasimanickam et al., 2004; Gilbert et al., 2005; Dubuc et al., 2010a; Valdmann et al., 2018). Among the affected parameters were pregnancy rate (Kasimanickam et al., 2004; Gilbert et al., 2005; Dubuc et al., 2010a), days open (Kasimanickam et al., 2004; Gilbert et al., 2005; Valdmann et al., 2018), postpartum anestrus, and AI per conception (Gilbert et al., 2005). Similar negative effects on pregnancy outcome have also been identified when CYTO was diagnosed at AI (Pascottini et al., 2017a). In contrast, a small number of studies have also reported no significant effect of CYTO on reproductive performance in dairy cattle reared under less intensive systems, including Holstein cattle reared in a pasture-based, extensive dairy farming system in Argentina (Plöntzke et al., 2010), and in small- and medium-sized herds of mainly Simmental in Austria (Prunner et al., 2014).

Norwegian Red has been the main breed of dairy cattle in Norway since 1935, and since the 1970s breeding programs have emphasized fertility and health, such that female fertility has been included in the total merit index in Norway since 1972 (Andersen-Ranberg et al., 2005). The population average interval from calving to first AI (**CFAI**) is 81.8 d, and treatments against reproductive disorders are currently low, with treatments against metritis and endometritis equal to 1.3/100 cow-years in 2018 (TINE Rådgiving, 2019). A pregnancy incidence of 62.9% and a calving rate of 56.3% has been reported in Norwegian Red (Garmo et al., 2008), and the 56 d nonreturn rate was 72.4% in 2018 (Geno SA, 2016). The prevalence and effect of CYTO have not been investigated in this high fertility breed. Such knowledge would be valuable to understand the basis of the successful reproductive capacities of this breed, and if the success is due to, or rather in spite of a low occurrence of this condition. Our hypothesis was that because of the high fertility in Norwegian Red, CYTO is not very prevalent, as neither metritis nor endometritis are highly reported issues in this breed.

The present study aimed to assess the occurrence of CYTO, as defined by presence of PMN in the endometrium at first AI postpartum in Norwegian Red cows and to establish associated risk factors. Additional aims were (1) to investigate how pregnancy to first AI is affected by CYTO and by other already known risk factors and (2) to investigate the risk for late embryo loss and abortion with regard to PMN status at first AI.

MATERIALS AND METHODS

A prospective cohort field trial was conducted between September 2017 and March 2019. The study unit was Norwegian Red cows presented for first AI after parturition (n = 1,648). The sample size was based on a difference in pregnancy to first AI of 10% between CYTO-positive and -negative animals, and the desired power and confidence interval (90% and 95%, respectively), and the true prevalence was expected to be 10%. In total 116 herds in the counties of Hedmark (n = 52) and Trøndelag (n = 64), with a range from 1 to 123 cows, were included in the study. The herds were selected based on the farmers' willingness to participate in the study. The animals were housed in 35 tiestall herds (17.5%) and 87 freestall herds (82.5%) of which 66 (69.1%) had an automated milking system and 21 a traditional milking parlor system (13.4%). The ethical approval for the present study was given by the Nor-

wegian Food Safety Authority with approval number 17/152686–1. A cytological sample was collected from the endometrium in connection with first AI in 1,738 cows. Of these, 90 samples were excluded due to poor quality, resulting in the study being based on 1,648 endometrial samples. The cows were selected for AI mainly on spontaneous estrus, detected by mucus, estrus behavior, electronic activity monitors, or a combination of these. There was no voluntary waiting period, but a general recommendation from the Norwegian Red breeding organization (Geno SA) of at least 42 d from CFAI.

Data from the Norwegian Dairy Herd Recording System

All herds used in the study were part of the Norwegian Dairy Herd Recording System (**NDHRS**), which routinely records data on parity; parturition and AI date; pregnancy controls; culling data; disease occurrence; calf sex; dystocia; milk yield (**MY**); concentrate allocation (**CA**); milk fat, protein, and lactose content; natural logarithm of somatic cell count (**LnSCC**); and urea. All feed and milk data were measured and analyzed monthly and reported to NDHRS.

Disease Treatment and Culling

Registrations of disease treatments were retrieved from NDHRS for the period from 30 d before parturition until first AI in subsequent lactation. All diagnoses and treatments registered in NDHRS are performed and reported by veterinarians according to predefined health codes (Animalia, 2020). Calving-related conditions and diseases were diagnosed in 241 of the 1,648 cows included in the study. There were 48 cases of obstetrical conditions: dystocia, 9; prolonged pregnancy, 3; uterine prolapse, 1; uterus torsion, 1; retained placenta, 21; early metritis, 12; and others, 1. In total, 52 cases of fertility disorders were reported before first AI: anestrus, 16; ovarian cysts, 8; silent heat, 22; endometritis, 2; and estrus synchronization, 4. Other conditions with treatment registered were mastitis, 59; ketosis, indigestion, or both, 32; lameness, bone, and claw disorders, 9; and others, 41.

In total, 327 of the included cows were culled during the study period: 68 pregnant, 156 nonpregnant, and 103 with uncertain pregnancy status. Thirteen of these cows were culled 6 to 29 d after first AI and were not included in statistical analysis with pregnancy as outcome. The reasons for culling were abortion, 21; low MY, 49; mastitis or high SCC or poor udder confirmation, 75; poor fertility, 86; bone and confirmation, 20; and others, 76.

AI Personnel

Eleven AI technicians and 3 veterinarians employed at GENO SA performed the sampling from the endometrium at first AI. The AI personnel were selected based on interest, experience, and competence, and before sampling, they participated in a one-day practical course. The course included a tutorial of body condition scoring and evaluations of the quality of their endometrial cytology samples. Five persons, both AI technicians and veterinarians, who in total performed only 7.5% (n = 124) of the AI and collected ≤46 endometrial cytology samples each, were counted as one group in the statistical analyses, due to small numbers of AI ranging from 7 to 46. The distribution of AI and endometrial samples between the other AI personnel was 22.5% (n = 370), 19.7% (n = 324), 15.5% (n = 255), 8.8% (n = 145), 6.9% (n = 114), 6.9% (n = 113), 4.9% (n = 80), 4.5% (n = 74), and 3.0% (n = 49).

BCS, Mucus Evaluation, and Endometrial Sampling on the Farm

Body condition was scored by the AI personnel at first AI on a scale from 1 (very thin) to 5 (obese), in increments of 0.25 (Edmonson et al., 1989), modified and adjusted for Norwegian Red according to Gillund et al. (1999).

A Metricheck device (Metricheck, Simcro) was used to investigate and score the appearance of the vaginal mucus. Samples of mucus were collected and evaluated from 1,442 of the cows. The instrument was advanced to the cervix to collect mucus and withdrawn. The AI personnel assessed the content in comparison to pictures of vaginal mucus characteristics and gave a score from 0 to 3: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤50% white or off-white mucopurulent material; score 3 = discharge containing ≥50% purulent material, usually white or sanguineous (Williams et al., 2005).

A cytotape device was mounted and used to collect cytology samples from the endometrium, according to the technique developed by Pascottini et al. (2015). Briefly, paper tape was glued to the sanitary sheaths (IMV technologies Split Universal sheaths 007494) covering the insemination gun. A protective disposal plastic tube (Continental plastic, Sheath protector tubes 262–728–4800) was mounted over the sampling device. Following the cleaning of the vulva and perineum, the protective tube was passed into the uterus. The endometrial samples were collected by penetrating the protective tube, rolling the tape against the endometrium, releasing the semen, withdrawing the tape into the

protective tube, and rolling the tape onto a microscope glass slide.

Microscopic Evaluations and PMN Assessment

The microscopic evaluation of the slides was performed by 3 veterinarians. A subset of 40 cytology slides were randomly chosen from the first 475 samples to calculate the interobserver reliability. Randomization was made using Sergeant, ESG, 2018, Epitools Epidemiological Calculators, Ausvet (available at http://epitools.ausvet.com.au). Calculations were made using Stata (Stata SE/15.1, Stata Corp.). Kendall's W was 0.72, which was assessed as high (Martin and Bateson, 2007). Calibration was performed by using example pictures and support from of a professional cytologist. The evaluation distribution between the 3 observers was 55.2% (n = 909), 31.4% (n = 518), and 13.4% (n = 221).

The slides were fixed and stained with Kruuse Dip Quick Stain (Jorvet, J0322A1, A2, A3, Jorgensen Laboratories) and examined under the 100× magnification in a light microscope. The amount of red blood cells (**RBC**) was scored from 0 to 3: 0 = none, 1 = low, 2 = moderate, 3 = high, as previously described by Pascottini et al. (2015). The slides were assigned a quality score based on proportion of nonfragmented cells; <50%, 50–75%, >75% (Pascottini et al., 2015). At 400× magnification, a total of 300 representative epithelial cells and PMN were counted in several fields and the proportion of PMN calculated.

Estrus Confirmation and Pregnancy Detection

Milk samples were collected by hand for progesterone analysis at the time of AI and 21 d post-AI. A Broad Spectrum Microtab tablet was added (D&F Control Systems Inc.), and the samples were stored frozen at −18°C before laboratory analysis. Milk progesterone was measured by enzyme immunoassay (Waldmann, 1993), which was modified by a second antibody coating technique. The specificity of the monoclonal antibody was described previously (Waldmann, 1999). For this method, the interassay coefficient of variation was 9.2 and 5.3%, at milk progesterone concentrations of 1.48 and 19.66 ng/mL, respectively, whereas the intraassay coefficient of variation was less than 10%. The threshold for progesterone content in estrus was ≤3.0 ng/mL, while >3.0 ng/mL indicated AI during diestrus. A progesterone content of ≤3.0 ng/mL 21 d after first AI was evaluated as return to estrus. Pregnancy to first AI was confirmed by rectal palpation by the AI personnel or by milk samples analyzed for pregnancy-associated glycoproteins (**PAG**) at 42 d after first AI. The PAG concentration was analyzed using an Idexx Milk Pregnancy Test (99–41209, Idexx Laboratories). A corrected optical density value of >0.250 was used as a cut-off to confirm pregnancy, whereas <0.1 indicated nonpregnancy. Results between 0.10 and 0.25 were recorded as re-check or uncertain. Cows presented for a subsequent AI within 18 to 42 d, and a new calf born later than 290 d after first AI were assessed not to be pregnant after first AI.

Late Embryonic Loss

Late embryonic loss was defined as having happened in cows with progesterone levels >3.0 ng/mL 21 d after the first AI, who were not pregnant on rectal palpation or PAG analysis ≤42 d after the first AI, or cows that were presented for a new AI ≥ 42 d after first AI, and with no recording on pregnancy status. Cows with >3 ng/mL at AI were excluded from the material in the late embryonic loss model.

Missing Observations

Due to some missing observations, there are variations in cow numbers for milk samples, data retrieved in the field, and from NDHRS. There were missing milk samples for progesterone analysis both at first AI (n = 160) and at d 21 (n = 201), but 1,287 cows were registered with progesterone data both at first AI and 21 d later.

Statistical Analysis

All statistical analyses were performed in Stata (Stata SE/15.1, Stata Corp.). Pearson's chi-squared test was used to test the univariate relationship between the different outcome variables and explanatory variables. Three logistic models were built: model 1 with CYTO based on endometrial samples, with no = 0, yes = 1, as the outcome. Model 2 with pregnancy ≥42 d after first AI, with yes = 0, no = 1, as the outcome. Model 3 with embryo loss, with yes = 0 or no = 1, as the outcome. To select the best cut-off point for the definition of CYTO in the logistic model with pregnancy as outcome, we constructed receiver operating characteristic (**ROC**) curves (Dohoo et al., 2009). The highest summation of sensitivity on 32.4% and corresponding specificity value on 74.9% was detected at the 3.0% level, Figure 1. The positive and negative predictive values was 46.6% and 62.2%, respectively, and the area under the curve was

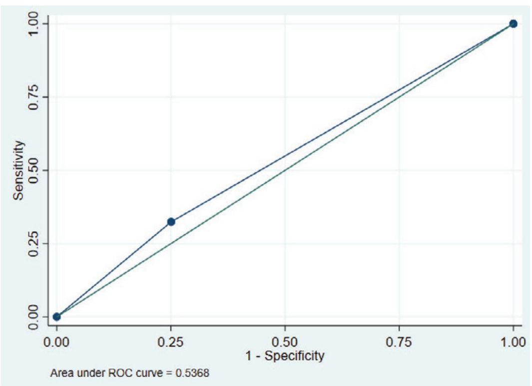

Figure 1. Receiver operating characteristics (ROC) curve with sensitivity of 32.4%, specificity of 74.9%, and area under the curve equal to 0.54 for cytological endometritis using cut-off at 3.0% polymorphonuclear cells in endometrial samples collected at first AI postpartum in Norwegian Red, n = 1,449.

0.54. In the logistic models with CYTO (model 1) and embryo loss (model 3) as outcomes, CYTO equal to 3.0% PMN was also used as the cut-off level.

Associations between the outcomes in the 3 models and the explanatory variables were tested separately using the random effects logistic model, *xtlogit*, in Stata SE/15.1 with herd included as the random effect in the models. The following explanatory variables were tested univariately: parity (first, >1), CFAI, test day MY, test day CA, urea, and the LnSCC closest to first AI, herd size, barn type (automated milking system, milk parlor, tied), season (December–February, March–May, June–August, September–November), BCS at AI (≤2.75 thin, 3.0–3.75 optimal, ≥4.0 fat), vaginal mucus (clear = score 0 and opaque = score 1 or 2 combined), RBC occurrence in endometrial samples (0 = none, 1 = low, 2 = moderate, 3 = high), calf size, as reported to NDHRS by the farmer based on experience (small, medium, large, twins), dystocia (none, some, large), obstetrical condition or fertility treatment before first AI, and finally milk fat, protein, and lactose % at test day closest to first AI. In addition, the occurrence of PMN as a continuous variable or CYTO defined by a 3.0% level (no = 0, yes = 1) was added as an explanatory variable in model 2 and model 3.

In all 3 models, explanatory variables with $P \leq 0.20$ when assessed separately were included in an extended multivariable model with herd included as random effects in the respective models. Co-linearity was assessed between explanatory variables by calculation of the Pearson correlation coefficient (Dohoo et al., 2009). Except for a correlation between CA and MY of 0.7, no co-linearity between explanatory variables was detected, and MY was selected to be included in a multivariable model due to biological relevance. The backward elimination procedure was applied for explanatory variables with $P > 0.05$ in the multivariable model, eliminating the variable with the highest P-value, re-running the model for each elimination of an explanatory variable. None of the interaction terms tested between the explanatory variables in the final models were associated with (1) CYTO in endometrial sample at first AI, (2) pregnancy to first AI, or (3) the occurrence of ED, and hence not included in either of the final models. The herd level residuals in the logistics models were reasonably normal. Only explanatory variables with $P \leq 0.05$ were retained in the final models. Number of cows with endometrial samples, BCS, milk yield, concentrate allocation, mucus evaluation, milk content samples, progesterone samples, and pregnancy diagnosis are presented in Appendix Table A1.

RESULTS

Descriptive Statistics: Herd Level

In the present study, the average herd size was 39 cows with a range from 13 to 110 cows. The average 305-d ECM production was 8,402.8 kg ± 83.3, whereas the average milk fat and protein content was 4.31% ± 0.03 and 3.45% ± 0.01, respectively. There were 736 first and 912 >1 parity cows included in the study.

Descriptive Statistics: Cow Level

The overall pregnancy incidence to first AI was 59.8% (866/1,449), and the corresponding values were 62.5% (412/659) and 57.5% (454/790) for first and >1 parity, respectively.

The overall average CFAI was 71.7 d ± 0.7, and the corresponding values were 70.1 d ± 1.0 and 72.9 d ± 1.0 for first and >1 parity, respectively. The proportion of double AI as a new AI recorded 1 to 4 d after first AI was 3.4% (56/1,648).

Average MY closest to test day was 31.6 kg ± 0.2 (n = 1,602), whereas the average CA was 11.2 kg ± 0.1 (n = 1,600). Average milk fat-, protein-, and lactose content at test day closest to the first AI was 4.08% ± 0.02, 3.27% ± 0.01, 4.71% ± 0.01, respectively, whereas the average test day LnSCC and urea was 3.89 ± 0.03 and 4.67 mmol ± 0.03, respectively (n = 1,586).

The distribution of AI (n = 1,648) by season was 32.5% (n = 535) from December to February, 28.0% (n = 461) from March to May, 22.9% (n = 377) from June to August, and 16.7% (n = 275) from September to November.

The distribution of calf size (n = 1,600) was 13.9% small (n = 223), 69.9% medium (n = 1,118), and 16.2% large (n = 259). Twins were recorded in 1.7% (28/1639) of the births, whereas dystocia was reported by the farmer in 148 cows (44 major and 104 minor).

The distribution of BCS (n = 1,637) was divided into 3 groups: 13.2% (n = 216) with a BCS ≤2.75, 75.7% (n = 1,240) with a BCS between 3.0 and 3.75, and 11.1% (n = 181) with a BCS ≥ 4.0. Fourteen cows were recorded with a BCS between 2.0 and 2.25. Vaginal mucus was assessed in 1,442 cows. In 93.1% (n = 1,343) of the animals, the mucus was given score of 0, whereas 6.2% (n = 89) had score of 1, and 0.7% (n = 10) had a score of 2. No sample was characterized with score 3. There was no difference in pregnancy to first AI with 60.4% (717/1,188) and 62.3% (48/77) for cows with clear and opaque mucus, respectively. For the 10 cows with opaque or mucopurulent mucus, 2 were pregnant, 4 nonpregnant, 1 had uncertain pregnancy status, and 3 cows were inseminated during the luteal phase.

CYTO and Descriptive Associations

The distribution of PMN at first AI (n = 1,648) is presented in Table 1. The CYTO was present in 28.0% (461/1,648) of the endometrial samples. Successful pregnancy, following the first AI, was recorded in 53.4% (217/406) and 62.2% (649/1,043) of cows positive and negative for CYTO, respectively, $P < 0.01$. The proportion of CYTO-positive samples was 26.7% (357/1,343) and 45.5% (45/99) for cows with clear and opaque mucus, respectively, $P < 0.01$.

The distribution of RBC in the endometrial samples (n = 1,648) was 57.8% (n = 953) without, 22.6% (n = 372) with low, 10.5% (n = 173) with moderate, and 9.1% (n = 150) with a high amount of RBC. Pregnancy following first AI was 59.8% (500/836) and 59.4% (364/613) for endometrial samples classified with or without RBC, respectively. Red blood cells were detected in 52.0% (239/460) and 38.4% (456/1,188) of the CYTO-positive and CYTO-negative samples, respectively, $P < 0.01$.

Insemination During the Luteal Phase

Progesterone concentration at AI was ≤3.0 ng/mL in 96.1% (1,430/1,488) of the cows. The AI in diestrus was detected by a progesterone concentration of >3.0 ng/mL in 3.9% (n = 58) of the cows, and these were excluded from the model with pregnancy as outcome. Among these cows, 32.8% (19/58) were CYTO positive. Fifteen cows with progesterone concentrations between 0.1 and 1.0 ng/mL at first AI were presented for a new AI 21 d later with progesterone concentrations between 7.8 and 27.9 ng/mL. In 40.0% (6/15) of these cows, CYTO was observed in the endometrial sample at AI. Thirteen of these cows were presented for a new AI later in lactation or registered as nonpregnant by pregnancy control.

Embryo and Fetal Loss

The proportion of late embryo loss was 8.6% (82/948) and was restricted to 49 herds, with 1 to 7 embryo losses in each herd.

Fetal loss was recorded in 24 cows that was pregnant 42 d after first AI and presented for a new AI later, such that abortion percentage was 2.8% (24/866). The CYTO was observed in 29.3% (24/82) and 12.5% (3/24) of the endometrial samples in cows with late embryo loss or fetal loss, respectively, and there was no difference in the chi-squared test, $P = 0.25$.

Model 1: Associations Between the Likelihood for CYTO in Endometrial Samples at First AI and Explanatory Variables

The univariable analyses on relationships between the CYTO in the endometrial samples at first AI and explanatory variables are presented in Table 2. The following variables were associated with CYTO: AI personnel, CFAI, BCS, vaginal mucus condition, RBC in sample, barn type, and season.

In the multivariable model presented in Table 3, the likelihood of CYTO in the endometrium at first AI was associated with following predictors: AI personnel, CFAI, vaginal mucus condition, RBC in sample, season, and barn type. The likelihood of CYTO decreased from an odds ratio of 0.99 to 0.86 by increasing the interval for first AI by 21 d, also meaning that the likelihood

Table 1. The occurrence of PMN counting 300 cells in endometrial samples from Norwegian Red cows at first AI (n = 1,648) and pregnancy to first AI (n = 1,449)

PMN %	n	%	Cumulative %	Pregnancy % (no./total)
No PMN	686	41.6	41.6	62.5 (383/613)
≤0.9	259	15.7	57.3	57.7 (128/222)
1.0–2.9	242	14.7	72.0	66.3 (138/208)
3.0–4.9	116	7.0	79.1	57.3 (59/103)
5.0–9.9	130	7.9	87.0	56.1 (64/114)
10.0–19.9	96	5.8	92.8	53.0 (44/83)
20.0–49.9	86	5.2	98.0	47.4 (36/76)
≥50.0	33	2.0	100.0	46.4 (14/30)
Total	1,648			1,449

Table 2. Univariable analyses describing associations between the occurrence of cytological endometritis (0 = no, baseline, 1 = yes) and explanatory variables in 1,648 Norwegian Red cows from 116 herds (herd is included as a random effect)

Variable	Level (n)	β	Odds ratio	SE	95% CI	*P*-value	N
Parity[1]	>1 parity (912)	—	1.0	—	—	0.76	1,648
	1 parity (736)	0.04	1.04	0.12	0.83–1.29		
AI personnel[1]	Several levels[2]	—	—	—	—	<0.01	1,648
CFAI interval[3]	—	−0.01	0.99	<0.01	0.99–1.00	<0.01	1,648
BCS[4] class[1]	≤2.75 (216)	—	1.0	—	—	0.04[5]	1,637
	3.0–3.75 (1,240)	−0.09	0.91	0.16	0.65–1.28	0.59	
	≥4.0 (181)	−0.58	0.56	0.14	0.34–0.92	0.02	
Vaginal mucus[1]	Clear (1,343)	—	1.0	—	—	<0.01	1,442
	Unclear (99)	0.84	2.32	0.51	1.51–3.56		
RBC[6] in sample[1]	No (953)	—	1.0	—	—	<0.01[5]	1,648
	Low (372)	0.40	1.49	0.21	1.14–1.96	<0.01	
	Moderate (173)	0.68	1.96	0.36	1.38–2.80	<0.01	
	High (150)	0.75	2.11	0.40	1.46–3.06	<0.01	
Calf size[1]	Small (223)	—	1.0	—		0.72	1,623
	Medium (1,118)	0.08	1.09	0.19	0.78–1.52	0.63	
	Large (259)	−0.06	0.94	0.20	0.62–1.43	0.77	
	Twins (23)	0.35	1.42	0.67	0.56–3.59	0.46	
Dystocia[1]	No (1,481)	—	1.0	—	—	0.85[5]	1,629
	Some (104)	−0.07	0.93	0.22	0.59–1.49	0.77	
	Large (44)	0.16	1.17	0.40	0.60–2.27	0.64	
Milk yield test day AI	—	<−0.01	1.00	0.01	0.98–1.01	0.85	1,602
Concentrate test day AI	—	0.03	1.03	0.03	0.98–1.08	0.22	1,600
Fat %	—	0.06	1.06	0.07	0.93–1.22	0.40	1,586
Protein %	—	0.04	1.05	0.23	0.68–1.60	0.84	1,586
Lactose %	—	0.33	1.39	0.43	0.76–2.53	0.29	1,586
LnSCC[7]	—	−0.06	0.94	0.05	0.86–1.04	0.22	1,586
Urea	—	<0.01	1.00	0.06	0.90–1.12	0.97	1,586
Season[1]	Dec–Feb (535)	—	1.0	—	—	<0.01[5]	1,648
	Mar–May (461)	−0.06	0.94	0.14	0.71–1.26	0.70	
	Jun–Aug (377)	−0.35	0.70	0.12	0.51–0.98	0.04	
	Sep–Nov (275)	0.38	1.46	0.24	1.06–2.00	0.02	
OCFT[1,8]	No (1,545)	—	1.0	—	—	0.94[5]	1,648
	Obstetrical (51)	0.79	1.05	0.33	0.56–1.98	0.87	
	Fertility (52)	−0.53	0.90	0.30	0.47–1.75	0.76	
Barn type[1]	AMS[9] (1,138)	—	1.0	—	—	<0.01[5]	1,648
	Milk parlor (221)	−0.15	0.86	0.17	0.58–1.27	0.45	
	Tied (289)	−0.66	0.52	0.10	0.36–0.75	<0.01	
Number cows	—	0.01	1.01	<0.01	1.00–1.01	0.18	1,648

[1]Categorical variable.
[2]Levels A–J for AI personnel.
[3]CFAI = interval from calving to first AI.
[4]BCS = level 1–5.
[5]Overall Wald test for categorical explanatory variables.
[6]RBC = red blood cells.
[7]LnSCC = natural logarithm somatic cell count.
[8]OCFT = obstetrical condition or fertility treatment before first AI.
[9]AMS = automated milking system.

for no CYTO increased odds ratio from 1.01 to 1.16 by increasing the interval to first AI by 21 d.

Model 2: Associations Between the Likelihood of Pregnancy to First AI and the Explanatory Variables

The univariable analyses on relationships between pregnancy after first AI and predictor variables are presented in Table 4. The following variables were associated with pregnancy to first AI: CYTO or the PMN occurrence as a continuous variable, parity, AI personnel, test day MY, test day CA, barn type, and obstetrical conditions or fertility treatments before first AI.

In the multivariable model presented in Table 5, the pregnancy success after first AI was associated with CYTO, AI personnel, test day MY, barn type, and obstetrical conditions or fertility treatments before first AI. The odds ratio for nonpregnancy increased by a factor of 1.51 for a cow with CYTO compared with a CYTO-negative cow.

Table 3. Multivariable model describing associations between the occurrence of cytological endometritis (0 = no, baseline, 1 = yes) and explanatory variables in 1,439 Norwegian Red cows from 116 herds (herd is included as a random effect in the model)

Variable	Level	β	Odds ratio	SE	95% CI	*P*-value
AI personnel[1]	A	—	1.0	—	—	<0.01[2]
	B	−1.54	0.21	0.07	0.12–0.39	<0.01
	C	−1.28	0.28	0.09	0.15–0.54	<0.01
	D	0.19	1.21	0.49	0.54–2.67	0.65
	E	−1.11	0.33	0.07	0.22–0.49	<0.01
	F	−0.79	0.46	0.09	0.31–0.68	<0.01
	G	−0.42	0.66	0.28	0.29–1.50	0.32
	H	−1.05	0.35	0.14	0.16–0.77	0.01
	I	−0.25	0.78	0.19	0.49–1.25	0.30
	J	−0.51	0.60	0.17	0.34–1.04	0.07
CFAI[3] interval		−0.01	0.99	<0.01	0.98–1.00	<0.01
Vaginal mucus[1]	Clear	—	1.0	—	—	0.03
	Opaque	0.50	1.65	0.38	1.05–2.60	
RBC[4] in sample[1]	No	—	1.0	—	—	<0.01[2]
	Low	0.25	1.28	0.20	0.94–1.74	0.11
	Moderate	0.66	1.93	0.38	1.32–2.83	<0.01
	High	0.58	1.79	0.37	1.18–2.70	<0.01
Barn type[1]	AMS[5]	—	1.0	—	—	0.01[2]
	Milk parlor	0.02	1.02	0.20	0.69–1.50	0.93
	Tied	−0.60	0.55	0.11	0.37–0.81	<0.01
Season[1]	Dec–Feb	—	1.0	—	—	0.03[2]
	Mar–May	−0.04	0.97	0.16	0.71–1.32	0.83
	Jun–Aug	−0.46	0.63	0.16	0.39–1.02	0.06
	Sep–Nov	0.30	1.35	0.24	0.96–1.90	0.09
Constant		<0.01	1.00	0.24	0.63–1.60	0.99

[1]Categorical variable.
[2]Overall Wald test for categorical explanatory variables.
[3]CFAI = interval from calving to first AI.
[4]RBC = red blood cells.
[5]AMS = automated milking system.

Model 3: Associations Between Late Embryo Loss and Explanatory Variables

In model 3, late embryo loss was only associated with the variable obstetrical conditions or treatment against fertility disorders before first AI. The odds ratio for late embryo loss was 4.37 (95% CI: 1.77–10.83, $P < 0.01$) in cows treated against reproductive disorders compared with pregnant cows without treatment, whereas for obstetrical conditions, there were no association with late embryo loss.

DISCUSSION

The present study demonstrated that Norwegian Red, a breed with high calving rates due to selection for fertility and health over the last 50 years, has a high prevalence of cows with CYTO at first AI. The condition had a significant negative effect on the fertility of the breed. However, pregnancy to first AI was still high, as presented in Table 1. The herds and cows included are highly comparable with the population of Norwegian Red according to management system, herd size, season for AI, and MY (TINE Rådgiving, 2019). Moreover, the present study is the first study of CYTO in Norwegian Red and is the largest field study of CYTO so far described in the literature, including 1,648 cows from 116 farms. Other comparable field studies involved between 383 and 1,044 individuals from 1 to 18 farms, mainly with Holstein breed (Dubuc et al., 2010a; Ribeiro et al., 2013; Pascottini et al., 2017a).

Sampling at AI or directly after AI has been published only in 2 studies (Kaufmann et al., 2009; Pascottini et al., 2017a). Thus, solid evidence for cut-off levels defining CYTO-positive animals at AI still remains to be documented. Both studies counted the proportion of PMN from 300 cells. Kaufmann et al. (2009) defined CYTO positive to include all cows with at least one PMN in the slides, whereas Pascottini et al. (2017a) concluded that optimal cut-off was 1% with sensitivity and specificity equal to 33.8% and 88.6%, respectively. The predicative positive and negative value was 89.2% and 32.4%, respectively, indicating that a CYTO-positive cow is very likely to not become pregnant, but also that a negative sample at AI was not at good indicator for a cow to be pregnant. The constructed ROC curve

in their study showed an area under curve (**AUC**) equal to 0.62. In the present study, the AUC was 0.54 with a sensitivity and specificity equal to 32.4% and 74.9%, respectively. The positive and negative predicative value was 46.6% and 62.2%, respectively, indicating that the cut-off has a modest ability to predict a CYTO-positive cow to become nonpregnant. However, the odds ratio of a CYTO-positive nonpregnant cow compared with a CYTO-negative pregnant cow was 1.51 and significant in the model presented in Table 5. Overall pregnancy to first AI was 59.8%, and 53.4% in CYTO-positive cows, which is high compared with similar studies (Gilbert et al., 2005; Galvão et al., 2009; Pascottini et al., 2017a) and could explain the modest AUC. Various studies

Table 4. Univariable analyses describing associations between nonpregnancy to first AI (0 = pregnant baseline, 1 = not pregnant) and explanatory variables in 1,449 Norwegian Red cows from 116 herds (herd is included as a random effect)

Predictor	Level (n)	β	Odds ratio	SE	95% CI	*P*-value	N
Parity[1]	>1 parity (790)	—	1.0	—	—	—	1,449
	1 parity (659)	−0.22	0.80	0.09	0.65–1.00	0.05	
AI personnel[1]	Several levels[2]	—	—	—	—	<0.01	1,449
CFAI[3] interval		<0.01	1.00	<0.01	1.00–1.00	0.87	1,449
PMN[4] continuous		0.01	1.01	0.01	1.01–1.02	<0.01	1,449
CYTO[1,5]	No (1,043)	—	1.0	—	—	—	1,449
	Yes (406)	0.40	1.50	0.18	1.18–1.91	<0.01	
BCS[1]	2.0–2.75 (188)	—	1.0	—	—	0.11[6]	1,439
	3.0–3.75 (1,096)	0.29	1.34	0.23	0.95–1.88	0.09	
	≥4.0 (155)	0.03	1.03	0.25	0.64–1.65	0.91	
Vaginal mucus[1]	Clear (1,188)	—	1.0	—	—	—	1,265
	Opaque (77)	−0.07	0.93	0.23	0.58–1.51	0.78	
RBC[7] in sample[1]	No (839)	—	1.0	—	—	0.39[6]	1,449
	Low (329)	−0.01	1.00	0.14	0.76–1.29	0.94	
	Moderate (152)	0.29	1.33	0.25	0.93–1.91	0.12	
	High (131)	−0.10	0.91	0.18	0.61–1.34	0.62	
Calf size[1]	Small (199)	—	1.0	—		0.56[6]	1,430
	Medium (989)	0.16	1.17	0.19	0.84–1.61	0.35	
	Large (224)	0.24	1.27	0.26	0.85–1.90	0.25	
	Twins (18)	0.54	1.72	0.77	0.59–4.08	0.28	
Dystocia[1]	No (1,307)	—	1.0	—	—	0.27[6]	1,434
	Some (88)	−0.01	0.99	0.23	0.63–1.56	0.98	
	Large (39)	0.54	1.72	0.58	0.89–3.32	0.11	
Milk yield test day AI		0.02	1.02	0.01	1.01–1.04	<0.01	1,406
Concentrate test day AI		0.07	1.07	0.03	1.02–1.12	<0.01	1,406
Fat %		0.03	1.03	0.07	0.90–1.17	0.69	1,394
Protein %		−0.04	0.71	0.15	0.47–1.08	0.11	1,394
Lactose %		−0.20	0.82	0.23	0.47–1.42	0.47	1,394
LnSCC[8]		0.02	1.02	0.05	0.93–1.11	0.72	1,394
Urea		0.05	1.05	0.06	0.95–1.16	0.38	1,394
Season[1]	Dec–Feb (464)	—	1.0	—	—	0.28[6]	1,449
	Mar–May (409)	0.10	1.11	0.16	0.84–1.46	0.48	
	Jun–Aug (338)	0.05	1.05	0.17	0.77–1.43	0.77	
	Sep–Nov (238)	−0.23	0.80	0.14	0.57–1.12	0.18	
OCFT[1,9]	No (1,360)	—	1.0	—	—	0.01[6]	1,449
	Obstetrical (44)	0.11	1.11	0.36	0.60–2.08	0.74	
	Fertility (45)	0.94	2.55	0.82	1.36–4.80	<0.01	
Milking system[1]	AMS[10] (1,000)	—	1.0	—	—	<0.01[6]	1,449
	Milk parlor (196)	−0.45	0.64	0.11	0.45–0.90	0.01	
	Tied (253)	0.58	1.78	0.26	1.34–2.37	<0.01	
Number cows		<−0.01	1.00	<0.01	0.99–1.00	0.51	1,449

[1]Categorical variable.
[2]Levels A–J for AI personnel.
[3]CFAI = interval from calving to first AI.
[4]PMN = polymorphonuclear neutrophils on a continuous scale.
[5]CYTO = cytological endometritis.
[6]Overall Wald test for categorical explanatory variables.
[7]RBC = red blood cells.
[8]LnSCC = natural logarithm somatic cell count.
[9]OCFT = obstetrical condition or fertility treatment before first AI.
[10]AMS = automated milking system.

Table 5. Multivariable model describing associations between nonpregnancy to first AI (0 = pregnant baseline, 1 = not pregnant) and explanatory variables in 1,406 Norwegian Red cows from 116 herds (herd is included as a random effect in the model)

Variable	Level	β	Odds ratio	SE	95% CI	*P*
CYTO[1,2]	No	—	1.0	—	—	—
	Yes	0.41	1.51	0.19	1.17–1.94	<0.01
AI personnel[1]	A	—	1.0	—	—	<0.01[3]
	B	0.16	1.18	0.28	0.75–1.86	0.48
	C	0.38	1.46	0.36	0.90–2.36	0.12
	D	0.57	1.77	0.43	1.10–2.86	0.02
	E	0.08	1.08	0.21	0.75–1.57	0.67
	F	−0.12	0.89	0.16	0.63–1.26	0.51
	G	−0.07	0.94	0.34	0.46–1.90	0.85
	H	−0.08	0.93	0.28	0.51–1.69	0.80
	I	1.05	2.87	0.71	1.77–4.65	<0.01
	J	0.46	1.59	0.45	0.91–2.76	0.10
Milk yield test day AI		0.02	1.02	0.01	1.00–1.03	0.01
Barn type[1]	AMS[4]	—	1.0	—	—	<0.01[3]
	Milk parlor	−0.48	0.62	0.11	0.43–0.89	0.01
	Tied	0.70	2.02	0.32	1.48–2.76	<0.01
OCFT[1,5]	No disease	—	1.0	—	—	0.04[3]
	Obstetrical	0.17	1.19	0.39	0.63–2.25	0.60
	Fertility	0.80	2.23	0.73	1.18–4.22	0.01
Constant		−1.45	0.24	0.07	0.13–0.41	<0.01

[1]Categorical variable.

[2]CYTO = cytological endometritis.

[3]Overall Wald test for categorical explanatory variables.

[4]AMS = automated milking system.

[5]OCFT = obstetrical condition or fertility treatment before first AI.

use ROC curves to establish the cut-off point for the diagnosis of cytological endometritis (Kasimanickam et al., 2004; Dubuc et al., 2010a; Madoz et al., 2013). In the mentioned studies including in this study, the sum of sensitivity and specificity is modest, which can be expected as reproductive success is affected by numerous other factors than the inflammatory status of the uterus (Kasimanickam et al., 2004).

The PMN in the endometrium is a first line of defense against bacterial infections after calving, which should reach its highest occurrence in the first month postpartum, and then a decline (Pascottini and LeBlanc, 2020). Early sampling resulted in a higher cut-off level for the diagnosis of CYTO (Madoz et al., 2013; Arias et al., 2018). Accordingly, we were expecting a low prevalence of CYTO in the present study where the sampling was performed at an average of 72 DIM. However, the prevalence of CYTO in our study population was higher than in other studies sampling at AI or at similar time period after parturition (Kaufmann et al., 2009; Dubuc et al., 2010a; Pascottini et al., 2017a). The average CFAI of 122 d, compared with 72 d in the present study, partially explains the lower prevalence of CYTO reported by Pascottini et al. (2017a). This assumption is also supported by the trend that a shorter CFAI was a risk factor for the occurrence of CYTO (Pascottini et al., 2017b). Kaufmann et al. (2009) sampled 4 h after AI on median 78 DIM using cytobrush and detected at least 1 PMN out of 300 counted cells (i.e., 0.3% PMN) in 42.8% of the cows. Dubuc et al. (2010a) sampled on average 56 DIM and diagnosed 13.8% of cows as CYTO positive, using cytobrush and a cut-off level of 5% PMN. In the present study, simulating cut-off at 0.3%, 1.0%, and 5.0% PMN, the proportion of CYTO-positive cows would be 58.4%, 42.7%, and 20.9%, respectively. In 20.6% of the samples, 1 to 3 PMN (0.3–1%) was found. Pascottini et al. (2017a) reported only 3 samples with 1 to 3 PMN, whereas 72% of the samples had no PMN. We suggest that the differences in detection of PMN may be explained by scanning and counting technique.

Pregnancy to first AI in the study population was 59.8% and comparable to the pregnancy incidence of 61.3% in Norwegian Red reported by Garmo et al. (2008), supporting the findings by Pascottini et al. (2017a) that pregnancy success is not affected by cytotype sampling of the endometrium at AI. However, Pascottini et al. (2017a) reported an overall conception rate of 43%, which is substantially lower compared with the present study.

In the present study, the odds ratio for nonpregnancy was 1.51 in cows with CYTO, whereas the corresponding odds ratio reported by Pascottini et al. (2017a) was 1.76, although AI was performed much later in their study. However, Kaufmann et al. (2009) found no dif-

ference in conception rate between animals free from PMN or with medium (>0–15%) or high (>15%) levels of PMN. Previous studies that reported no correlation between PMN in the endometrium and pregnancy outcome suggest that their results might have been due to one or more of the following contributing factors: a lower bacterial load, a more thorough reproduction management in their study designs, or a more effective immune system in the animals (Plöntzke et al., 2010; Prunner et al., 2014). It is reasonable to believe that breed is an important factor because fertility trait has been included in Norwegian Red breeding program since 1972 (Andersen-Ranberg et al., 2005). Earlier literature suggests that CYTO is a result of a nonoptimal uterine immune function (Cheong et al., 2011; Wagener et al., 2017; Pascottini and LeBlanc, 2020). Although mechanisms for good reproductive performance in Norwegian Red are not fully known, the high pregnancy rate in animals with CYTO, as well as the low incidence of clinical cases of endometritis, may indicate that Norwegian Red has a beneficial uterine immunology.

Opaque vaginal discharge was detected in few cows, and very few (0.7%) cows were diagnosed with clinical endometritis according to the definitions by Sheldon et al. (2006). Dubuc et al. (2010a) reported clinical endometritis in 12% of the cows using Metricheck in Holstein cows. Metricheck has been shown to better detect clinical endometritis than other methods such as vaginoscopy and gloved hand technique (Pleticha et al., 2009). Hence, several studies might have underestimated the condition. Individuals with clinical endometritis have generally been excluded in studies of subclinical endometritis, due to the interference between the definition of the conditions (Kasimanickam et al., 2004; Kaufmann et al., 2009; Plöntzke et al., 2010). In some studies that investigated CYTO (Dubuc et al., 2010a; Pascottini et al., 2017a), the higher prevalence of positive animals might be explained by inclusion of individuals with opaque vaginal discharge. However, because only 10 cows in the present study suffered from endometritis, the results of CYTO were not affected by this condition. This is supported by low number of treatments against metritis and endometritis of 1.3/100 cow-years in the NDHRS (TINE Rådgiving, 2019).

The risk of contaminating the endometrial samples with PMN from the vagina and cervix was reduced by the plastic sheet covering the insemination gun. Opaque mucus in the Metricheck test was associated with CYTO in endometrial samples, but not with pregnancy outcome. Dubuc et al. (2010a) found that only 38% of the individuals with purulent vaginal discharge diagnosed by Metricheck also suffered from CYTO, when it was diagnosed by cytobrush, which contradicted earlier beliefs that purulent vaginal discharge normally comes from the endometrium. The only other study of risk factors for CYTO at AI used visual registration of the perineum, which was not sufficient to diagnose purulent vaginal discharge or its relation to CYTO (Pascottini et al., 2017b).

Red blood cells in the endometrial samples were associated with CYTO, but not with the pregnancy success after first AI, and hence were not related to postestrus bleeding. The occurrence of RBC could be due to sensitive mucosa, by sampling method, or perhaps a tighter cervix in Norwegian Red compared with Holstein. However, PMN was not necessarily found close to RBC in the slides, rather between epithelial cells in the focus areas. Pascottini et al. (2015) used amount of blood as a slide quality control and did not consider RBC as a risk factor for CYTO (Pascottini et al., 2017b), but suggested it could change the CYTO diagnosis due to low amounts of PMN circulating in blood. However, a comparison between cytotape and cytobrush techniques revealed agreement in PMN occurrence despite increased amount of RBC in cytobrush samples (Pascottini et al., 2015).

Season for AI has been evaluated as a risk factor for CYTO in several studies. For example, Pascottini et al. (2017b) found that heat stress was a risk factor for CYTO at AI, whereas Prunner et al. (2014) found no correlation between season and the occurrence of PMN. The present study found lower risk for CYTO in the summer and spring time compared with winter season. In Norway, the cold summer climate reduces the risk for heat stress compared with warmer countries. Fertility is better in the summer months (Geno SA, 2016), perhaps due to outdoor management and more daylight (Reksen et al., 1999). Nevertheless, pregnancy success was not affected by season in the present study.

Barn type was associated with CYTO, where tiestall herds had less CYTO-positive cows. In contrast, Prunner et al. (2014) reported higher PMN occurrence in tiestalls than calving pens. Regardless of CYTO status, the pregnancy success to first AI was higher for cows in freestall herds, hence not largely affected by the higher incidence of CYTO. These results correlate with earlier evidence of a higher reproductive success in Norwegian Red managed in freestalls (Simensen et al., 2010), as well as a calculation from NDHRS data where the nonreturn rate on d 56 for freestalls and tiestalls was 72.3 (n = 85,793) and 67.8 (n = 55,562), respectively (Geno Breeding and AI Association, Hamar, Norway, personal communication).

A shorter CFAI interval was a risk factor for CYTO. This finding was supported in one earlier study performed in average 68 DIM (Cheong et al., 2011), whereas another study only found such correlation after 124 DIM (Pascottini et al., 2017b). These data support

the use of lower cut-offs to diagnose CYTO at AI than what has been used in most studies that sampled at earlier time points. The length of CFAI did not affect pregnancy results in the present study even though the interval was approximately 10 d shorter than the population average of 82 d (Geno SA, 2016).

Low or decreasing BCS has previously been shown to contribute to a higher risk for CYTO (Wagener et al., 2017; Valdmann et al., 2018), whereas other studies did not report this association (Cheong et al., 2011; Pascottini et al., 2017b). In the present study, BCS at first AI was not associated with CYTO or pregnancy outcome, which could be explained by few cows with low BCS at AI. In a previous study, loss in BCS from CFAI was related to pregnancy success in Norwegian Red (Gillund et al., 2001). Hence, measurement of BCS loss from parturition to AI would be a more relevant measure. Norwegian Red is a dual-purpose breed selected for both milk and meat, and has a relatively thick muscle layer. The backfat thickness has been measured to be less than half of that measured in Holstein dairy cows at the same BCS score (Gillund et al., 1999). Most of the cows (75.2%) in this study were in optimal body condition (3.0–3.75), which may have contributed to a high rate of embryo survival despite the relatively high frequency of CYTO. Earlier literature supports an association between a nonoptimal BCS and poor blastocyst development in vitro (Snijders et al., 2000; Armstrong et al., 2001).

Negative energy balance and systemic inflammation plays an important role in control of uterine immune function and the development of CYTO in Holstein cows (Dubuc et al., 2010b; Cheong et al., 2011; Pascottini and LeBlanc, 2020). Parameters such as nonesterified acids, BHB, or haptoglobin that measure levels of negative energy balance or systemic inflammation have not been published in Norwegian Red.

Our definition of embryo loss did not consider fertilization failure. Fertilization rate is described to be 90 to 100% for heifers and probably a bit lower and more variable in high-producing dairy cows (Diskin et al., 2016). The study population showed a lower occurrence of embryo loss compared with global estimates (Sreenan et al., 2001). Cows treated for fertility disease before first AI had a much higher risk of losing an embryo (odds ratio = 4.37), whereas obstetric conditions did not affect the outcome. Embryo quality is dependent on the microenvironment of the uterus (Evans and Walsh, 2012; Gilbert, 2012; Leroy et al., 2017), which in turn may be altered by many factors. Medical treatment as such may alter the metabolism of the uterus, or the disease leading to treatment may alter it. Earlier literature describes an association between both insufficient and exaggerated blood progesterone levels preceding AI, and high embryo mortality rate (Diskin et al., 2016). In this context, Lamming and Darwash (1998) earlier reported that a persistent corpus luteum could alter the progesterone levels and affect embryo survival rate negatively. The association between treatment for fertility disease and late embryo loss found in the present study may not be a result of the treatment itself, but rather connected to the treated disorders that can cause imbalances in the hormone cycle.

Interestingly, CYTO at first AI did not affect late embryo survival, suggesting that late embryo loss is mainly influenced by other factors yet to be investigated. Earlier literature supports that an inflamed environment has a negative effect on embryo quality in vitro (Hill and Gilbert, 2008). However, in one in vivo study of superovulated cows the embryo survival rate was higher in cows whose proportion of PMN had a slight increase from AI to flushing at d 7, compared with those with no PMN at the 2 occasions (Drillich et al., 2012). The present study, together with the previous study of Drillich et al. (2012), indicates that some extent of inflammation, as measured by PMN in the uterine endometrium, may not be of major concern regarding pregnancy outcome.

CONCLUSIONS

The overall pregnancy incidence to first AI was high and the negative association with CYTO was significant, although its biological effect appears to be modest. The risk for CYTO was associated with CFAI, abnormal mucus condition, RBC, season, AI personnel, and barn type. Risk factors for nonpregnancy to first AI were CYTO, AI personnel, barn type, MY, and obstetrical conditions or fertility treatments before first AI. The occurrence of embryo loss was low compared with global estimates and late embryo loss was not related to CYTO at first AI. Our results suggest that even if Norwegian Red cows show a fairly high prevalence of PMN in uterine cytology, it does not seem to have a major effect on fertility. It appears that the inclusion of fertility in Norwegian red breeding programs for almost 50 years has resulted in improved production traits, with genetically advantageous uterine immunology possibly being one of the mechanisms behind this observation. Further studies are necessary to reveal the genetics behind these traits, a hot topic considering a recent change from progeny testing to genomic selection.

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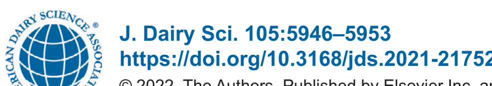
APPENDIX

Table A1. Number of cows with endometrial samples, BCS, milk yield, concentrate allocation, mucus evaluation, milk content samples, progesterone samples, and pregnancy diagnosis

Item	Number of cows
Endometrial samples	1,648
BCS	1,637
Mucus evaluation	1,442
Milk yield recorded	1,602
Concentrate recorded	1,600
Test day milk content; protein, fat, lactose, urea, and SCC	1,586
Progesterone first AI	1,488
Progesterone 21 d after first AI	1,287
Progesterone at first AI and 21 d	1,287
Pregnancy controls, manual rectal palpation, or pregnancy-associated glycoprotein analysis	1,449

Paper II



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Heritability of subclinical endometritis in Norwegian Red cows

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ABSTRACT

Subclinical endometritis (SCE) is highly prevalent in dairy cows, causing negative effects on reproductive outcomes and the producer economy. Genetic selection for animals with better resilience against uterine disease should be prioritized due to both sustainability and animal welfare. Therefore, the aim of the present study was to estimate the heritability of SCE in the Norwegian Red (NR) population. Moreover, future perspectives of the condition as a fertility phenotype for breeding are discussed. A total of 1,642 NR cows were sampled for SCE at the time of artificial insemination, using cytotape. The percentage of polymorphonuclear cells (PMN) in each sample was established by cytology, through the counting of 300 PMN and epithelial cells. The mean percentage of PMN was 5%. Different trait definitions were examined, and SCE was defined as binary traits, based on the following cut-off levels of PMN: Cyto0 = PMN >0, Cyto3 = PMN >3%, Cyto5 = PMN >5%, Cyto10 = PMN >10%, and Cyto20 = PMN >20%. The mean ranged from 0.07 (Cyto20) to 0.59 (Cyto0). We also analyzed PMN as a continuous variable using percent PMN. Information on the animals and herds was obtained from the Norwegian Dairy Herd Recording System. The pedigree of cows with data included a total of 24,066 animals. A linear animal model was used to estimate the heritability. The only trait definition that had an estimated genetic variance larger than the standard error was Cyto5, with an estimated heritability of 0.04. For all other definitions, the genetic variance was not significantly different from zero. A cut-off level of 5% PMN has been established as a general threshold for the definition of SCE in earlier literature. The standard errors of the estimated variance components were relatively large, and results should be interpreted with caution. However, the current study indicates that SCE is heritable at a similar level to that of clinical endometritis and metritis, and has potential as a future fertility phenotype to be used for breeding purposes. A more feasible method to diagnose SCE is needed to establish larger data sets.

Key words: Norwegian Red, subclinical endometritis, cytological endometritis, heritability

INTRODUCTION

Subclinical endometritis (**SCE**) was initially identified as a measurable fertility problem in 2004 (Kasimanickam et al., 2004) and has been the target of a growing body of research during the last decade. The condition consists of a persistent presence of PMN in the endometrium postpartum, exceeding a naturally occurring first line of defense. Although the cause or mechanism has yet to be fully explained (Sheldon et al., 2019), it is well known that this condition has a major negative influence on dairy cattle fertility (Kasimanickam et al., 2004; Gilbert et al., 2005; Wagener et al., 2017).

The diagnosis of SCE is based on the collection of a cytology sample from the uterine endometrium or lumen and a subsequent analysis of the reproductive performance. Cytological endometritis (**CYTO**) is defined by the presence of PMN on the endometrial surface or lumen (Dubuc et al., 2010; Pascottini et al., 2016), whereas the term SCE includes both the definition of CYTO and reduction in reproductive performance in the absence of clinical symptoms of endometritis (Kasimanickam et al., 2004; Sheldon and Owens, 2017). The distinction between the two definitions is relevant because several studies have failed to find any negative correlation between cytological endometritis and reproductive outcome (Plöntzke et al., 2010; Prunner et al., 2014), and in those cases the term SCE would not be correct. However, the use and definition of these terms are not consistent in the literature, and a great deal of overlap also occurs. To diagnose SCE in a study population, a cut-off level for the percentage of PMN in the cytology sample must be defined (reviewed by Wagener et al., 2017).

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Several management-related risk factors for SCE have been identified, including bedding and housing factors (Cheong et al., 2011; Prunner et al., 2014; Diaz-Lundahl et al., 2021). Previous diseases such as ketosis and metritis (Cheong et al., 2011) or endometritis (Ribeiro et al., 2013) have also been associated with the condition, in addition to metabolic stress peripartum (Hammon et al., 2006; Kaufmann et al., 2010). Hence, improving targeted management factors or taking action to prevent other postpartum diseases would lower the prevalence of SCE and improve fertility. However, recent reviews within the field of uterine health highlight that tolerance of disease appears to be more important than avoiding pathogenic bacteria or resistance to infection in the birth canal (Sheldon et al., 2019; Pascottini and LeBlanc, 2020). Furthermore, dysregulation of the immune system has been postulated to have a central role in the pathogenesis of SCE specifically, affecting its ability to limit or avoid excessive inflammation (Pascottini and LeBlanc, 2020). This concept suggests a genetic component to SCE. A better understanding of this theory could be helpful in future breeding programs aimed at improving immune function, provided that the trait is heritable.

Clinical uterine disease has genetic components, with heritability estimates published for both metritis (Zwald et al., 2004; Parker Gaddis et al., 2014; Gernand and König; 2017) and endometritis (Distl et al., 1991; Shabalina et al., 2020; May et al., 2022). May et al. (2022) performed a genome-wide association study to identify genomic loci associated with clinical uterine disease. Interestingly, they found no overlap in SNP or candidate genes for different stages of endometritis and metritis. Equally, Pascottini et al. (2020) found a distinction in uterine microbiota between cows with clinical endometritis and SCE. With two very different approaches, these results suggest clear differences between clinical endometritis and SCE, which supports a separate investigation of the pathogenesis and genetics behind these conditions. To the authors' knowledge, no studies on the heritability of SCE have been published.

Norwegian Red (**NR**) is the main dairy cattle breed in Norway. The breeding strategy has emphasized health and fertility in the total merit index since the 1970s (Andersen-Ranberg et al., 2005). Norwegian Red cows are notable for their good reproductive performance, with a reported pregnancy incidence of 62.9% and calving rate of 56.3% (Garmo et al., 2008), and a more recently reported 56-d nonreturn rate of 72.5% (Geno SA, 2016). With the implementation of genomic selection in dairy cattle breeding, interest in new phenotypes that can add information on fertility and health has been increasing. In an earlier study, Diaz-Lundahl et al. (2021) found a high prevalence of SCE (28%, using 3% PMN as threshold) in NR at first AI after calving, and a significant negative effect on pregnancy to AI. Even though pregnancy to first AI was high in NR cows both positive (53.4%) and negative (62.2%) for SCE, the study confirmed that fertility problems due to this condition represent an opportunity for improvement. The objectives of the current study were therefore (1) to estimate the heritability of SCE in the NR population, (2) to examine how to define the trait based on PMN levels, and (3) to discuss future perspectives of this condition as a possible new fertility phenotype to be used for breeding purposes.

MATERIALS AND METHODS

Herds and Animals

The animals included were enrolled in a previous study (Diaz-Lundahl et al., 2021). A cytology sample from the uterine endometrium was collected from 1,738 NR cows at first AI after parturition, from 122 farms in Trøndelag and Hedmark counties in Norway, between September 2017 and March 2019. Out of the 1,738 cows initially included, 1,642 had sufficient sample quality and available animal history to be included in the present study. The inclusion criterion was cows presented for first AI. The ethical approval for animal use and care was given by the Norwegian Food Safety Authority, with approval number 17/152686–1.

Sampling

At the time of estrus after parturition, an AI technician or veterinarian ($n = 14$) was called out to perform the sampling. To monitor the number of cows with opaque vaginal discharge equivalent to clinical endometritis, a Metricheck (Simcro) was used to collect mucus from the vagina at the level of the cervix. The assessment was performed in 1,442 cows, using a scale from 0 to 3 (score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤50% white or off-white mucopurulent material; score 3 = discharge containing ≥50% purulent material, usually white or sanguineous), according to Williams et al. (2005). Cows with all levels of mucus score were included in the analyses.

In accordance with the cytotape method developed by Pascottini et al. (2015), the sanitary sheath of the insemination gun was covered at the top with a piece of paper tape. The sampling was further protected by a disposable plastic tube to avoid contamination from the vagina and cervix. The vulva and perineum were

cleaned, and the protective tube was passed through the cervix and into the uterus. Following penetration of the protective tube, the sanitary sheath with the paper tape was rolled against the endometrium to collect cells. The semen was then released, and the sample was withdrawn into the protective tube before it was removed from the reproductive tract. The paper tape with the sample was rolled onto a microscopic slide and sent to the laboratory for fixing and staining with Kruuse Dip Quick Stain (Jorvet, J0322A1, A2, A3, Jorgensen Laboratories) and microscopic analysis.

Trait Definition

To diagnose CYTO, a total of 300 representative epithelial cells and PMN were counted under 400× magnification in several fields of the microscopic slides, followed by a calculation of the PMN proportion. For estimation of heritability, the trait was defined as 0 (negative for CYTO) or 1 (positive for CYTO). Five binary CYTO traits were scored as 0 or 1 based on different cut-off levels for percentages of PMN: Cyto0 = PMN >0, Cyto3 = PMN >3%, Cyto5 = PMN >5%, Cyto10 = PMN >10%, and Cyto20 = PMN >20%. We also analyzed PMN as a continuous variable, using both percentage PMN and the natural logarithm for percentage PMN + 1 (logPMN). Information on both individual animals and herds was available from the Norwegian Dairy Herd Recording System. The pedigrees of cows with data were traced as far back as 8 generations and included a total of 24,066 animals. A total of 393 sires had daughters with data, and the number of daughters per sire varied from 1 to 93. All the 1,642 cows with phenotypes had at least one parent known, and 1,618 of them had both parents known.

Statistical Analyses

We used the software DMU with the (AI)REML procedure (Madsen and Jensen, 2013) for the estimation of variance components. The following linear animal model was used for all traits:

$$Y_{ijklmn} = \text{AIpersonnel}_i + \text{BarnType}_j + \text{Parity}_k + \text{Season}_l + \text{DIMAI}_m + \text{animal}_n + e_{ijklmn},$$

where Y_{ijklmn} is an observation of the trait (Cyto0, Cyto3, Cyto5, Cyto10, Cyto20, percentage PMN, or logPMN) for animal n, performed by AI personnel i, in a herd with barn type j, for a cow in parity k, in season l, and days in milk (DIMAI) class m. The following fixed effects were included in the model, based on known risk factors for SCE: AIpersonnel_i (i = 1, . . . , 14, for different persons), BarnType_j (j = 1, 2, for tiestall or freestall, respectively), Parity_k (4 classes: 1, 2, 3, and >3), Season_l (4 seasons: December–February, March–May, June–August, and September–November), and DIMAI_m (interval from calving to first AI, grouped in 10 classes of 10 d each, where the first interval is <40 d and the last is >119 d). The random effects were animal_n; the additive genetic effect of animal n, $N \sim \left(0, \mathbf{A}s_a^2\right)$, where $\mathbf{A}$ is the additive genetic relationship matrix and s_a^2 is the additive genetic variance; and residual e_{ijklmn}, $N \sim \left(0, \mathbf{I}s_e^2\right)$, where $\mathbf{I}$ is an identity matrix and s_e^2 is the residual variance.

RESULTS

Descriptive Statistics

The cows and herds included were representative of the NR population considering milk yield, herd size, management system, and season of insemination (TINE Rådgivning, 2019). Descriptive statistics are presented in Table 1. The average herd size was 39 (range 13–110) cows, and the average interval from calving to first AI was 72 d. Further descriptive statistics on cow and herd level were presented in an earlier publication (Diaz-Lundahl et al., 2021). The frequencies of the alternative traits for subclinical endometritis are presented in Table 2.

Heritability

The estimated variance components and corresponding heritability are presented in Table 3. The only trait definition that had an estimated genetic variance larger

Table 1. Descriptive statistics for the data set used to estimate heritability of subclinical endometritis in Norwegian Red cows

Variable	Level	Number of cows[1]	Percentage
Barn type	Tiestall (n = 35)	289	17.6
	Freestall (n = 87)	1,353	82.4
Mucus score	0	1,343	93.1
	1	89	6.2
	2	10	0.7
	3	0	0
Parity	1	731	44.5
	2	465	28.3
	3	260	15.8
	>3	186	11.3
Season of calving	Dec–Feb	611	37.2
	Mar–May	188	11.5
	Jun–Aug	269	16.4
	Sep–Nov	574	34.7

[1]For mucus score, the number of investigated animals was 1,442. For all other variables, the number of cows was 1,642.

Table 2. Number of Norwegian Red cows (n = 1,642) positive for subclinical endometritis, and mean frequency using alternative trait definitions[1]

Trait definition[2]	n positive	Mean	SD
PMN_%		5.03	12.34
logPMN		0.89	1.14
Cyto0	962	0.59	0.49
Cyto3	461	0.28	0.45
Cyto5	333	0.20	0.40
Cyto10	215	0.13	0.34
Cyto20	119	0.07	0.26

[1]The binary cytological endometritis (Cyto) traits were based on different cut-off levels for the presence of PMN in cytological samples, collected at first AI after calving, by counting 300 cells. Binary Cyto traits were scored as 0 or 1 based on different cut-off levels for percentage PMN: Cyto0 = PMN >0, Cyto3 = PMN >3%, Cyto5 = PMN >5%, Cyto10 = PMN >10%, and Cyto20 = PMN >20%.

[2]PMN_% = percentage of PMN; logPMN = natural logarithm for percentage PMN + 1.

than the standard error was Cyto5. Cyto5 had an estimated heritability of 0.04, with standard error 0.035.

DISCUSSION

In the present study, we report the first heritability estimate of SCE, a condition with a high prevalence and significant economic impact in the dairy industry. In addition to contributing to the general understanding of SCE, our results are particularly valuable for future genetic improvement of NR, at a time when the breeding strategy has changed from progeny testing to genomic selection. Despite well-functioning phenotypes in the past and notably high reproductive success, interest in new phenotypes that can be related to other aspects of cow fertility has been increasing. The present study indicates a genetic component of SCE in NR cows, with a heritability of 0.04 for Cyto5. Although the heritability was low, similar low heritability estimates have been reported for other health traits (e.g., Heringstad and Østerås, 2013). Nevertheless, the results from the present study should be interpreted with caution, as the standard errors of the estimates were relatively high.

The fixed effects included in the model were based on risk factors for CYTO identified in our earlier publication (Diaz-Lundahl et al., 2021). We also tried to modify the model in the following ways: (1) herd instead of AI personnel, and defined as fixed or as random effect; (2) parity in 2 groups instead of 4; (3) month and year of calving (19 classes) instead of season in 4 classes; and (4) changing DIMAI to a regression effect instead of a class variable. None of these alternatives had any effect on the results. In this first genetic analysis of subclinical endometritis, we chose to analyze all traits using the same linear animal model, although a threshold liability model might have been theoretically more appropriate for the binary CYTO traits. Linear models are robust and are often the model of choice for routine genetic evaluations. We therefore found it appropriate to use this approach for this initial genetic analysis of a new trait, to explore its potential.

Alternative Cut-Offs to Predict Pregnancy Outcome

To investigate the best threshold for the diagnosis of CYTO in the prediction of pregnancy to AI in NR, Diaz-Lundahl et al. (2021) constructed a receiver operating characteristic curve. The highest summation of sensitivity and specificity was seen at 3% PMN. Accordingly, this was used as the cut-off to define CYTO and estimate the prevalence in the NR population, which was 28% (Diaz-Lundahl et al., 2021). However, a 5% PMN level also had a negative effect on pregnancy to AI, with a slightly lower summation of sensitivity and specificity. Hence, by defining the trait SCE at a 5% cut-off, the prediction of reproductive outcome is

Table 3. Estimated variance components with associated standard errors and heritability for different definitions of the trait subclinical endometritis[1]

Trait definition[2]	s_a^2	SE	s_e^2	SE	h^2	SE
PMN_%	1.7131	3.7390	142.519	6.1663	0.012	0.026
logPMN	0.0377	0.0396	1.1274	0.0541	0.032	0.034
Cyto0	0.0032	0.0065	0.2183	0.0099	0.014	0.029
Cyto3	0.0000	0.0047	0.1849	0.0080	0.000	0.026
Cyto5	0.0062	0.0054	0.1465	0.0072	0.041	0.035
Cyto10	0.0035	0.0037	0.1048	0.0050	0.032	0.033
Cyto20	0.00001	0.0015	0.0648	0.0027	0.0002	0.023

[1]Variance components: s_a^2 is the additive genetic variance, and s_e^2 is the residual variance. Heritability: $h^2 = s_a^2 / s_a^2 + s_e^2$.

[2]PMN_% = percentage of PMN; logPMN = natural logarithm for percentage PMN + 1. Binary cytological endometritis (Cyto) traits were scored as 0 or 1 based on different cut-off levels for percentage of PMN. Cyto0 = PMN >0, Cyto3 = PMN >3%, Cyto5 = PMN >5%, Cyto10 = PMN >10%, and Cyto20 = PMN >20%.

marginally reduced. However, the current study defined a 5% cut-off as the only trait definition with estimated genetic variance significantly different from 0. Earlier literature also supports a 5% PMN level as a general cut-off for SCE for the purpose of a common standard, but at 21 to 62 d after calving (Madoz et al., 2013). A 5% cut-off for the definition of SCE would result in a prevalence of about 20% in the NR population (Table 2).

Heritability of Uterine Disease

The heritability of clinical uterine disease has been published previously. For metritis, heritability ranges from 0.03 to 0.08 based on threshold models (Zwald et al., 2004; Heringstad, 2010; Parker Gaddis et al., 2014), and was 0.06 using a linear model (Gernand and König, 2017). Few studies have estimated the heritability of clinical endometritis. May et al. (2022) used records of 14,810 Holstein cows and a linear model to estimate the heritability of different clinical stages of endometritis based on the appearance of the vaginal discharge in the first 100 d after parturition. The heritability for all stages combined was 0.03, and the highest heritability ($h^2 = 0.08$) was seen in pyometra. Another recent study, using records of 114,060 Holstein cows, reported a heritability for clinical endometritis from a linear model of 0.06 to 0.07, 0.04 to 0.05, and 0.06 for first, second, and third lactation, respectively (Shabalina et al., 2020). One older study found a heritability of 0.01 for clinical endometritis using a linear model based on veterinary records from 46,159 cows (Distl et al., 1991). We must mention that estimates of disease heritability between different studies cannot be directly compared. The results vary depending on available information, chosen model, trait definition, breed, and size of the study population.

In NR, only one previous study has been published regarding the heritability of uterine disease. Heringstad (2010) analyzed several common fertility-related diseases and disorders in NR, using a multivariate threshold liability model. The estimated heritability of metritis was 0.03, close to the results of the studies mentioned previously. A distinct difference between the investigated populations mentioned for Holstein and NR was that the NR population had a remarkably lower incidence of clinical uterine disease. May et al. (2022) found signs of uterine disease (metritis, endometritis, or both) in 26.7% of all cows, recorded during the first 100 DIM. For NR, no directly comparable study currently exists, but veterinary records of endometritis or metritis with subsequent treatment were registered for 1.5 cows per 100 cow-years in 2020 (TINE Rådgivning, 2021).

Prevention or Treatment of SCE

Despite knowledge about risk factors, the etiology of SCE is poorly understood. When diagnosed at AI, SCE was found to have a minor effect on the reproductive outcome in NR (Diaz-Lundahl et al., 2021) compared with Holsteins (Pascottini et al., 2017). The theory about the central role of immune regulation in SCE could explain this: NR might have a beneficial immune regulation in the uterus. The understanding of PMN as proinflammatory or repairing agents, the determining factors of their actions, and a possible switch between these states, might be central in how SCE affects the animal. Furthermore, it appears that inflammatory disease outside the uterus (such as mastitis) has an unfortunate effect on uterine immune function (Pascottini and LeBlanc, 2020). Mastitis has been included in the NR breeding objectives since 1978, and significant genetic improvement has been achieved (Heringstad and Østerås, 2013).

One suggestion on how to combat SCE has been through medication regimens that modulate immune function, such as administration of nonsteroidal anti-inflammatory drugs or interleukins peripartum. Further investigation is required, to evaluate these treatments (Pascottini and LeBlanc, 2020). Attempts have also been made to treat SCE as a clinical uterine inflammation, using $PGF_{2\alpha}$ analogs and intrauterine antibiotics, but with differing results (reviewed in Wagener et al., 2017). Routine antibiotic treatment of SCE-positive cows, which, in many herds, may include a high percentage of the animals, would not be ethically defendable. As an alternative to medical treatment, genetic selection for animals with better resilience against uterine disease should be prioritized. This would improve both sustainability and animal welfare. A review by Sheldon et al. (2019) states that the genetic scope in the prevention of uterine health in general is limited. We would like to reason that the genetic potentials are under strong development due to recent advances within the field. Also, expanded genetic insight is a vital component in the prevention of uterine health problems, including SCE. Moreover, considering recent updates in the breeding strategy, this is the right time to investigate whether new phenotypes could help to predict and improve fertility with the genetic tools available.

Although the current study used data only from NR, it would be interesting to expand investigations on the genetics of SCE to breeds or herds where the reproductive outcome is more clearly affected by SCE. In breeds with a tradition of a strong genetic selection for milk yield, tolerance for inflammation in the birth

canal is much lower now than it was 50 years ago, with increased metabolic stress probably being one of the causal factors (Sheldon et al., 2019). Metabolic stress is also negative for the active mechanisms of PMN (Hammon et al., 2006). It might be that breeds with large differences in the matter of metabolic stress need different approaches to handle SCE.

Subclinical Endometritis As a Fertility Trait

Our genetic evaluation of NR is based on data from the Norwegian Dairy Herd Recording System, operated by the dairy cooperative TINE. The system includes about 98% of Norwegian dairy herds, containing 92% of all dairy cows (TINE Rådgivning, 2021). Individual health recording of all veterinary treatments is integrated. The diagnosis is set and reported by a veterinarian, using defined health codes (Heringstad and Østerås, 2013). Retained placenta, metritis, cystic ovaries, and silent heat are the 4 most common fertility-related diseases or disorders in NR. These are included in the total merit index as a composite trait "other disease," which currently has a relative weight of 0.3% (Geno SA, 2021). The number of cases per 100 cow-years in NR in 2020 was 1.09 for silent heat, 1.71 for cystic ovaries, 1.68 for retained placenta, and 1.54 for metritis (TINE Rådgivning, 2021). Compared with this, the prevalence of SCE is high. Hence, SCE has economic relevance for farmers, although the effects of the other fertility-related diseases might be more severe for reproductive outcomes in the affected animals. This, together with the findings of the present study, provides motivation to further investigate whether SCE could be incorporated as a new trait in the breeding program. However, SCE as a trait encounters one major disadvantage: the possibility for efficient data collection is still poor. Notably, the cytologic evaluation requires time-consuming cell counting. Alternative and more feasible methods to diagnose SCE would certainly be beneficial, with the prospect of building larger data sets and more accurate information and preventive measures. This would also allow estimation of the genetic correlation with other fertility and production parameters. Different attempts to diagnose SCE in a more feasible way have been published. This includes the measurement of total proteins, optical density, pH, and leukocyte esterase in endometrial fluids or cells collected from the endometrium mixed with a neutral fluid (Cheong et al., 2012; Couto et al., 2013; Van Schyndel et al., 2018). The leukocyte esterase test, using test stripes designed for urine samples, has given the most promising results (Cheong et al., 2012; Van Schyndel et al., 2018). Hence, it constitutes a future possibility, provided that refinements of the test are successfully performed.

Limitations of the Study

The present study included animals with all levels of vaginal mucus scores. We have observed the same reasoning in other studies investigating the prevalence of CYTO (Dubuc et al., 2010; Pascottini et al., 2017). Mucus scores were registered, to ensure that our material would not include a large proportion of animals with clinical uterine disease. As only 0.7% (n = 10) could be defined as having purulent vaginal discharge in the present study, which might indicate clinical endometritis (Diaz-Lundahl et al., 2021), this was not considered to have influenced our results.

The present study included cows from all lactations, and about half of the sampled individuals were in first lactation. With more data, it would be of interest to examine whether the trait is genetically the same across parities. By making a distinction between first and subsequent lactations, the accuracy of our calculations might have been stronger, because animals highly affected by SCE might have been culled after the first lactation. Several genetic studies of clinical uterine disease have made such distinctions (Zwald et al., 2004; Shabalina et al., 2020; May et al., 2022). Furthermore, a recently published paper suggested that the optimal PMN thresholds and sampling time points were different in the first lactation than in dairy cows in subsequent lactations (Druker et al., 2022).

Although the current study was based on the largest published data set on SCE, the number of records was still low in the context of estimating heritability of disease in dairy cattle.

CONCLUSIONS

Results indicate that SCE is heritable. Although the estimated heritability was low, in common with other uterine health traits, SCE shows potential as a future fertility phenotype to be used for breeding purposes. It is recognized that a more feasible method to diagnose SCE is needed, to establish larger data sets.

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Paper III

The microbiota of uterine biopsies, cytobrush and vaginal swabs at artificial insemination in Norwegian Red cows

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Abstract

The individual resistance or tolerance against uterine disease in dairy cattle might be related to variations in the uterine tract microbiota. The uterine tract microbiota in dairy cattle is a field of increasing interest. However, its specific taxonomy and functional aspects is under-explored, and information about the microbiota in the endometrium at artificial insemination (AI) is still missing. Although uterine bacteria are likely to be introduced via the vaginal route, it has also been suggested that pathogens can be transferred to the uterus via a hematogenous route. Thus, the microbiota in different layers of the uterine wall may differ. Norwegian Red (NR) is a high fertility breed that also has a high prevalence of subclinical endometritis (SCE), an inflammation of the uterus that has a negative effect on dairy cattle fertility. However, in this breed the negative effect is only moderate, raising the

question of whether this may be due to a favorable microbiota. In the present study we investigated the endometrial microbiota in NR at AI by biopsy and cytobrush samples, and comparing this to the vaginal microflora. The second objective was to describe potential differences at both distinct depths of the endometrium, in healthy vs SCE positive NR cows. We sampled 32 lactating and clinically healthy Norwegian red cows in their second heat or more after calving, presented for first AI. First, we obtained a vaginal swab and a cytobrush sample, in addition to a cytotape to investigate the animal's uterine health status with respect to SCE. Secondly, we acquired a biopsy sample from the uterine endometrium. Bacterial DNA from the 16S rRNA gene was extracted and sequenced with Illumina sequencing of the V3-V4 region. Alpha and beta diversity and taxonomic composition was investigated. Our results showed that the microbiota of endometrial biopsies was qualitatively different, richer, and more even than that of cytobrush and vaginal swab samples. The cytobrush samples and the vaginal swabs shared a similar taxonomic composition, suggesting that vaginal swabs may suffice to sample the surface-layer uterine microbiota at estrus. The current study gave a description of the microbiota in the healthy and SCE positive NR cows at AI. Our results are valuable as we continue to explore the mechanisms for high fertility in NR, and possible further improvements.

Keywords: Subclinical endometritis, microbiota, Norwegian Red, 16S, Uterine biopsy

1. Introduction

The optimization of dairy cattle fertility is crucial for production efficiency [1, 2] and to reduce emissions per unit of milk [3]. One factor with a major negative impact on fertility is early embryo death [4], which is affected by a suboptimal uterine environment [5]. Certain changes in the uterine microbiota cause uterine disease. For instance, there is an association between dysbiosis and the development of metritis and purulent vaginal discharge [6, 7]. The individual capacity of developing resistance or tolerance against uterine disease might also be related to variations in the uterine tract microbiota [8].

Even though the uterine tract microbiota in dairy cattle is a field of increasing interest, it is under-explored in terms of specific taxonomy and functional aspects [8, 9]. Reasons for this might include the challenge in accessing the tissue in a sterile manner in living animals, and the expected low microbial mass. Our understanding of the uterine microbiome has changed with the introduction and development of 16S rRNA metagenomic sequencing. However, a challenge of microbiome studies from low-biomass sites such as the uterine tract is the introduction of contaminants, both during handling and from laboratory reagents. As part of current recommendations, a blank extraction control should be included [10]. Many studies have not included negative controls and might have erroneously appointed contaminants as microbiota present in body sites of low expected microbial biomass [11]. One example is the question of a human placental microbiota: With the initial understanding of this body site being sterile, a metagenomic study presented the presence of a unique microbiome [12]. However, one recent study showed that when accounting for naturally occurring microbiota in DNA extraction kits and well-to-well contamination, no such microbiome could be confirmed [13]. With contradicting results regarding body sites of expected low microbial mass, it is highly relevant to continue the exploration of the reproductive tract microbiota in both healthy and diseased animals, along with refined

68 methodological recommendations. Such data could help us to establish microbial biomarkers and
69 dysbiosis indexes that could improve dairy cattle fertility [8].

70 To investigate the uterine microbiota, it is common to use flush samples, swabs, or cytobrush samples.
71 One previous study investigated the microbiome of endometrial biopsies, arguing that the deeper layers
72 of the endometrium might possess a different microbiota than the uterine lumen. They speculated that
73 the findings could reveal more invasive bacteria with associations to different diseases or disorders [14].
74 It has also been suggested that pathogens can be transferred to the uterus via a hematogenous route
75 from the gut [15], and those bacteria might be more abundant in the deeper cell layers of the uterus
76 that can be reached by a biopsy sample.

77 There are a vast number of studies investigating the microbiome related to metritis, endometritis, or
78 purulent vaginal discharge [7]. Subclinical endometritis (SCE) is another condition that affects fertility in
79 dairy cattle that has been explored during the last 20 years. The condition consists of a persistent
80 presence of polymorphonuclear cells in the post-partum endometrium, exceeding a naturally occurring
81 first line of defense [16]. According to the definition, SCE occurs when there are no symptoms of clinical
82 disease, when the cytological changes occur at a pre-defined level (elevated PMN; referred to as
83 cytological endometritis (CYTO)), and when fertility is reduced. Only two studies have investigated the
84 microbiome related to this condition [17, 18]. Both studies concluded that SCE is not associated with
85 changes in the uterine microbiome. Hence, the current hypothesis states that SCE is mainly affected by
86 uterine immune regulation [17]. However, these studies only investigated the superficial endometrial
87 microbiota at set timepoints post-partum. Information about the microbiome at artificial insemination
88 (AI) is still missing, both concerning the superficial layers compared to deeper layers of the
89 endometrium, as well as potential associations with SCE at this point of the production cycle.

90 Norwegian Red (NR) is the main dairy cattle breed in Norway. Female fertility has been included in the
91 total merit index in Norway since 1972 [19]. As a result, Norwegian Red cows are notable for their good
92 reproductive performance. The breed has a reported pregnancy incidence of 62.9% and calving rate of
93 56.3%, both to first AI [20], and a more recently reported 56-d nonreturn rate (NRR) of 72.9% [21].
94 Recently, we found that NR had a high prevalence of SCE at AI with only a moderate effect on fertility
95 compared to Holstein [22]. One study showed an indication of this condition having a heritable
96 component in NR [23], but the majority of the variation was not explained by this factor. Hence, it is not
97 known if the relatively positive outcome from SCE in NR is due to a beneficial immune regulation, certain
98 traits of the microbiota, other factors, or combinations of such. One step towards a better
99 understanding of this question, was to investigate the uterine microbiota at AI. The main objective of
100 the current study was to investigate the endometrial microbiota in NR at AI, by comparing the deep
101 layer to the superficial layer of the endometrium and considering associations to the vaginal microflora.
102 The second objective was to describe potential differences at these distinct depths of the endometrium,
103 in healthy vs SCE positive NR cows.

104
2. Materials and Methods

105
2.1 Experimental design and study population

106 The present observational cross-section study was conducted at The Animal Production Experimental
107 Centre, NMBU in Ås, Norway, from October 2017 to March 2018. Ethical approval was provided by the
108 Norwegian Food Safety authority with approval ID 11732. The study unit was lactating NR cows in their
109 second heat or more after calving, presented for first AI. The reproductive tract samples were collected
110 from 32 cows on different days according to their natural heat, as detected by activity monitors and
111 visual inspection. Before sampling, all animals were clinically examined by one of three veterinarians.
112 The following parameters were controlled: General appearance, desire to feed, mucous membrane
113 color, rectal temperature, heart rate, respiration rate, and udder appearance. To evaluate the vaginal
114 mucus characteristics, a mucus sample was collected using a Metricheck (Simcro, Hamilton, New
115 Zealand) that was advanced to the level of the cervix and withdrawn. The evaluation was conducted
116 according to a scale from 0 to 3 [24]. Only healthy animals with vaginal mucus score 0 (clear or
117 translucent mucus) were included in the study. Body condition scoring at the sampling day was
118 registered by DeLaval Delpro (DeLaval, Ski, Norway) on a scale from 1-5 with 0.1 intervals, which was
119 based on the NR body condition scoring system [25].

120 To evaluate the milk progesterone level and hence confirm the heat status, a milk sample was collected
121 from each animal, by hand from one teat. A Broad Spectrum MicroTabs tablet was added (D&F Control
122 Systems Inc., Dublin, USA), and the samples were stored frozen at −20°C before laboratory analysis. The
123 progesterone concentration was measured using an enzyme immunoassay [26], modified by a second
124 antibody coating technique. The specificity of the monoclonal antibody for this method was described
125 previously: The intraassay coefficient of variation was <10%, while the inter-assay coefficient of variation
126 was 9.2% and 5.3%, at milk progesterone concentrations of 1.48 and 19.66 ng/mL, respectively [27]. In

127 the present study, a progesterone concentration of >3.0 ng/mL was considered to indicate that the cow
128 was not in estrus.

129 2.2 Uterine sampling and diagnostic method for cytological endometritis

130 Sampling from the uterus was performed by two different veterinarians who had practiced the
131 procedure together on organs from slaughterhouses. The sampling was performed in two phases. In the
132 first step, we obtained a vaginal swab and a cytobrush sample. Here, we also used a paper tape
133 (cytotape) to investigate the animal's uterine health status with respect to SCE. The second step
134 consisted of acquiring a biopsy sample from the uterine endometrium. A dual-purpose instrument for
135 the collection of a diagnostic sample for cytological endometritis and a cytobrush sample was prepared
136 in the laboratory, according to the method developed by Pascottini et al. [28]. A cytobrush (535010, Jan
137 F.Andersen A/S, Jevnaker, Norway) was attached to the stylet of a sterile stainless steel insemination
138 gun. Cytotape was glued around the top of the same gun, and the device was covered with a disposable
139 plastic tube (Sheath protector tubes, Continental plastic, Delavan, USA). Before sampling, the vulva and
140 the perineum were cleaned with lukewarm water and Hibiscrub (Mölnlycke Health Care AB, Göteborg,
141 Sweden) and dried with paper towels. Avoiding any contact with the external genitalia, a sterile cotton
142 swab was used to collect bacteria from the vagina, and further deposited into a sterile Eppendorf tube.
143 The dual-purpose instrument was then inserted into the vaginal canal and advanced through the cervix
144 to the uterine body. The top of the protective tube was penetrated, uncovering the insemination gun
145 with the cytotape. First, the cytotape was rolled against the endometrium to collect cells, giving it a
146 slight pressure with a finger from the rectum. Secondly, the cytobrush was released into the uterine
147 body and rotated towards the uterine wall in the same manner. The cytobrush was then retracted into
148 the insemination gun, and the insemination gun was again pulled back into the protective plastic tube.
149 After that, the tube was carefully drawn back out of the reproductive canal.

150 The sampling device was transported to the laboratory. To avoid contamination, the plastic tube was
151 dried off with paper and cut off at the top using sterile scissors, and the sample was taken out on the
152 clean, cut, end. The cytobrush sample was directly transferred to a sterile Eppendorf tube, and instantly
153 frozen in liquid nitrogen. It was further stored in a freezer at -80° Celsius. The same freezing conditions
154 were used for the vaginal swabs. The cytotape was rolled against a glass slide and air dried, followed by
155 a fixation and staining using Dip Quick Stain (Jorvet, J0322A1, A2, A3 Jorgensen Laboratories, Loveland,
156 USA). After this, the sample was evaluated at 400X magnification in a bright field microscope, counting
157 in total 300 representative PMN and epithelial cells in several fields, and calculating the proportion of
158 PMN. A threshold of 5% PMN was used to diagnose SCE [29].

159 After the SCE diagnosis was set, the second phase of sampling was initiated. Again, the vulva and
160 perineum were washed and dried in the same manner. A sterile biopsy forceps (Kruuse biopsy
161 instrument, 141700 Kruuse, Norway) was covered with a sanitary sleeve (340842 Kruuse, Drøbak,
162 Norway) and introduced into the reproductive tract without touching the external genitalia. The forceps
163 were advanced into the uterine body and the sanitary sleeve was penetrated at the top by pulling it
164 back. With the pressure from a finger on the rectal side, the forceps were pressed against the
165 endometrium to cut off the biopsy. The forceps were then withdrawn from the reproductive tract. Any
166 tissue or mucus on the outside of the forceps was cut off with a sterile surgical blade, and the closed
167 instrument was cleaned of with a paper towel drenched in 70% ethanol. The biopsy was transferred to a
168 sterile Eppendorf tube using a new sterile surgical blade, and instantly frozen in liquid nitrogen, then
169 transferred to a freezer holding -80° Celsius.

170 After sampling, each animal was artificially inseminated with cryopreserved semen. Information about
171 the NRR at 56 days was retrieved from the breeding company Geno SA.

172 ## 2.3 DNA extraction, qPCR and sequencing

173 Thawing and DNA extraction from biopsy, vaginal swabs and cytobrush samples was performed in 13
174 rounds with 4-5 samples at a time, using the QIAamp Cador mini Pathogen kit (QIAGEN, Hilden,
175 Germany), according to the manufacturer's recommendations. As a negative control for the extraction
176 process, DNA was extracted from DEPC water, and the resulting material was sequenced in the same
177 manner as the rest of the samples. Each extraction round contained one negative extraction control. The
178 negative controls from three different rounds were pooled before sequencing. The biopsies had a
179 weight of 12-25 mg per sample. For efficient lysis of tissue, biopsies were pretreated enzymatically using
180 protocol T2 as described by the manufacturer. In this step, ATL mixed with Proteinase K was added,
181 followed by vortexing and an overnight incubation at 56°C. The negative controls were also subjected to
182 this pretreatment. For further lysis of bacteria, biopsy samples, negative controls, cytobrush and swab
183 samples were subjected to the pretreatment B1, before continuing with the remaining extraction
184 procedure. Nanodrop (Nanodrop 1000, Thermo Fisher Scientific, Waltham, USA) was used to assess DNA
185 quality using the 260/280 and 260/230 ratios, and a Qubit fluorometer with the dsDNA HS Assay kit (0,1-
186 120 ng/µl, Thermo Fisher Scientific, Waltham, USA) was used to assess DNA concentration.

187 The bacterial count in the different sample types was estimated using qPCR quantification and
188 compared to a dilution series of a standard with known 16S rRNA copy number. For this estimate, a
189 subgroup of 30 samples (the first 30 samples that we extracted DNA from) were individually subjected
190 to this analysis (20 biopsies, 5 cytobrush and 5 vaginal swabs). Copies of the 16S rRNA gene were
191 quantified using a previously described primer set (forward primer: 5'-TCCTACGGGAGGCAGCAGT-3';
192 reverse primer: 5'-GGACTACCAGGGTATCTAATCCTGTT-3') [30] in a total reaction volume of 20 µl on a
193 Mx3005p Real-Time PCR System (Agilent Technologies, Santa Clara, USA). Each reaction contained: SYBR
194 GreenER qPCR Supermix Universal Kit (Invitrogen, Waltham, USA), 0.2 µM of each primer, 50 nM ROX

195 dye and 2 µl of template DNA. The cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C
196 followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C, and dissociation for 1 min at 95°C, 30 sec at
197 55°C and 30 sec at 95°C.

198 To explore the bacterial microbiota, the V3-V4 hypervariable regions of the bacterial 16S gene were
199 targeted using the 341F/785R primer pair [31]. In addition to the biopsies, vaginal swabs and cytobrush
200 samples, pooled negative extraction controls, negative control (sterile water) and positive control
201 (ZymoBIOMICS Microbial Community DNA Standard II (Zymo Research, Irvine, USA)) were included.
202 Amplification of the 16S V3-V4 region was performed based on the 2-step PCR procedure described in
203 the Illumina application note
204 (https://support.Illumina.com/documents/documentation/chemistry_documentation/16s/16s-
205 metagenomic-library-prep-guide-15044223-b.pdf, 05.01.2023). 32+8 cycles were used. Library size was
206 checked using an Agilent Tape station 4200 with High Sensitivity reagents (Agilent, Santa Clara, USA).
207 Sequencing libraries of expected size (~630 bp) were pooled and size selected on a gel. Sequencing was
208 performed on an Illumina MiSeq, using V3 reagents with 2 x 300 bp reads. 30 % PhiX control library was
209 added to the 16S libraries, and cluster density was reduced to 80% of regular levels. Base calling was
210 performed using Real Time Analysis Software (RTA) version 1.18.54, followed by bclfastq v2.18.0.12 to
211 demultiplex the raw data and produce fastq files.

212 2.4 Statistical analyses

213 The raw sequences were deposited in the SRA archive (NCBI) with bioproject ID PRJNA841790.
214 Bioinformatic analyses to obtain taxonomy and diversity data were performed using the QIIME2 pipeline
215 version 2021.8 [32]. The DADA2 plugin [33] was applied for filtering, denoising and chimera removal.
216 The demultiplexed sequences were trimmed at 18 base pairs at the 5' end for all reads, and at 300 and
217 255 base pairs at the 3' end for forward and reverse reads, respectively. This decision was based on a

218 Phred score with lowest median of 28 and lowest value of 18 in two base pair positions. The resulting
219 high-quality sequences were clustered to amplicon sequence variants (ASVs).

220 The bacterial taxonomic analyses were performed using a Naïve Bayes classifier trained on SILVA
221 reference sequences version 138.1 [34] that was preprocessed using the rescript plugin [35]. The
222 classifier was further adapted to the investigated region of 16S using the q2-feature-classifier [36]. Once
223 the taxonomy file was generated, the sequences were filtered for mitochondria, chloroplasts, and
224 archaea. Additionally, all features that did not reach a classification of at least phylum level were
225 removed as we suspected them to be a result of host-specific DNA (q2-taxa plugin) [32]. The resulting
226 features were used in downstream analyses. For the generation of the taxa barplot figure in QIIME2, the
227 features were filtered to retain only those that appeared in at least two samples and at least at a
228 frequency of 4000. This was done for the purpose of visual clarity to retain the most highly represented
229 features.

230 For phylogenetic diversity analyses, a phylogenetic fasttree [37] was generated by aligning the ASVs with
231 MAFFT [38], which integrated the mask method [39]. Further, the alpha rarefaction plot was generated
232 to find the optimal rarefaction depth and investigate whether the sequencing was deep enough.

233 To study the alpha diversity, we used Chao1 [40], Pielou's Evenness [41] and Shannon metrics [42] in the
234 QIIME2 pipeline at a rarefaction depth of 27.500 at which all samples were included. Pairwise
235 comparisons between sample types and SCE status were calculated using the Kruskal-Wallis analysis of
236 variance after multiple testing correction with Benjamini/Hochberg (non-negative) FDR adjustment.
237 Bray-Curtis measure of dissimilarity [43] and Weighted unifrac [44] in the QIIME2 pipeline were used as
238 estimates for beta diversity. The latter, but not the first, takes phylogeny into consideration. Based on
239 the beta diversity, the differences between sample types and the SCE status were calculated using
240 PERMANOVA. An adjusted p-value (q-value) of < 0.05 was considered significant.

241 To investigate which taxa that best could explain the differences between the groups, we used Linear
242 discriminant analysis effect size (LDA-LEfSe) [45] through the online Galaxy tool
243 (https://huttenhower.sph.harvard.edu/galaxy/ 14.11.2022). The class was sample type, and the subclass
244 was SCE status. A significance level of 0.05 was used for factorial Kruskal-Wallis test among classes and
245 the pairwise Wilcoxon test between subclasses, and the effect size threshold was set to 3.0. The strategy
246 for multi-class analysis was one-against-all. We included the negative extraction controls in the analyses
247 in order to eliminate potential contaminating taxa from the comparison.

248 # 3. Results

249 ## 3.1 Descriptive statistics

250 Twenty-four animals were included in the study, of which three cows were positive for subclinical
251 endometritis. Supplementary Table 1 provides an overview of the descriptive statistics. Out of all
252 included animals, only one was considered to have been sampled and inseminated outside of heat,
253 based on milk progesterone levels. The median DIM was 53 (41 - 67) with one outlier sampled at day
254 170 after parturition. The body condition score ranged between 3.1 and 4.1 with a median of 3.8. At 56
255 days after AI, 10 out of the 24 sampled animals did not enter a new estrus with a subsequent
256 insemination, resulting in a NRR of 41.7%.

257 The sequencing provided a median of 113098 (12970 – 214853) raw sequences per sample. After data
258 cleaning, which included denoising, chimera removal and taxonomic filtering, a median of 60537 (9011-
259 91454) high quality sequences per sample were kept for further analyses. The alpha rarefaction plot
260 confirmed that the sequencing depth was sufficient to describe the bacterial microbiome, as it leveled
261 out for all sample types.

262 ## 3.2 Quantification by qPCR

263 Supplementary figure 1 shows the number of genome copies estimated by qPCR for a subgroup of 30
264 samples. Results showed that the bacterial load was highest in the vagina (13.4 - 6126.3 genome
265 copies/µl, median 706), and lower in the cytobrush (1.3 - 16.1 genome copies/µl, median 7.5) and
266 biopsy samples (1.4 - 340.9 genome copies/µl, median 18.6). One cytobrush sample had a very high
267 number of gene copies compared to the others (78048.1 copies/ µl). Using Grubb's test
268 (https://www.graphpad.com/quickcalcs/grubbs1/ 06.01.2023) this sample was detected as an outlier (p
269 < 0.05) and was not included in the figure.

270
3.3 Alpha and beta diversity analysis

271 Figure 1 shows the alpha diversity measurement for each sample type. The Chao1 measurement
272 showed no difference in richness between the different sample types, while both Pileou's evenness and
273 Shannon showed a difference between biopsy and each of the two other sample types ($q < 0.0008$ for
274 both). Supplementary Table 2 presents the outcome from Kruskal-Wallis calculations for each pairwise
275 comparison. No difference in alpha diversity was seen between SCE positive and negative individuals.

276 The beta diversity is visualized in PCoA plots (Figure 2). For Bray Curtis, the three axes explained 43.1 %
277 of the total differences between the samples, while for Weighted unifrac the corresponding number was
278 74.1%. For both measurements, there was a clear clustering of the biopsy samples compared to the
279 other sample types, which were more scattered in general in all dimensions. The three biopsy samples
280 belonging to SCE positive animals were clustered together, but that cluster did not differ visually from
281 the other biopsy samples. The PERMANOVA calculations for the pairwise comparison (Supplementary
282 Table 3), revealed a difference between biopsy and the other two sample types ($q = 0.0015$). There was
283 no difference between cytobrush and vaginal swab based on the Weighted unifrac diversity
284 measurement. In contrast, cytobrush vs vaginal swab showed a difference with the Bray-Curtis
285 dissimilarity measurement. For the SCE-status, there was no difference in beta diversity for either of the
286 measurements. There was no clustering of samples based on the lab extraction round.

287 The negative extraction controls had a low total richness, and a high evenness meaning that the
288 microbiota consisted of an even mix of few taxa, see Supplementary Figure 2. For the beta diversity,
289 these samples were distributed together with the biopsy samples, but also clustered at one end.

290

291 ## 3.4 Taxonomy composition and differential abundance analysis

292 In total, 319 bacterial genera were identified. To simplify visualization and interpretation, the dataset
293 was filtered to show only those ASVs appearing in at least two samples and at a frequency of 4000
294 highlighting the 29 most abundant bacterial genera. Their relative abundances in the different samples
295 are shown in Figure 3. From this outcome, the dominant represented phyla were Proteobacteria,
296 Firmicutes, Actinobacteriota and Bacteroidota. The differential abundance analysis of the complete
297 dataset (Figure 4) showed that Bacilli had a higher abundance in the vaginal swabs and the cytobrush
298 samples, compared to the biopsy samples. In the biopsy samples, Clostridia, Bacteroidia and
299 Bacteroidota were among the enriched taxa.

300 The bacterial genus with the highest overall abundance in all samples combined was *Streptococcus*,
301 which was present in 16 out of 18 cytobrush samples and all vaginal samples. In 11 out of these samples,
302 *Streptococcus* represented more than half of the relative abundance, and up to 99.5%. It was barely
303 detected in the biopsy samples (< 0.8%). The differential abundance analysis confirmed that
304 Streptococcus was enriched in the cytobrush and vaginal swab samples compared to the biopsy
305 samples. Escherichia-Shigella had a similar pattern with highest abundance in the vaginal swabs.
306 Mycoplasma was also more abundant in the cytobrush samples (identified in 3 samples) and the vaginal
307 swabs (identified in 3 samples).

308 In the 23 biopsies, we found a high abundance of *Oscillospiraceae UCG-005* in 17 samples (relative
309 abundance 0.6 - 44.7%) and *Bacteroidetes_vadinHA17* in 15 samples (0.3 - 17.8%). Other taxa with high
310 relative abundance in the biopsies were genera *Ruminococcus*, *Bacteroides*, *Alysiella* and four different
311 genera of the family Lachnospiraceae. The mentioned taxa were not present in the negative extraction
312 controls, and barely in the cytobrush and vaginal swab samples.

313 The negative extraction controls showed a variety of taxa that were also present in the other sample
314 types. They were dominated by genera *Massilia*, *Burkholderia*, *Polaromonas*, and *Flavobacterium*. The
315 first three were also present in high abundance in the other sample types. *Massilia* was the second
316 highest represented genus in all samples combined. It was present in all negative extraction controls
317 (relative abundance 32.1 - 58.0%), in all biopsies (1.7 - 89.5%), 15 out of 18 cytobrush samples (0.2 -
318 72.4%), and 4 out of 13 vaginal swab samples (3.9 - 46.6%). *Burkholderia* and *Polaromonas* were the
319 fifth and sixth most abundant genera in general, and had a similar pattern to *Massilia* with respect to
320 appearance in the different sample types. *Flavobacterium* represented up to 15.5% of the relative
321 abundance in the negative extraction controls, but it was barely present in the other sample types (<
322 2.1%).

323

324 # 4. Discussion

325 ## 4.1 Ecologic diversity and bacterial load: sample type

326 The present study investigated the microbiota of the reproductive tract in NR cows at AI, using three
327 different sample types. We demonstrated that the microbiota of endometrial biopsies is qualitatively
328 different, richer, and more even than that of cytobrush and vaginal swab samples. There were more
329 inter-individual differences in the microbiota of the cytobrush and vaginal samples, than in the biopsy
330 samples. We also found that the microbiota from cytobrush samples and vaginal swabs had no
331 significant difference in alpha or beta diversity and a similar taxonomic composition. This result suggest
332 that the vagina and uterus share a common microbiota, at least when the cow is presented for AI at
333 natural heat. Following this, one may also question the necessity of taking a cytobrush sample instead of
334 a vaginal swab in future investigations of uterine microbiota and its correlation to different disorders
335 and diseases, at least in periods where the anatomical restriction between vagina and uterus is weak,
336 such as in estrus [8]. The microbiota of a biopsy sample, however, could reveal important information
337 that is not captured through the other two sample types. There is some evidence suggesting the
338 transmission of pathogens from the gut to the uterus via the hematogenous route [15]. Possibly, deeper
339 layers have a higher load of bacteria descended from this route while the superficial endometrium and
340 vagina are more likely to be affected by extrinsic and ascending pathways. It is important to remember
341 that the microbial biomass in the biopsy samples and the other two sample types may differ. Our results
342 from the biopsies have to be interpreted with caution, as earlier literature shows that when the starting
343 microbial mass is low, the proportion of contaminant bacterial DNA increases [46].

344 One previous study compared the uterine microbiota using biopsies and flush samples at week 1, 4 and
345 7 post-partum. In concordance with our study, they concluded that the microbiota of the biopsy
346 samples was richer and had a higher evenness. They also hypothesized that the richness of the uterine

347 microbiota would decrease as the cow got closer to completing the involution process [14]. The present
348 study implies that the uterine biopsies still have a rich microbiota far after the involution process and at
349 the normal timepoint for AI in NR. Furthermore, the number of days between calving and sampling (41-
350 170) did not seem to influence the microbial composition in the present study. This result is interesting
351 from a practical point of view, as current recommendations from Geno are that AI may start from day 42
352 [47].

353 Quantification by qPCR of a subgroup of samples showed that the median bacterial load was much
354 higher in the vaginal swabs compared to cytobrush and biopsy. This is not surprising and concords with
355 results from the female genital tract by Chen et al. [48]. In that study, both the endometrium and the
356 vagina were sampled with sterile swabs. Our results also support our hypothesis that the contamination
357 from the vagina to the uterine samples during the sampling process in field in the current study was low.
358 The bacterial load was similar and of low biomass in the biopsies and the cytobrush samples. The one
359 earlier study that investigated bovine uterine biopsies by 16S analysis did not perform any quantification
360 [14], and neither did the two studies that investigated the microbiota in healthy cows and cows with SCE
361 [17, 18].

362 4.2 Ecologic diversity: SCE diagnosis

363 Along with collecting three different sample types from the reproductive tract, the individuals in the
364 present study were investigated for SCE. This design was initially set up to calculate the differences in
365 microbiota related to this condition in NR, but it also provided important knowledge of the uterine
366 status in our study population in general. Instead of using the term SCE, CYTO has been suggested as a
367 more appropriate alternative when referring to cytological changes in the endometrium alone.
368 However, the two expressions are not used in a consequent manner in literature. We chose to use SCE
369 in the present article, as it is more commonly used and better coordinates with the two earlier studies of

370 the microbiome related to PMN in the uterus. Interestingly, very few individuals were positive for SCE in
371 the investigated herd, compared to the earlier presented prevalence level of 28% [22]. The low
372 prevalence in the present study marks a limitation in the statistical analysis of how the microbiota is
373 affected by SCE, and this part of the study should be considered as descriptive. The prevalence
374 discrepancy also confirms that herd factors, management and seasonal variability affect the occurrence
375 of SCE [22, 49, 50]. The three biopsies from SCE positive animals formed a cluster in the beta diversity
376 PCoA visualization which indicates that there are similarities between them, although this cluster did not
377 seem to separate from the remaining biopsy samples. Our results coincide with studies from Wang et al.
378 [18] and Pascottini et al. [17], who both concluded that there was no difference between SCE positive
379 and healthy cows at 30 and 10/21/35 days postpartum, respectively. These two studies used uterine
380 flush samples or cytobrush samples, but neither used biopsies. The microbiota from the deeper layers of
381 the uterus might have a correlation with SCE even if the superficial bacterial population does not show
382 such a correlation. The mechanism for the presence of PMN in the endometrium may well be more
383 affected by invasive bacteria or bacteria present in the deeper layers of the endometrium than the
384 superficial layers. Likewise, our study pointed to differences between the different sample types. By all
385 means, studying uterine biopsies could influence the current understanding of SCE as a condition
386 affected mainly by the immune regulation of the animal [16, 51]. However, while a biopsy might provide
387 important information, the sampling is less feasible to perform on at high throughput. Furthermore,
388 taking a biopsy from the uterus at AI might itself affect fertility, which is relevant for routine diagnostics
389 or in studies where downstream fertility is a response variable. In the present study, fertility was most
390 likely affected by the biopsy sampling. Few sampled animals (41.7%) maintained their pregnancy at 56
391 days after AI, comparing our results to the average non-return-rate in NR of 72.9% [21]. Recently,
392 Ramirez-Garzon et al [52] published a review on the effect of endometrial sampling procedures on the
393 subsequent pregnancy rate in cattle. They concluded that endometrial biopsy does not have a negative

394 effect on fertility. However, their paper did not include evaluations of biopsies taken the same day as
395 performing the AI, which was the method used in the current study.

396 ### 4.3 Taxonomic composition

397 The most highly represented phyla in the present study were Proteobacteria, Firmicutes,
398 Actinobacteriota and Bacteroidota. Our results concord with earlier studies of the bovine reproductive
399 tract using next generation sequencing in healthy cows or cows with SCE [14, 18, 53]. Interestingly, all
400 the mentioned studies also found a high abundance of Fusobacteriota which was not detected at all in
401 the present study. Fusobacteriota is associated with the development of metritis [7, 53, 54] and
402 purulent vaginal discharge [6]. NR has a uniquely low occurrence of metritis and endometritis based on
403 a low registered treatment rate in the Norwegian Dairy Herd Recording System of 1.3 treatments per
404 100 cow-years [55]. Likewise, Diaz-Lundahl et al [22] found purulent vaginal discharge in only 10 out of
405 1,648 NR cows when sampling was performed with Metricheck at AI. The reason for the differences in
406 the abundance of Fusobacteriota might also be partly due to that sampling in the mentioned studies
407 was performed earlier after parturition.

408 The cytobrush samples and vaginal swabs presented a less even microbiota, which in some of the
409 samples was dominated by only a few taxa, with particularly the class Bacilli highly abundant.
410 *Streptococcus* appeared in almost all cytobrush and vaginal samples, but barely in the biopsy samples.
411 The family Enterobacteriaceae and the genus *Escherichia-Shigella* was detected mainly in the vaginal
412 swabs. Both *Streptococcus* and Enterobacteriaceae have been identified among the most abundant taxa
413 in earlier microbiome studies of the bovine vagina [56]. Wang et al [18] found more *Streptococcus* in
414 healthy cows than SCE positive cows. We did not detect that difference. The taxonomic composition of
415 the vaginal microbiota appears to differ significantly between individuals [8]. This was also indicated in
416 our data. On the genera level, *Bacteroides*, *Aggregatibacter* and *Streptobacillus* are typically highly

417 abundant. *Lactobacillus,* which is the most common vaginal genus in humans, is also commonly
418 detected in the bovine vagina [8]. Interestingly, the biopsies in the present study had some abundance
419 of *Bacteroides*, while it was barely detected in the cytobrush samples, and missing in the vaginal swabs.
420 The same pattern was seen for other taxa of the phylum Bacteroidota. *Aggregatibacter* and
421 *Streptobacillus* were not present in a rich number in our samples in general, although one cytobrush
422 sample had a high abundance of *Aggregatibacter* (85.3%). *Lactobacillus* genus was not represented
423 among the 29 most highly abundant genera. However, there was a high composition of unclassified
424 Bacilli class in the vaginal samples.

425 One previous study showed that there are differences in the vaginal microbiota in different phases of
426 the estrus cycle in buffalo [57]. Microbial variations throughout the estrus cycle might be a relevant
427 cofounding factor when comparing microbiome with fertility outcome in cattle. In our study, the estrus
428 status of each individual was confirmed by milk progesterone measurement, and all animals except for
429 one had a confirmed heat. This animal did not show a deviant pattern in beta diversity or taxonomy.

430 The bacterial composition of the negative extraction controls was dominated by *Massilia* (family
431 Oxalobacteriaceae*)*, *Burkholderia*, *Polaromonas* (which was also highly abundant in other sample types,
432 especially biopsy) and *Flavobacterium* (mainly in negative controls). It is uncertain whether these taxa
433 represent a kit contamination, if they are actually also present in the reproductive tract, or a
434 combination of the two. This question is not only the reality for the current study, but a general concern
435 when studying microbiota in low biomass samples using next generation sequencing. Negative
436 extraction controls can produce a vast number of sequences and represent a high number of taxa due to
437 kit microflora [46, 58]. Well-to-well contamination is common and further complicates the matter, while
438 barcode leakage is indicated to be of lesser importance [59]. An earlier study lists possible
439 contaminating taxa that appeared in different DNA extraction kits over several years, and *Massilia*,

440 *Burkholderia*, *Polaromonas* and *Flavobacterium* are all among the mentioned taxa [60]. Interestingly,
441 Bacteroides and Lachnospiraceae were found in a high abundance in negative extraction controls by
442 Karstens et al. [46]. In the current study, these taxa were present in the biopsies but not in the negative
443 extraction controls. It has been stated that DNA extraction kit contaminants depends on different kits or
444 lots, and also the laboratory in which the samples are processed [60]. We did not observe a clustering in
445 the beta diversity plot, nor any obvious differences in the taxonomy, based on lab extraction round.

446 Of particular concern with low microbial biomass samples, contaminants may play an outsize role, due
447 to less competition from genuine biological material during amplification. We reason that the taxa that
448 distinguish the biopsies from the other sample types, are the ones appearing in the biopsies without
449 detection in the negative extraction controls. Taking this into consideration, Clostridia and Bacteroidia
450 were among the enriched classes in the biopsies compared to the other sample types. Further,
451 *Oscillospiraceae UCG-005*, *Bacteroidetes_vadinHA17*, *Marvinbryantia*, *Ruminococcus*, *Bacteroides*,
452 *Alysiella* and three different genera of the family Lachnospiraceae were highly abundant. There is some
453 level of concordance between our results and the results from Knudsen et al [14], who compared the
454 taxonomy in biopsy samples with a superficial uterine sample. They also found a high abundance of
455 Bacteroidia in biopsies at 4- and 7-weeks post-partum, and a higher abundance of *Ruminococcus* in
456 biopsies at week 7. Likewise, they found a high abundance of Streptococcaceae, but only at week 1
457 post-partum. Even though it was present at a higher abundance in flush samples, it still appeared at a
458 fair abundance in the biopsies, which it did not in the present study. Further, the family
459 Mycoplasmataceae was more abundant in the superficial uterine samples in both studies. The
460 concordance and the fact that these taxa were not present in the negative extraction controls in our
461 study, strengthens the assumption that they are not a result of a random contamination.

462 ### 4.4 Limitations

463 After this study was initiated, Pascottini et al [61] showed that primiparous cows presented a different
464 composition of uterine bacteria than multiparous cows. This was not considered in the present study
465 and the inclusion of that factor might have affected our outcomes. The most important limitation of this
466 study is related to the use of negative controls. There are large variations in the literature of 16S studies
467 over time and whether this is used or not, and how the results are interpreted, implemented, and
468 presented. The one earlier study investigating the microbiota of uterine biopsies did not mention a
469 negative extraction control [14], and neither did the more recent study by Pascottini et al., although
470 they used sampling blanks as input for Decontam R package to remove ASVs found in control samples
471 from the dataset [17]. Kim et al [10] recommended including negative controls for the full pipeline when
472 investigating low biomass material, and to present the results alongside with the samples. The correct
473 use of sequences appearing in negative controls is still an ongoing discussion [46]. To deal with the issue,
474 some consider all taxa appearing in negative controls as contamination and eliminate them from the
475 dataset. Such an approach might be too strict and might eliminate taxa with a biological relevance [46,
476 62]. Other methods consist of removing low abundance taxa or taxa that are common contaminants or
477 passing the dataset through programs such as Decontam or SourceTracker. A more recently published
478 method is to use a mock microbial community of known composition as a positive control, which will
479 support the interpretation of possibly contaminating taxa and the findings in a negative extraction
480 control [46]. This approach would have been a good option in the present study. However, the method
481 was published after performing the current study. We did not use a negative sampling control (for
482 instance, a swab in open air in the barn). This is recommended for low biomass samples to be able to
483 detect contamination from the environment [10] and was for example used by Pascottini et al. [17].
484 Regarding a positive control, we used the swab from the vagina and a positive sequencing control, as
485 suggested by Kim et al. [10] when other types of positive controls are not suitable or cost-effective.

486 In the current study, we filtered the data because we suspected that some sequences were the result of
487 host DNA. Such filtering might skew the outcome and shift the taxonomic composition and the
488 community diversity and presents a common limitation in the study of microbiome data [11]. This might
489 be even more important when analyzing data of low microbial mass. Another important concern is that
490 no region of 16S rRNA can differentiate between all bacteria. Some regions are more likely to better
491 distinguish a certain set of bacteria than other regions, and vice versa [63]. There might be some
492 discrepancies when our results from the V3-V4 region are compared with studies that used other
493 combinations of 16S regions, such as Pascottini et al. [17] (V4) or Knudsen et al. [14] (V1-V2). One study
494 from 2016 suggested that V4-V6 regions are the most reliable to represent the full 16S rRNA [64], but
495 this is also an ongoing discussion within the field of microbiota studies [11].

496 Another concern regarding 16S analyses is that it is not known whether the outcome of such studies
497 comes from viable bacteria or bacterial remnants. As such, relative proportions of viable and non-viable
498 bacteria may differ between both sample types and different anatomical locations. However, Pascottini
499 et al. [17] partly investigated this matter by doing aerobic and anaerobic bacterial culturing of uterine
500 cytobrush samples that were also investigated using 16S rRNA analyses. They found a concordance
501 between the culture results and the most highly abundant bacteria found in 16S analyses [17],
502 suggesting that 16S analysis of these uterine samples probably does reflect mostly live bacteria.

503 4.5 Conclusions and future perspectives

504 The microbiota of endometrial biopsies was qualitatively different, richer, and more even than that of
505 cytobrush and vaginal swab samples. It remains to be seen whether microbiota from biopsy samples
506 could be correlated to different disorders and diseases even when superficial cytobrush samples are not.
507 Moreover, the cytobrush samples had a similar taxonomic composition to what could be found in
508 vaginal swabs at estrus, suggesting that vaginal swabs may suffice to sample the surface-layer uterine

509 microbiota, although this conclusion also requires further validation. The current study gave a

510 description of the microbiota in the healthy and SCE positive NR cows at AI. The results from the present

511 study are valuable as we continue to explore the mechanisms for high fertility in NR, and possible

512 further improvements.

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696 Tables and figures

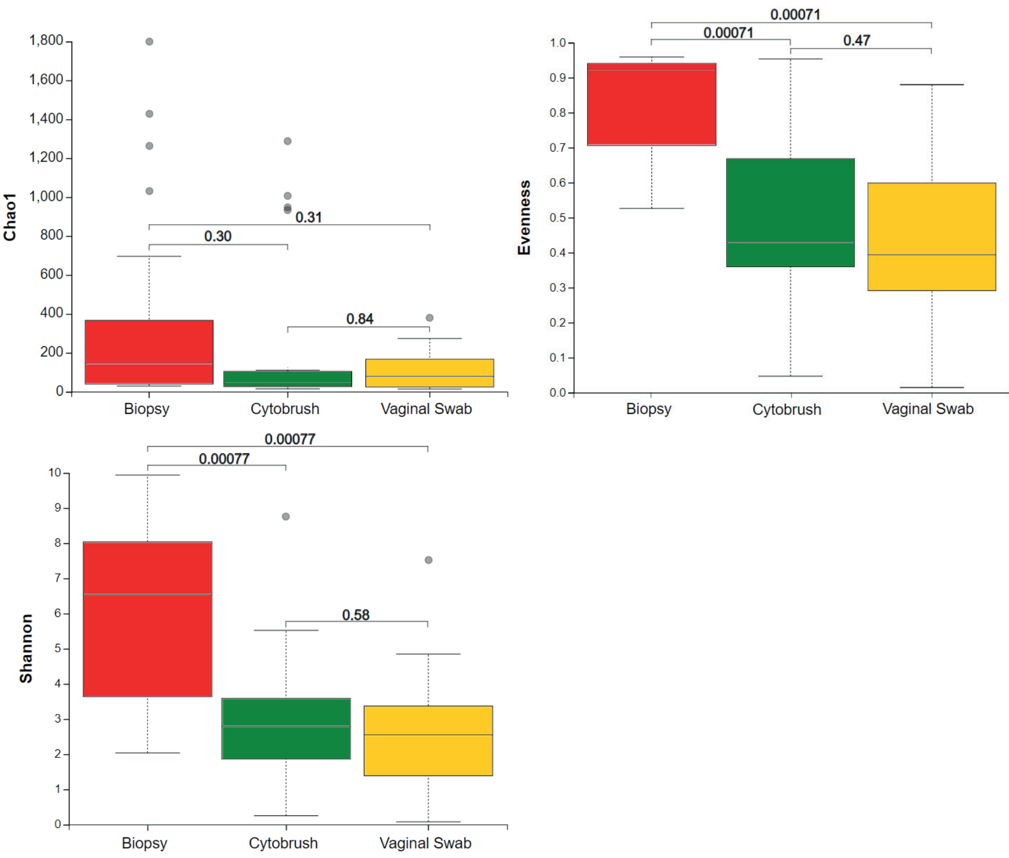
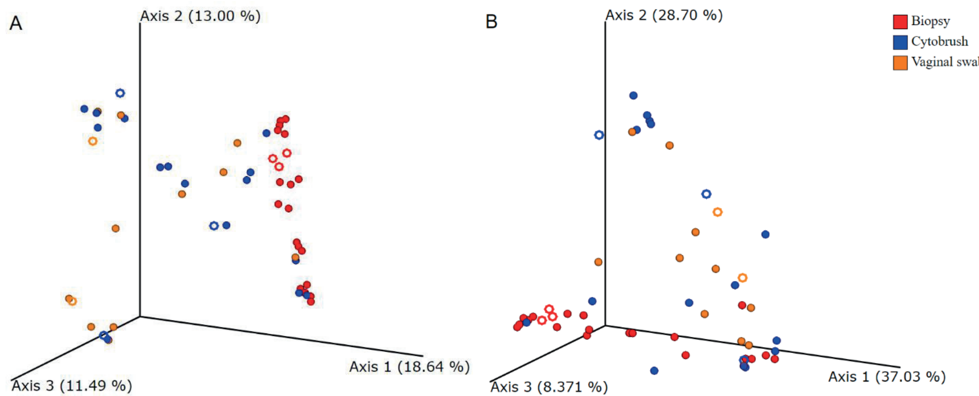


Figure 1: *Alpha diversity for bacterial ASV's from uterine biopsy and cytobrush, and vaginal swab, sampled from NR cows at AI.*

698



699 ***Figure 2:*** *Beta diversity (principal coordinate analysis) of the microbiota in uterine biopsies and cytobrush, and vaginal swabs,*
700 *sampled from NR cows at AI. A: Bray Curtis. B: Weighted Unifrac. Samples from SCE positive animals are marked with a ring.*

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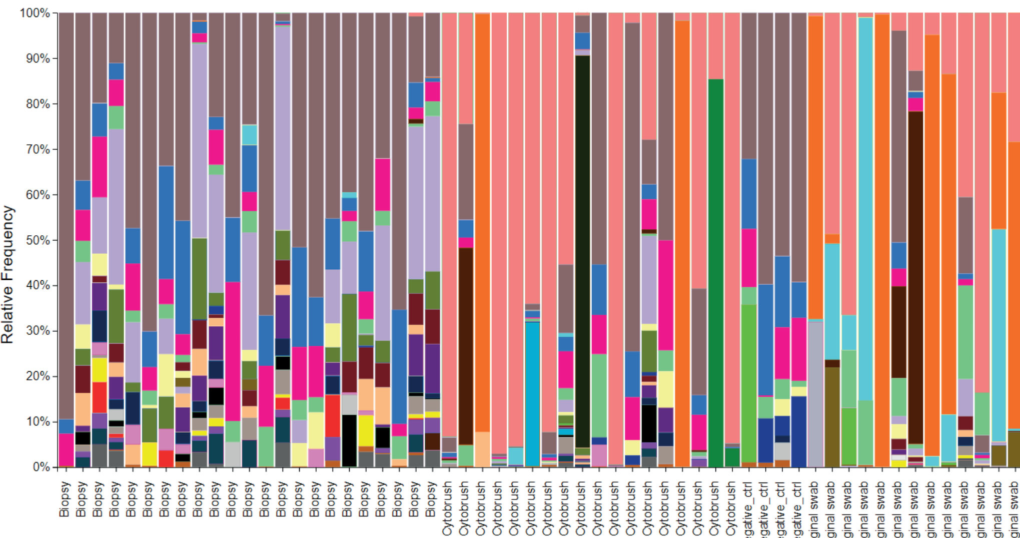

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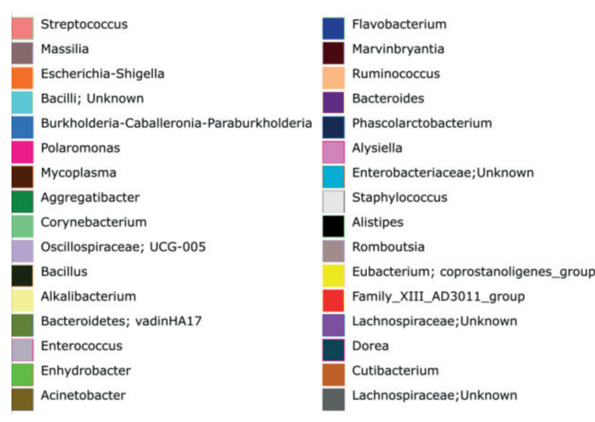

Figure 3 *Taxonomy stacked bar charts for biopsy, cytobrush, negative extraction control and vaginal swabs sampled from NR cows at AI. The figure represents the most highly abundant taxa at genus level, filtered to at least 4000 ASVs, appearing in minimum 2 samples.*

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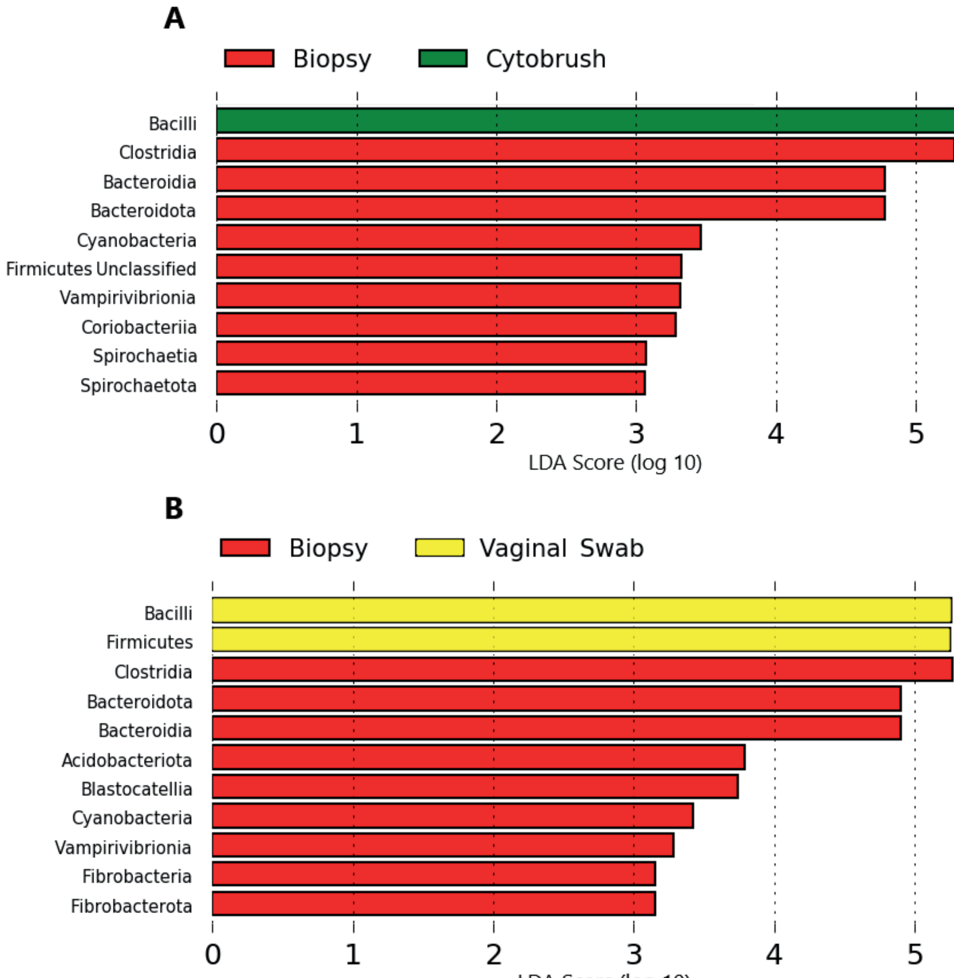

Figure 4: *Logarithmic LDA score for biopsy vs cytobrush (A) and biopsy vs vaginal swab (B) sampled from NR cows at AI.*

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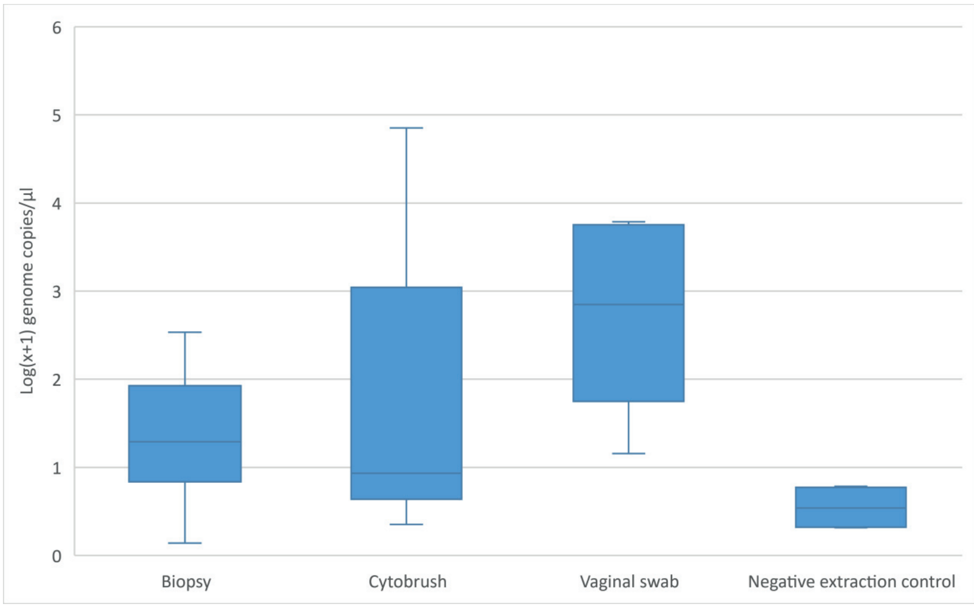

717

718 ***Supplementary Figure 1:*** *Log(x+1) of 16S rRNA genome copies per µl in a subgroup of 30 samples (20 biopsies, 5 cytobrush*
719 *samples and 5 vaginal swabs) sampled from NR cows at AI. One cytobrush sample with a copy number of 78048.1 was detected*
720 *as an outlier and was removed from the figure.*

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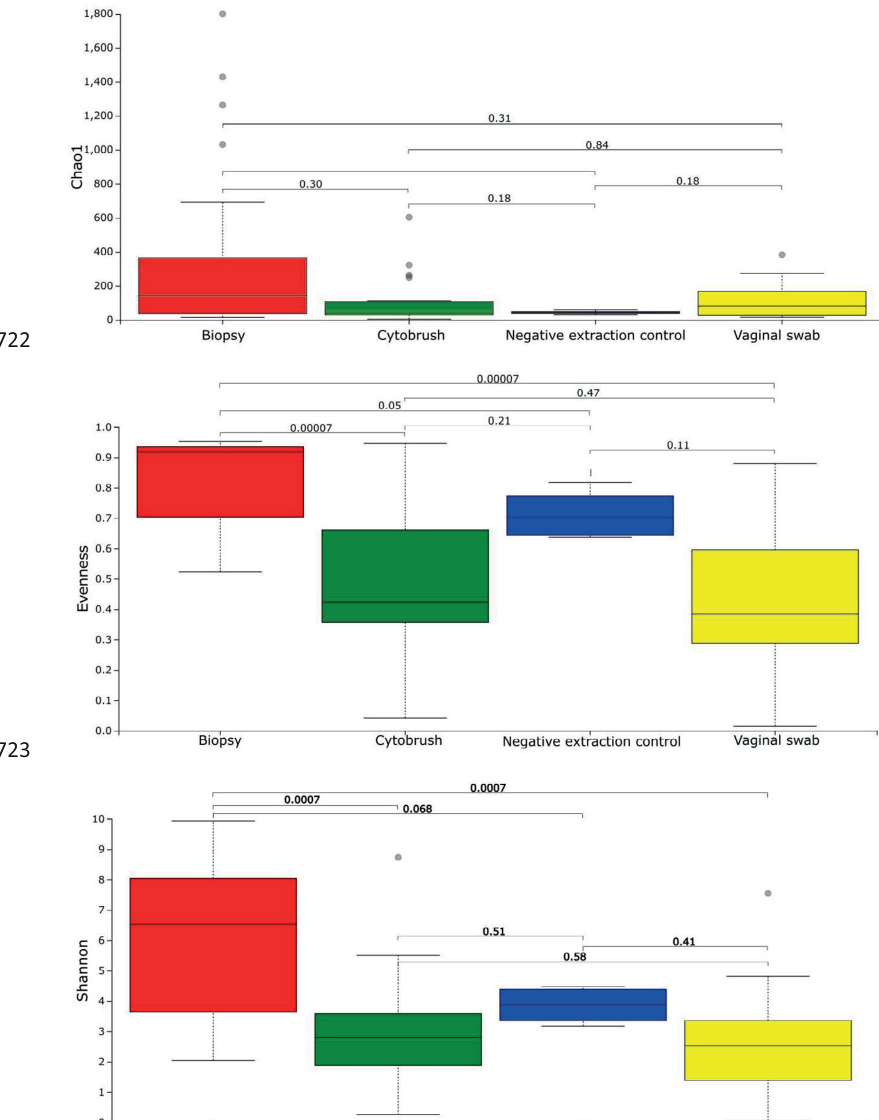

725 ***Supplementary figure 2*** *Alpha diversity for bacterial ASV's for uterine biopsy and cytobrush, negative extraction control and*
726 *vaginal swab, sampled from NR cows at AI.*

727

728 ***Supplementary table 1*** *Descriptive statistics for the sample types that represented each cow, the diagnosis of subclinical*
729 *endometritis (SCE) and the number of polymorphonuclear cells (PMN) when counting 300 cells from a cytology slide. Three*
730 *different sample types were collected from NR cows at AI: B = endometrial biopsy, C = Cytobrush from the endometrium, V =*
731 *Vaginal swab*

Sample type	Number of animals	Number of SCE positive	PMN count per SCE positive animal
B	23	3	39, 12, 12
C	18	3	39, 12, 12
V	13	2	39, 12
B and C	18	3	39, 12, 12
B and V	12	2	39, 12
B, C and V	9	2	39, 12

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734 ***Supplementary Table 2:*** *Pairwise comparison of the alpha diversity between sample types or SE-diagnosis, with Kruskal-Wallis.*
735 *H=diversity value (or, effect size). q-value= Benjamini Hochberg corrected p-value.*

Metric	**Group 1**	**Group 2**	**H**	**p-value**	**q-value**
Shannon	Biopsy	Cytobrush	12.577640	0.000390	0.000767
		Vaginal swab	12.072946	0.000512	0.000767
		Negative extraction control	5.359420	0.020611	0.068702
	Cytobrush	Vaginal swab	0.314103	0.575174	0.575174
		Negative extraction control	0.360000	0.548506	0.506729
	Vaginal swab	Negative extraction control	0.930769	0.334663	0.418329
Chao1	Biopsy	Cytobrush	2.739369	0.097903	0.293710
		Vaginal swab	1.608620	0.204686	0.307029
		Negative extraction control	6.626928	0.010045	0.100448
	Cytobrush	Vaginal swab	0.040105	0.841276	0.841276
		Negative extraction control	2.290258	0.130188	0.179939
	Vaginal swab	Negative extraction control	3.181356	0.074483	0.179939
Evenness	Biopsy	Cytobrush	17.228433	0.000033	0.000071
		Vaginal swab	16.544066	0.000048	0.000071
		Negative extraction control	5.869565	0.015405	0.051350
	Cytobrush	Vaginal swab	0.641026	0.519231	0.471170
		Negative extraction control	1.777778	0.182422	0.202692
	Vaginal swab	Negative extraction control	4.069231	0.043671	0.109178
Shannon	SCE positive	SCE negative	0.048024	0.826539	0.826539
Chao1	SCE positive	SCE negative	0.432345	0.51084	0.510840
Evenness	SCE positive	SCE negative	0.261462	0.609117	0.609117

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739 *Supplementary table 3 Pairwise comparision of the beta diversity calculated by PERMANOVA between sample types or SCE-*
740 *diagnosis. q-value= Benjamini Hochberg corrected p-value.*

SCE diagnosis, pairwise						
Metric	**Group 1**	**Group2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
Weighted Unifrac	SCE positive	SCE negative	54	0.563282	0.678	0.678
Bray-Curtis	SCE positive	SCE negative	54	0.974616	0.465	0.465
Sample type, pairwise						
Metric	**Group 1**	**Group2**	**Sample size**	**pseudo-F**	**p-value**	**q-value**
Weighted unifrac	Biopsy	Cytobrush	41	8.275838	0.001	0.0015
		Vaginal swab	36	9.377037	0.001	0.0015
		Negative extraction control	27	9.792818	0.001	0.0033
	Cytobrush	Vaginal swab	31	1.038841	0.353	0.3530
		Negative extraction control	24	4.912167	0.004	0.0080
	Vaginal swab	Negative extraction control	19	5.185061	0.002	0.0050
Bray-Curtis	Biopsy	Cytobrush	41	3.623646	0.001	0.0015
		Vaginal swab	36	6.056833	0.001	0.0015
		Negative extraction control	27	2.183685	0.027	0.0540
	Cytobrush	Vaginal swab	31	1.832312	0.045	0.0450
		Negative extraction control	24	2.961306	0.001	0.0033
	Vaginal swab	Negative extraction control	19	6.167003	0.001	0.0033
Sample type all (biopsy, cytobrush, vaginal swab)						
Weighted unifrac			53		0.001	
Bray-Curtis			53		0.001	

741

742 ## Author contribution
743

744 Sofia Diaz-Lundahl: Conceptualization, Data curation, Formal analysis, Investigation, Methodology,
745 Validation, Visualization, Writing - original draft

746 Simen Foyn Nørstebø: Conceptualization, Data curation, Investigation, Methodology, Resources,
747 Supervision, Validation, Visualization, Writing - review and editing.

748 Thea Blystad Klem: Conceptualization, Investigation, Methodology, Resources, Writing - review and
749 editing.

750 Gregor Duncan Gilfillan: Data curation, Formal analysis, Methodology, Resources, Writing - review and
751 editing.

752 Marianne Dalland: Data curation, Formal analysis, Resources, Writing - review and editing.

753 Per Gillund: Conceptualization, Funding acquisition, Project administration, Resources, Writing - review
754 and editing.

755 Anette Krogenæs: Conceptualization, Funding acquisition, Investigation, Methodology, Project
756 administration, Resources, Supervision, Validation, Writing - review and editing.

Paper IV

ORIGINAL RESEARCH
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Gene Expression in Embryos From Norwegian Red Bulls With High or Low Non Return Rate: An RNA-Seq Study of in vivo-Produced Single Embryos

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During the last decade, paternal effects on embryo development have been found to have greater importance than previously believed. In domestic cattle, embryo mortality is an issue of concern, causing huge economical losses for the dairy cattle industry. In attempts to reveal the paternal influence on embryo death, recent approaches have used transcriptome profiling of the embryo to find genes and pathways affected by different phenotypes in the bull. For practical and economic reasons, most such studies have used *in vitro* produced embryos. The aim of the present study was to investigate the differences in the global transcriptome of *in vivo* produced embryos, derived from sires with either high or low field fertility measured as the non-return rate (NRR) on day 56 after first AI of the inseminated cows. Superovulated heifers (n = 14) in the age span of 12–15 months were artificially inseminated with semen from either high fertility (n = 6) or low fertility (n = 6) bulls. On day seven after insemination, embryos were retrieved through uterine flushing. Embryos with first grade quality and IETS stage 5 (early blastocyst), 6 (blastocyst) or 7 (expanded blastocyst) were selected for further processing. In total, RNA extracted from 24 embryos was sequenced using Illumina sequencing, followed by differential expression analysis and gene set enrichment analysis. We found 62 genes differentially expressed between the two groups (adj.p-value<0.05), of which several genes and their linked pathways could explain the different developmental capacity. Transcripts highly expressed in the embryos from low fertility bulls were related to sterol metabolism and terpenoid backbone synthesis, while transcripts highly expressed in the high fertility embryos were linked to anti-apoptosis and the regulation of cytokine signaling. The leukocyte transendothelial migration and insulin signaling pathways were associated with enrichments in both groups. We also found some highly expressed transcripts in both groups which can be considered as new candidates in the regulation of embryo development. The present study is an important step in defining the paternal influence in embryonic development. Our results suggest that the sire's genetic contribution affects several important processes linked to pre-and peri implantation regulation in the developing embryo.

Keywords: bull fertility, paternal influence, Norwegian Red bulls, RNA-seq, gene expression, bovine preimplantation embryos, embryo mortality, *in vivo* produced embryos

INTRODUCTION

Embryo mortality is an issue of concern in dairy cattle breeding, being the most common cause for failed pregnancy (Diskin et al., 2006), with negative consequences for milk and food production and corresponding economic impact. The majority of embryo mortality occurs within 16 days from breeding, and probably within the first 8 days for cows with a high milk yield (Diskin et al., 2016). A former commonly accepted theory stated that early embryo development was exclusively regulated by the mother, based on the fact that the female gamete is much larger than the male gamete and consequently had the capacity to house the necessary regulating factors such as transcripts and proteins (Immler, 2018). As it was revealed that breeding for high milk yield could have an inverse effect on reproductive outcome, complying with the earlier decline in fertility observed worldwide, the cow became the main target for studies related to embryo mortality (Kropp et al., 2014). In contrast, previous investigations on the father's contribution to fertility mainly focused on morphological assessments of the spermatozoa's ability to reach and fertilize the oocyte (Moldenhauer et al., 2003; Daigneault, 2020). More recent evidence demonstrates that both parents contribute to embryo programming, through genetic and epigenetic components, and *via* RNAs and proteins directly deposited within the zygote (Gross et al., 2019; Daigneault, 2020; Wu and Sirard, 2020). Thus, both male and female fertility can be defined as the capacity of fertilization and continued embryo and fetal development until birth. Consequently, the contribution from both parents can be responsible for embryo death. Separate investigation of male fertility is crucial, as the correlation of genetic progress in the fertility of the bull and the cow is low (Taylor et al., 2018).

Recent advances in biotechnology have initiated an understanding of genetic control of the embryo, investigating different levels of genomics and epigenomics through single-embryo analysis at different developmental stages and qualities (Huang et al., 2010; Graf et al., 2014; Kropp et al., 2017). Jiang et al. (2014) investigated *in vivo* produced embryos from three different species and found that the bovine embryo is a better model for human embryonic development than the mouse embryo, implying that studies on the bovine embryo are highly relevant beyond the field of veterinary science. In cattle, the major embryonic gene activation (EGA) occurs at the 8-cell stage (Graf et al., 2014). Studies have revealed that the 2-4 cell bovine embryo consists of both maternal-specific and paternal-specific transcripts (Gross et al., 2019). These transcripts have the potential to affect embryonic development, both at that specific stage and in later developmental stages. It has also been demonstrated that the father contributes on the epigenomic level, with mechanisms such as chromatin structure alterations and DNA-methylation differing between high and low fertility bulls (Kropp et al., 2017), affecting the fate of gene transcription in the embryo. The exact function of paternally delivered transcripts or their regulation of genes that control embryo development remains largely unclear.

Norwegian Red (NR) is the main dairy breed in Norway. The breeding program has had a strong emphasis on fertility and health since the 1970s. The Norwegian Dairy Herd Recording System is well-established and includes information on fertility outcome that can be used for investigations related to bull fertility. The breeding strategy for NR was recently changed from progeny testing to genomic selection, which results in a faster breeding progress. The bulls are now in semen production at an earlier age, and identification of reliable markers for the bull fertility is of increased interest. By comparing different phenotypes in the father with the outcome in the embryo, one can reveal genes and pathways that are affected by the bull's contribution, which in a longer perspective could support the prediction of bull fertility. At the blastocyst stage, Kropp and colleagues demonstrated that *in vitro* produced embryos from bulls of different fertility had different gene expression (Kropp et al., 2017). Another study investigated the transcriptome of IVF blastocysts derived from the same father animal at either 10, 12 or 16 months of age. Using microarray data, they found several genes to be differentially expressed depending on the age of the father (Wu et al., 2020). *In vitro* production of embryos offers a valuable research tool with a high level of feasibility and accuracy. However, even under detailed control, an *in vitro* system may affect or alter the gene expression through stressors that are unnatural for an embryo. Hence, theories established by *in vitro* studies need to be considered in an *in vivo* approach. The current study aimed to explore the differences in gene expression, on a whole transcriptomic scale, of *in vivo* produced single blastocyst embryos derived from two groups of Norwegian red bulls with high or low non-return rate.

MATERIAL AND METHODS

Animals

The present study used frozen semen from 12 NR bulls, divided into two groups based on fertility. The bulls were selected from a database of 470 NR bulls, born between 2010 and 2014, all with at least 500 registered first inseminations (AI). Fertility was recorded as the non-return rate (NRR) at 56 days after first AI of the inseminated cows, and varied from 49.3 to 80.5 with an average of 72.5 (s.e. = 3.5). The 12 selected bulls had a record of 661–901 first artificial inseminations and represented the highest and lowest fertility among all registrations. Bulls in the high fertility (HF) group had a NRR of 78.7–80.5 ($n = 6$), while the low fertility group (LF) had an NRR of 49.3–62.1 ($n = 6$). The reason for the difference in fertility was not known, and the semen was no longer in commercial use. The semen had passed standard testing requirements performed by Geno SA[1], the breeding organization for NR cattle, before commercial use; Macroscopic evaluation, a concentration threshold of 390 million cells per ml, at least 70 and 50% motile spermatozoa pre-freezing and post-thawing, respectively, and a threshold of at least 83–90% morphologically normal spermatozoa depending on the specific deviation (personal communication, Geno SA).

[1]www.geno.no.

For embryo production *in vivo*, we used 14 NR heifers in the age span of 12–15 months. In order to reduce individual differences and the maternal effect to a minimum, all animals came from the same genetic line with 28 years of targeted breeding, with high fertility and a low occurrence of clinical mastitis as target traits (Heringstad and Larsgard, 2010). They were free from disease or medical treatments according to their health records for the last 6 months before the sampling, they had no earlier inseminations and at least two visually registered estrus cycles. They were held indoors in the same free-range barn and received the same feeding throughout the study. Their body condition scorings were considered normal with an individual variation of 3.5–4.0, using a scale from 1-5 with increments of 0.25 (Edmonson et al., 1989), modified and adjusted for NR according to Gillund et al. (1999).

The ethical approval for the present study was provided by the Norwegian Food Safety authority with approval ID 11732. The combination of bull and heifer was randomized with block randomization; heifers were listed according to age, and every second animal was appointed a randomly chosen HF bull or LF bull, respectively. Randomization was performed using Sergeant, ESG, 2018, Epitools Epidemiological Calculators, Ausvet[2]. Semen from the two bulls with the lowest NRR were appointed to be used for two different heifers.

Embryo Production and Collection

Embryo production was performed at The Animal Production Experimental Centre, NMBU in Ås, Norway in the spring of 2017. A protocol for synchronization and superovulation was developed for young NR heifers. The animals were synchronized with an intramuscular (i.m) injection of 2 ml cloprostenol 0.25 mg/ml (Estrumat vet., MSD Animal Health, Intervet International B.V., Nederland) twice at a 12-day interval and the following heat was visually detected. On day 9 after the first signs of standing heat, the 4-day administration with decreasing amounts of follicle stimulating hormone (FSH; Follitropin 500 IE and lutinizing hormone 500 IE, Pluset® vet, Laboraotiros Calier, Barcelona, Spain) started.; Two i.m. injections were given daily (day one 2.0 ml, day two and three 1.5 ml and day four 1.0 ml). On the fourth day, a 2.0 ml i.m. injection of cloprostenol 0.25 mg/ml was administered in the morning and in the evening to induce luteolysis. The heat occurred after 2 days and AI was performed two or three times with 12 h in between depending on length of heat behavior.

Embryo flushing was performed 7 days after first AI. The ovaries were controlled for superovulation response using both manual palpation and rectal ultrasound, followed by an administration of epidural anesthesia. A Foley catheter was used to flush each uterine horn at least five times with ViGRO™ Complete Flush Solution (Vetoquinol, Lure Cedex, France. Previously Bioniche Animal Health, United States). The fluid was retrieved in an embryo collection filter (Emcon, Panningen, Netherland).

The embryos were transferred from the filter to a petri dish with SYNGRO ™ holding solution (Bioniche Animal Health, United States) and evaluated under a magnifying loupe at 40x magnification by three veterinarians according to the IETS guidelines (IETS-manual 3rd edition, IETS bovine *in vivo* embryo slide set tutorial, 2010). All embryos were then cleaned with phosphate buffered saline, moved to separate sterile mini tubes containing 1U/µl RNAsin in nuclease free water (RNasin Ribonuclease Inhibitor, Promega corporation, WI, United States), instantly frozen in liquid nitrogen, and stored in a −80°C freezer.

Embryos derived from HF sires were referred to as HF embryos, while those from the LF sires were referred to as LF embryos.

RNA Extraction

Embryos with first grade quality and IETS stage 5 (early blastocyst), 6 (blastocyst) or 7 (expanded blastocyst) from both HF and LF groups were selected for further processing. RNA isolation was performed using the RNAqueous-Micro Total RNA isolation Kit (Thermo-Fisher Scientific, MA, United States) according to the producer's instructions with some modifications; In order to break the zona pellucida and cell walls, embryos placed in 100 µl of the kit lysis solution were submitted to five cycles of 2 min freeze in liquid nitrogen and 2 min thaw in a 50°C water bath, followed by an incubation at 42°C overnight. The next day 50 µl of ethanol was added and isolation was continued according to the protocol. RNA was eluted twice in 6.0 and 6.5 µl elution solution, and the pooled eluate was treated with DNase1 as described by the producer. Production of cDNA was performed using the SMART-Seq v4 Ultra Low Input RNA Kit for sequencing (TaKaRa Bio Europe, Göteborg, Sweden) according to the producer's protocol, using 10.5 µl of input RNA and 18 cycles of amplification. The Agencourt AMPure XP kit (Beckman Coulter, IN, United States) was used to purify the amplified cDNA as described in the Smart-Seq kit protocol. The cDNA concentration and quality were measured by Qubit fluorometer using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, MA, United States) and Agilent TapeStation D1000 using High Sensitivity reagents (Agilent, Santa Clara, CA, United States), respectively.

cDNA samples with concentrations between 0.262 ng/µl and 17.1 ng/µl (**Supplementary Table S1**) and sufficient quality according to the TapeStation profiles (**Supplementary Figure S1**) were selected for sequencing. In total, cDNA from 24 embryos were sent for sequencing at the Norwegian Sequencing Centre[3], i.e., 13 embryos from four HF bulls and 11 embryos from three LF bulls. The total distribution between embryo stages 5, 6 and 7 were 1, 12 and 11 embryos, respectively. In the LF group, the embryo stages were 6 ($n = 5$) and 7 ($n = 6$), and in the HF group, the stages were 5 ($n = 1$), 6 ($n = 7$) and 7 ($n = 5$) (**Supplementary Table S1**).

[2]http://epitools.ausvet.com.au.

[3]www.sequencing.uio.no.

Sequencing and Data Analysis

Sequencing libraries from cDNA were prepared using SMARTer ThruPLEX DNA-prep kit (TaKaRa Bio United States Inc., San Jose, CA, United States) using unique indexes. Libraries were pooled and 150 bp paired end sequencing was performed on one lane of HiSeq 4,000 (Illumina, United States). Raw reads were processed using BBDuk (part of BBMap v34.56) (Bushnell, 2014) (parameters: ktrim = r k = 23 mink = 11 hdist = 1 tbo tpe qtrim = r trimq = 15 maq = 15 minlen = 36 forcetrimright = 149) to remove/trim low-quality reads and adapter sequences. Cleaned reads were aligned to the *Bos taurus* genome (ARS-UCD1.2; ENSEMBL release 95) using hisat2 v2.1.0 (Pertea et al., 2016) and the resulting sam files were converted to bam format using samtools v1.2. Reads mapping to the genes (ARS-UCD1.2; ENSEMBL release 95) were counted using featureCounts v1.4.6-p1 (Liao et al., 2014). Differential expression analysis was performed using DESeq2 v1.22.2 package (Love et al., 2014) in R v3. In brief, the counts were normalized followed by outlier detection (Cook's distance), dispersion estimation (fittype: parametric) and statistical testing (hypothesis testing: Wald test). Independent filtering was performed which discarded 14,355 genes due to very low count values. Finally, multiple testing was performed using the Benjamini-Hochberg method. Genes with the adj.*p*-value less than 0.05 were considered to be significantly differentially expressed.

Gene Set Enrichment Analysis

A gene set enrichment analysis of the DEGs was performed using the g:GOSt function in g:profiler version e104_eg51_p15_3922dba (Raudvere et al., 2019), which also integrated results from KEGG Pathway database, WikiPathways and Reactome. Separate analysis was performed for the genes highly expressed in the HF embryos and for the genes highly expressed in the LF embryos. A custom gene list consisting of 12,826 genes that were detected (adj.*p*-value not equal to "NA") in the DE-analysis was used as background list for statistical domain scope (**Supplementary Table S2**). To correct for multiple testing, we used the Benjamini-Hochberg FDR algorithm and a threshold level of 0.05 for significance. For further functional analysis and visualization, we used the online version of Pathview v1.3.2[4] (Luo et al., 2009; Luo and Brouwer, 2013; Luo et al., 2017).

The bovine genome is not as well studied and annotated as the human genome. To further enrich results interpretation, DEGs and the background list for statistical domain were converted to human ENSEMBL orthologs from bovine ENSEMBL IDs, by g:profiler and run though gene ontology analysis in g:profiler and pathway analysis in Pathviews, as described above.

[4]https://pathview.uncc.edu/.

TABLE 1 | Bulls with high (HF) or low (LF) fertility, and number of embryos with sufficient material to sequence. NRR 56 = non return rate on day 56.

Bull fertility category	Bull ID	NRR 56	Number of embryos sequenced
HF	A	80.5	0
	B	79.7	0
	C	79.3	4
	D	79.3	2
	E	78.8	4
	F	78.7	3
LF	G	62.1	0
	H	61.9	8
	I	61.7	1
	J	56.8	0
	K	56.5	2
	L	49.3	0

RESULTS

Output From Embryo Collection and Laboratory Work

All the heifers responded well to the superovulation protocol with normal size ovaries and no un-ovulated follicles. Sampling was normal in all heifers except for one, where collection from one of the uterine horns was not performed due to practical challenges. In total, 73 embryos were collected from 8 heifers inseminated with 6 LF bulls (=30 embryos) and 4 heifers inseminated with 4 HF bulls (=43 embryos), with an individual distribution of 1–19 embryos per animal. In the LF group, 17 of the 43 embryos collected had developed to the blastocyst stage, compared to 20 out of 30 in the HF group. From two heifers, no embryos could be found at collection. An overview of embryos sequenced from the different sires is given in **Table 1**. After the collection of embryos, Geno SA conducted an independent investigation of bulls that had previously been included in their breeding program, by analyzing genomic profiles, i.e., SNP data. That process revealed that one bull (K) had a deletion on chromosome 12.

Output From Sequencing and Mapping

The sequencing resulted in an average of 12.5 million paired end reads per sample and more than 98% passed quality check. Read information and alignment statistics are provided in **Supplementary Table S1**. Raw sequence reads were uploaded to the NCBI SRA database with bioproject ID number: PRJNA762262.

The distribution of gene expression in the individual blastocysts was visualized by principal component analysis plots (**Supplementary Figure S2**). Two embryos (number 48 and 66) were shown as outliers and were removed from further analyses. These two samples also had an inferior cDNA quality according to the TapeStation profiles, which justified our decision. Due to this, the lowest cDNA concentration for samples analyzed in DESeq2 was 1.64 ng/μl. Embryos of IETS stages 5, 6 and 7 did not show strong signs of clustering within these groups in any of the three dimensions. The embryo of IETS stage 5 (from a HF bull) did not stand out as outlier or represent

TABLE 2 | Differentially expressed genes (mRNA) between embryos produced from low fertility (LF) and high fertility (HF) bulls. L = mean normalized count values in LF embryos. H = mean normalized count values in HF embryos.

ENSEMBL ID	Gene symbol	L	H	Log2 fold change	Adj.*p*-value	Functional description
Highly expressed in LF embryos						
ENSBTAG00000014046	*BPI*	573	0	10.90	1.72E-03	Bactericidal permeability increasing protein
ENSBTAG00000003305	*NCF1*	16	0	5.81	6.69E-03	Neutrophil cytosol factor 1
ENSBTAG00000047563	*CLDN9*	142	11	3.69	6.76E-03	Claudin 9
ENSBTAG00000026893	*EXOC3L4*	496	52	3.26	1.70E-03	*Bos taurus* exocyst complex component 3 like 4
ENSBTAG00000049434	Non-annotated gene	238	30	2.99	2.52E-03	
ENSBTAG00000051376	Non-annotated gene	33	6	2.43	2.52E-03	
ENSBTAG00000013854	*CALML5*	963	213	2.17	7.14E-10	Calmodulin like 5
ENSBTAG00000011839	*HMGCS1*	1949	471	2.05	6.13E-04	*Bos taurus* 3-hydroxy-3-methylglutaryl-CoA synthase 1
ENSBTAG00000054516	*CYP17A1*	293	76	1.95	2.60E-02	Steroid 17-alpha-hydroxylase/17,20 lyase
ENSBTAG00000017819	*PMVK*	310	93	1.74	1.21E-02	Phosphomevalonate kinase
ENSBTAG00000004905	*KRT19*	24,981	7,880	1.67	2.83E-02	Keratin 19
ENSBTAG00000003068	*MSMO1*	3,085	1,031	1.58	6.49E-04	Methylsterol monooxygenase 1
ENSBTAG00000006305	*AK1*	184	65	1.51	4.30E-03	*Bos taurus* adenylate kinase 1
ENSBTAG00000017864	*PRPH*	904	326	1.47	2.52E-03	*Bos taurus* peripherin
ENSBTAG00000055207	*SCD*	1,324	478	1.47	4.39E-03	*Bos taurus* stearoyl-CoA desaturase
ENSBTAG00000004075	*IDI1*	1,616	618	1.39	2.60E-04	*Bos taurus* isopentenyl-diphosphate delta isomerase 1
ENSBTAG00000012432	*FDFT1*	2,967	1,145	1.37	1.05E-02	*Bos taurus* farnesyl-diphosphate farnesyltransferase 1
ENSBTAG00000007840	*HMGCR*	1,178	465	1.34	4.30E-03	*Bos taurus* 3-hydroxy-3-methylglutaryl-CoA reductase
ENSBTAG00000004881	*MTHFD2*	1,085	440	1.30	2.84E-03	*Bos taurus* methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2, methenyltetrahydrofolate cyclohydrolase
ENSBTAG00000014127	*PTGS2*	7,075	2,949	1.26	4.30E-03	*Bos taurus* prostaglandin-endoperoxide synthase 2
ENSBTAG00000055124	Non-annotated gene	53	23	1.23	2.76E-03	
ENSBTAG00000003948	*FDPS*	1,516	708	1.10	2.52E-03	Farnesyl diphosphate synthase
ENSBTAG00000003100	*SMTN*	209	100	1.05	2.76E-02	*Bos taurus* smoothelin
ENSBTAG00000004982	*GPLD1*	969	470	1.04	1.53E-02	*Bos taurus* glycosylphosphatidylinositol specific phospholipase D1
ENSBTAG00000032914	*SLC11A2*	1,023	513	1.00	2.51E-02	*Bos taurus* solute carrier family 11 member 2
ENSBTAG00000006471	*OSBPL11*	198	102	0.96	2.75E-02	Oxysterol binding protein like 11
ENSBTAG00000014227	*NDFIP2*	2,381	1,229	0.95	4.30E-03	*Bos taurus* Nedd4 family interacting protein 2
ENSBTAG00000019246	*SC5D*	605	319	0.92	4.56E-02	*Bos taurus* sterol-C5-desaturase
ENSBTAG00000055014	*SH3BGRL2*	2,429	1,352	0.85	3.07E-02	*Bos taurus* *SH3* domain binding glutamate rich protein like 2
ENSBTAG00000044015	*RBM12*	285	172	0.73	2.73E-02	*Bos taurus* RNA binding motif protein 12
ENSBTAG00000012317	*PNP*	3,762	2,302	0.71	4.78E-02	*Bos taurus* purine nucleoside phosphorylase
ENSBTAG00000016896	*HERPUD1*	531	327	0.70	2.52E-03	Homocysteine inducible ER protein with ubiquitin like domain 1
ENSBTAG00000017258	*ACSL3*	6,014	3,780	0.67	2.70E-02	*Bos taurus* acyl-CoA synthetase long chain family member 3
ENSBTAG00000011899	*USP4*	1,339	858	0.64	6.95E-03	Ubiquitin specific peptidase 4
Highly expressed in HF embryos						
ENSBTAG00000031825	Non-annotated gene	0	45	8.02	2.60E-04	
ENSBTAG00000046257	*GIMAP4*	1	68	5.85	3.57E-02	*Bos taurus* GTPase, *IMAP* family member 4
ENSBTAG00000014560	*HLX*	0	9	5.72	3.80E-02	*Bos taurus* H2.0 like homeobox
ENSBTAG00000030882	*hsd20b2*	7	81	3.48	1.85E-02	*Bos taurus* estradiol 17-beta-dehydrogenase 12-like (LOC508455)
ENSBTAG00000015836	Non-annotated gene	50	351	2.82	1.91E-04	
ENSBTAG00000010123	*APOE*	22	135	2.65	2.36E-02	Apolipoprotein E
ENSBTAG00000014596	*EFHD1*	153	823	2.43	1.70E-03	EF-hand domain family member D1
ENSBTAG00000027444	*SVIL*	12	60	2.37	4.98E-02	*Bos taurus* supervillin
ENSBTAG00000054434	Non-annotated gene	6	25	2.08	3.33E-02	
ENSBTAG00000033429	*FAM229B*	7	26	1.96	4.92E-02	*Bos taurus* family with sequence similarity 229 member B
ENSBTAG00000049950	Non-annotated gene	9	32	1.86	4.92E-02	
ENSBTAG00000026758	Non-annotated gene	22	77	1.83	2.60E-02	
ENSBTAG00000017094	*SHMT1*	63	209	1.72	2.73E-02	Serine hydroxymethyltransferase 1
ENSBTAG00000038384	*KRT5*	434	1,210	1.48	1.21E-02	Keratin 5
ENSBTAG00000054234		228	632	1.47	2.86E-02	

(Continued on following page)

TABLE 2 | (*Continued*) Differentially expressed genes (mRNA) between embryos produced from low fertility (LF) and high fertility (HF) bulls. L = mean normalized count values in LF embryos. H = mean normalized count values in HF embryos.

ENSEMBL ID	Gene symbol	L	H	Log2 fold change	Adj.*p*-value	Functional description
Highly expressed in LF embryos						
	Non-annotated gene					
ENSBTAG00000003568	*CLDN10*	417	1,120	1.43	4.44E-03	*Bos taurus* claudin 10, transcript variant 2
ENSBTAG00000004386	*SOCS1*	105	274	1.39	4.30E-03	Suppressor of cytokine signaling 1
ENSBTAG00000012511	*BAD*	306	787	1.36	1.72E-03	*Bos taurus BCL2* associated agonist of cell death
ENSBTAG00000003043	*GNG2*	148	373	1.33	2.09E-02	G protein subunit gamma 2
ENSBTAG00000006086	*MMP28*	110	278	1.33	1.72E-03	Matrix metallopeptidase 28
ENSBTAG00000022028	*DERL3*	114	257	1.17	2.52E-03	*Bos taurus* derlin 3
ENSBTAG00000013922	*MOSPD1*	196	435	1.15	4.47E-02	*Bos taurus* motile sperm domain containing 1
ENSBTAG00000020528	*PCOLCE*	126	273	1.12	1.01E-02	*Bos taurus* procollagen C-endopeptidase enhancer
ENSBTAG00000003222	*ASNS*	287	574	1.00	4.18E-02	Asparagine synthetase (glutamine-hydrolyzing)
ENSBTAG00000010740	*CLTB*	2,843	5,223	0.88	4.21E-02	Clathrin light chain B
ENSBTAG00000052249	Non-annotated gene	41	71	0.80	2.60E-02	
ENSBTAG00000021111	*POU5F1*	10,804	18,443	0.77	2.26E-03	*Bos taurus POU* class 5 homeobox 1
ENSBTAG00000017932	*CCDC84*	183	286	0.65	3.75E-02	*Bos taurus* coiled-coil domain containing 84

the highest or lowest value in any aspect of the PCA. Embryos from LF bulls compared to HF bulls clustered in the second dimension, but not in the first and third dimension.

Differentially Expressed Genes

Among the 14,744 annotated genes that were detected during the analysis, there was a significant difference in the expression of 62 genes; 28 genes had a higher expression in the HF group, and 34 genes a higher expression in the LF group (Log2-foldchange > 0.64 adj.*p*-value < 0.05), see **Table 2**.

An overview over the significant pathways and gene ontology (GO)-terms found in g:profiler is provided in **Table 3**. The full DE-list revealed three significantly affected pathways in Pathview, represented by 9 different genes; terpenoid backbone synthesis (4 genes represented), insulin signaling pathway (3 genes represented) and leukocyte transendothelial migration (2 genes represented).

Genes that were highly expressed in the LF group (n = 34) were associated with 29 GO-terms for biological processes and pathways, most prominently biosynthetic process- and metabolism of sterol, steroids, isoprenoids and cholesterol. Pathway analysis in KEGG, Reactome and WikiPathways through g:profiler showed enrichment of terpenoid backbone synthesis, and metabolism and biosynthesis of cholesterol, steroids and lipids. The individual transcript showing the biggest difference between the groups, with higher expression in the LF group, was mRNA coding for bactericidal permeability increasing protein (gene symbol *BPI*, log2fold change = 10.897).Another transcript, with higher expression in the LF group was mRNA coding for neutrophil cytosol factor 1 (gene symbol *NCF1*, log2 fold change = 5.812), a protein engaged in leukocyte transendothelial migration and a NADPH oxidase regulator. The leukocyte transendothelial migration pathway was further represented by the gene claudin 9 (*CDLN9*, log2 fold change = 3.688), the third most up-regulated transcript in the LF group and coding for cell adhesion molecules (CAMs).

Genes that were highly expressed in the HF group (n = 28) were associated with only one GO-term through Corum in g:profiler, the G protein complex. The biggest foldchange was represented by a non-annotated gene (ENSBTAG00000031825, log2fold change = 8.016) followed by *GIMAP4* (log2fold change = 5.850) and *HLX* (log2fold change = 5.724).

Non-Annotated Genes

Out of all significant DEGs in the DE-list, nine genes were described as non-annotated genes, of which seven did not have any pathway and GO-terms associated. Three of the non-annotated genes were highly expressed in LF embryos, and six in HF embryos (**Table 2**). Some of these represented a very high level of difference between the LF and HF groups, with the highest log2fold change of 8.016 and 2.823 in the HF group, and 2.994 and 2.425 in the LF group.

Human Orthologs

To enrich results interpretation, we used human orthologs, as the human genome is better studied and annotated. Out of the 62 bovine genes from the DE-list, g:profiler found 58 human orthologues (**Supplementary Table S3**). Only four genes did not have a human orthologue. The Pathview program pathways represented by these orthologues matched the pathways for the bovine genome. Out of the 29 GO-terms or pathways linked to genes highly expressed in the LF group, 27 were enriched in the human orthologs. Out of these 27, 16 terms or pathways were each associated with one or more genes in the human orthologs compared to the original material. In 6 other enriched processes or pathways, the same number of genes represented both the cattle and human ortholog outcome. Fourteen terms or pathways that did not appear in the original material, were shown in the analysis of orthologs. These pathways were strongly related to, or represented a higher hierarchy of, the same pathways that had already been identified. Only two pathways in the human

TABLE 3 | Significant pathways and gene ontology terms found in g:profiler for the differentially expressed genes between LF embryos and HF embryos.

Pathway or gene ontology term	Pathway ID	Adj. *p*-value	Genes represented
Highly expressed in LF embryos			
sterol biosynthetic process	GO:0016126	1.51E-08	*HMGCS1, PMVK, MSMO1, IDI1, FDFT1, HMGCR, SC5D*
cholesterol biosynthetic process	GO:0006695	1.42E-06	*HMGCS1, PMVK, IDI1, FDFT1, HMGCR, SC5D*
secondary alcohol biosynthetic process	GO:1902653	1.42E-06	*HMGCS1, PMVK, IDI1, FDFT1, HMGCR, SC5D*
steroid biosynthetic process	GO:0006694	8.33E-06	*HMGCS1, PMVK, MSMO1, IDI1, FDFT1, HMGCR, SC5D*
sterol metabolic process	GO:0016125	9.39E-06	*HMGCS1, PMVK, MSMO1, IDI1, FDFT1, HMGCR, SC5D*
lipid biosynthetic process	GO:0008610	3.63E-05	*HMGCS1, PMVK, MSMO1, SCD, IDI1, FDFT1, HMGCR, PTGS2, FDPS, SC5D, ACSL3*
isoprenoid biosynthetic process	GO:0008299	5.45E-05	*HMGCS1, PMVK, IDI1, HMGCR, FDPS*
isoprenoid metabolic process	GO:0006720	6.19E-05	*HMGCS1, PMVK, IDI1, FDFT1, HMGCR, FDPS*
organic hydroxy compound biosynthetic process	GO:1901617	2,59E-04	*HMGCS1, PMVK, MSMO1, IDI1, FDFT1, HMGCR, SC5D*
cholesterol metabolic process	GO:0008203	4,00E-04	*HMGCS1, PMVK, IDI1, FDFT1, HMGCR, SC5D*
secondary alcohol metabolic process	GO:1902652	5,45E-04	*HMGCS1, PMVK, IDI1, FDFT1, HMGCR, SC5D*
alcohol biosynthetic process	GO:0046165	8,84E-04	*HMGCS1, PMVK, IDI1, FDFT1, HMGCR, SC5D*
steroid metabolic process	GO:0008202	9,62E-04	*HMGCS1, PMVK, MSMO1, IDI1, FDFT1, HMGCR, SC5D*
lipid metabolic process	GO:0006629	5,31E-03	*HMGCS1, PMVK, MSMO1, SCD, IDI1, FDFT1, HMGCR, PTGS2, FDPS, GPLD1, SC5D, ACSL3*
small molecule metabolic process	GO:0044281	2,17E-02	*HMGCS1, PMVK, AK1, SCD, IDI1, FDFT1, HMGCR, MTHFD2, PTGS2,* Non-annotated gene: ENSBTAG00000014127, *GNG2, SC5D, PNP, ACSL3*
cellular lipid metabolic process	GO:0044255	4,60E-02	*HMGCS1, PMVK, SCD, IDI1, FDFT1, HMGCR, PTGS2, FDPS, GPLD1, ACSL3*
small molecule biosynthetic process	GO:0044283	4,94E-02	*HMGCS1, PMVK, SCD, IDI1, FDFT1, HMGCR, PTGS2, SC5D*
Metabolic pathways	KEGG:01100	2,91E-08	*HMGCS1,* Non-annotated gene: ENSBTAG00000054516, *PMVK, MSM O 1, AK1, SCD, IDI1, FDFT1, HMGCR, MTHFD2, PTGS2*, Non-annotated gene: ENSBTAG00000014127, *GNG2, FDPS, GPLD1, SC5D, PNP, ACSL3*
Terpenoid backbone biosynthesis	KEGG:00900	8,78E-08	*HMGCS1, PMVK, IDI1, HMGCR, FDPS*
Steroid biosynthesis	KEGG:00100	4,59E-04	*MSMO1, FDFT1, SC5D*
PPAR signaling pathway	KEGG:03320	1,10E-02	*HMGCS1, SCD, ACSL3*
Cholesterol biosynthesis	REAC:R-BTA-191273	3,13E-08	*MSMO1, IDI1, FDFT1, HMGCR, SC5D*
Metabolism of steroids	REAC:R-BTA-8957322	2,02E-05	*MSMO1, IDI1, FDFT1, HMGCR, SC5D*
Metabolism of lipids	REAC:R-BTA-556833	8,38E-04	*MSMO1, IDI1, FDFT1, HMGCR, PTGS2, SC5D, ACSL3*
Metabolism	REAC:R-BTA-1430728	1,72E-02	*MSM O 1, IDI1, FDFT1, HMGCR, MTHFD2, PTGS2, SC5D, PNP, ACSL3*
Cholesterol Biosynthesis	WP:WP1070	2,61E-16	*HMGCS1, PMVK, MSMO1, IDI1, FDFT1, HMGCR, FDPS, SC5D*
SREBP signalling	WP:WP3194	1,25E-05	*HMGCS1, IDI1, FDFT1, HMGCR, FDPS*
SREBF and miR33 in cholesterol and lipid homeostasis	WP:WP3137	2,14E-02	*HMGCS1, HMGCR*
Statin Pathway	WP:WP1041	4,16E-02	*FDFT1, HMGCR*
Highly expressed in HF embryos			
G protein complex (CACNA1A, GNB1, GNG2)	CORUM:3216	4,99E-02	*GNG2*

orthologs, Omega-9 FA synthesis and Cuteneous photosensitivity, were not related but these were only represented by two and three genes, respectivley. For the genes that were highly expressed in the HF group, the analysis of human orthologs did not give any GO-terms or pathways.

DISCUSSION

The present study is the first to compare the complete gene expression of *in vivo* produced embryos from sires with high and low field fertility, measured as high or low NRR respectively. Gene expression differed significantly between the two groups, and we identified several pathways affected by the field fertility of the bull. There was consensus between the different databases used by g: profiler (KEGG, Reactome, Corum and Wikipathways). Our findings were further strengthened by analysis of human orthologues, which were related to almost identical pathways.

We collected 30 embryos from four high fertility bulls and 43 embryos from six low fertility bulls, with individual differences of 1–19 embryos per bull. The deletion found in one LF embryo leads to embryonic or fetal death in homozygous conceptuses, and was described by Kadri et al. (2014). The embryos from LF sires showed a tendency of greater variation in developmental stage, where only 39.5% (17/43) had developed to the blastocyst stage, compared to 66.6% (20/30) in the HF group. Some earlier

studies support the positive relationship between embryo cleavage or blastocyst rate *in vitro*, and field fertility in the bull (Zhang et al., 1997; Ward et al., 2001; O'Callaghan et al., 2021), while others do not (Kropp et al., 2017).

We identified 62 genes differentially expressed between embryos produced from low fertility and high fertility bulls. This seemingly low number is comparable to the findings in two similar studies of male contribution to embryo development. Both studies used RNA-sequencing of *in vitro* produced embryos, identifying 65 differentially expressed genes for the blastocyst stage and the 2-4 cell stage embryos, respectively (Kropp et al., 2017; Gross et al., 2019). Another study, that compared morphologically degenerative embryos on day 8 to normally developed blastocysts, found 47 differentially expressed genes (Huang and Khatib, 2010), all suggesting that a change in only a slight number of transcripts can be responsible for gross changes in the embryo.

Enriched Pathways and Gene Ontology Terms

Embryos derived from LF bulls showed a higher genetic expression corresponding to a more active metabolism. These results are in correlation with earlier literature, proposing an association between a high survival rate in the embryo, and a lower level of metabolism (Leese, 2002; Baumann et al., 2007; Leese et al., 2007). One of the pathways that was highly expressed in the LF group was the terpenoid backbone biosynthesis (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021) (**Supplementary Figure S3**), which initiates the production of sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids (Buhaescu and Izzedine, 2007; Miziorko, 2011). The products derived from this pathway play an essential role in various cellular processes such as cell growth and differentiation, and cell signaling (Goldstein and Brown, 1990). One of the continuations of this pathway; steroid biosynthesis (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021) (**Supplementary Figure S4**) was also highly expressed in the LF group. The increased activity in these connected pathways was represented by nine transcripts in the present study. The first three, *HMGCS1*, *HMGCR* and *PMVK* all code for enzymes in the production of mevalonate. HMGCR has been described as a rate limiting enzyme and is of major importance for the entire downstream process (Goldstein and Brown, 1990). Both HMGCR and HMGCS1 underlie well-studied, vast mechanisms of regulation. One of those mechanisms is an end-product feedback system that allows any absence of sterol isoprenoids to activate the transcription of the *HMGCR* gene through a family of transcription factors called sterol regulatory binding proteins (SREBP) (Brown and Goldstein, 1997; Buhaescu and Izzedine, 2007).

The next two genes represented in these pathways, *DID1* and *FDPS*, encode enzymes that catalyze the further descendance of metabolites towards steroid biosynthesis while four other genes, *FDFT1*, *MSMO1*, *SC5DL* and *CYP17A1* encode enzymes that lead the metabolism down to the biosynthesis of cholesterol (and several other steroids) and steroid hormones. One study demonstrated that an increased SREBP activity not only acted on *HMGCR* and *HMGCS1*, but also increased the mRNA expression of several enzymes along the entire pathway of cholesterol production (Sakakura et al., 2001). Based on this information, we speculate that the high expression of the mentioned enzymes in the LF embryos, could be a result of any dysfunction of the pathways related to sterols, or any exaggerated degradation or demands of its products. Cholesterol is essential for the developing embryo, as it forms part of the cell membrane, and acts in cell signaling crucial for developmental patterning, in collaboration with the hedgehog gene family (Porter et al., 1996; Roux et al., 2000). It can be toxic in too large quantities and its production entails high metabolic costs for cells to produce. Hence, its production is under strict regulation (Sharpe and Brown, 2013). The complete knockout of the *HMGCR* gene in mouse embryos resulted in the recovery of morphologically normal blastocyst but no later developmental stages. This suggest that at least some of HMGCR products are essential for development from the blastocyst stage, either prior to implantation, or for the implantation process itself (Ohashi et al., 2003). Equally, *CYP17A1* disruption leads to early embryonic lethargy in murine embryos (Bair and Mellon, 2004). Hence, a suboptimal level of cholesterol and/or its precursors in the LF embryos would lower their developmental potential. This marks a difference between LF and HF embryos that could explain at least part of the reason for low field fertility in the LF bulls.

Interestingly, the sterol biosynthetic process and cholesterol pathway have been highlighted in the comparison between morphologically similar *in vitro* and *in vivo* produced embryos (Driver et al., 2012). *In vitro* embryos have a reduced developmental potential from the zygote to blastocyst stage, and a lower success in embryo transfer (Rizos et al., 2008). Driver et al. (2012) performed a transcriptome study in stage 7 blastocysts, where the *in vitro* group had an increased expression in 11 genes related to the cholesterol pathway. These genes included *HMGCS1*, *HMGCR*, *PMVK*, *IDI1* and *FDFT1* which are all identical to our findings. The present study only analyzed *in vivo* produced embryos, but equal to the study by Driver et al. (2012), it compared the transcripts of embryos with a hypothetical difference in developmental potential. The fact that the results of the two studies are in agreement, confirms the central role of the mentioned pathways for successful embryo development.

The leukocyte transendothelial migration pathway (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021) (**Supplementary Figure S5**) was represented by two of the most highly expressed genes in the LF group. Interestingly, the pathway was also represented by one gene with a high expression in the HF group. Earlier literature pointed to several similarities between leukocyte transendothelial migration and human implantation, stating that both processes use the same mechanisms of adhesion, molecular interaction and migration (Genbacev et al., 2003; Dominguez et al., 2005). Hence, one explanation for the low bull fertility, might be through an effect on the control of implantation. The leukocyte transendothelial migration pathway was represented by *NCF1* and *CLDN9* in the LF group, and *CLDN10* in the HF group. Claudins (CLDN9 and

CLDN10) also have a role in embryo development, independent of their role in this pathway. The claudin gene family forms part of tight junctions, which are transmembrane compounds with functions in the maintenance of apical-basal polarity and cell adhesion (Gupta and Ryan, 2010). Tight junctions are crucial for morphogenesis (Furuse and Moriwaki, 2009), and a loss of function-study revealed that some claudins are essential for the formation of the murine blastocyst (Moriwaki et al., 2007). In a review of claudin function in embryogenesis, the authors hypothesized that the combined expression of claudin, or the "claudin signature," is critical to embryonic tissues (Gupta and Ryan, 2010). The specific importance of a high expression of *NCF1* is uncertain. NCF1, also known as p47phox, takes part in the production of reactive oxygen species (ROS) through its role in NADPH oxidase (Babior, 2004). The change in expression of *NCF1* might point to a change in the redox state (reviewed by Harvey et al., 2002) in any of the two embryo groups. Changes in the embryo redox state through the limited accumulation of ROS is naturally occurring, enabling developmental progress in the embryo (Dennery, 2007). However, it also controls programmed cell death (Pierce et al., 1991), and in excess, oxidative stress is embryotoxic (Dennery, 2007). Consequently, one possible causative factor of the poorer outcome for the LF embryos could be through a lower competence in the regulation of redox activity. This hypothesis is supported by the findings in one earlier study of the sire's contribution to embryo development (Kropp et al., 2017).

Another pathway differing between the HF and LF groups was the insulin signaling pathway (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021) (**Supplementary Figure S6**). Two genes were abundant in the HF group: *SOCS1* and *BAD*. *SOCS1* encodes an enzyme which inhibits the action of the insulin receptor (Mooney et al., 2001), hence suppresses the full signaling pathway, which contains PI3K-Akt signaling. PI3K-Akt signaling has a central role in embryo survival, regulating differentiation and cell growth, proliferation, anti-apoptosis and calcium metabolism (Leese and Brison, 2015, p.184). The enzyme encoded by *SOCS1* also has several roles in the negative feedback mechanism of cytokine signaling (Krebs and Hilton, 2001). Cytokines are produced by the embryo itself, as well as the female reproductive tracts as a mediator of maternal-embryo communication. They affect a vast range of processes, again related to cell differentiation and cell survival (Leese and Brison, 2015, p.173–174). *SOCS1* expression is a product of both interferon-γ and interleukin-4 and the protein encoded by *SOCS1* has a negative feedback on these cytokines (Fujimoto and Naka, 2003). Interferon-γ has several important roles in embryo development, but excess production is detrimental (Leese and Brison, 2015, p.193), implying that a well-functioning regulatory mechanism is beneficial for the embryo. *BAD*, on the other hand, encodes an enzyme that is inhibited by the activation of the insulin signaling pathway, so a higher expression could be a result of the elevated activity in SOCS1. BAD is an antagonist of apoptosis, which is interesting since a higher apoptotic cell ratio indicates a lower developmental competence in the embryo (Maddox-Hyttel et al., 2003). Certainly, the lower degree of apoptosis in the HF group would make a logical explanation for a higher developmental potential. The apoptotic cell ratio is inversely correlated to early cleavage in zygotes (Byrne et al., 1999), which is again positively correlated to bull fertility (Ward et al., 2001). However, one study intended to demonstrate a direct association between bull fertility and apoptotic cell ratio, but failed to do so (Vandaele et al., 2006).

Other Transcripts

Some of the of the most highly expressed genes in the two groups were not represented in any pathways. *GIMAP4*, encoding a small GTPase active in the immune system (Heinonen et al., 2015) was highly expressed in the HF embryos. It regulates cytokine secretion in the early human $CD4^+$ Th lymphocytes and initiates the secretion of interferon-γ (Heinonen et al., 2015). GIMAP4 is also an important regulator of calcium signaling (Schnell et al., 2006), a process which in recent years has been shown to have several functions in the pre- and peri implantation period (Leese and Brison, 2015, p.158–164). To the authors knowledge, the exact role of *GIMAP4* in embryo development has not yet been defined. *HLX* was another highly expressed gene in the HF embryos. Similar to SOCS1, HLX is also a regulator of cytokines, allowing trophoblast proliferation and the development of the placenta (Rajaraman et al., 2010). This, again, proposes an association between the paternal contribution and the mechanism for implantation in our material.

Another interesting finding in the HF group was the higher expression of *POUF51*. This gene encodes the transcription factor Oct4, which is essential for pluripotency and the formation of an intracellular matrix (Nichols et al., 1998). Our results could denote that HF embryos are more competent in this matter, and are more likely to develop beyond the blastocyst stage. The highest DE seen in the HF group was of a non-annotated gene; ENSBTAG00000031825. Its homologue *C19orf12* (e value 0.0) has recently been shown to be important in neuronal development in zebrafish embryos, as a downregulation of the gene had severe effects on brain morphology and resulted in embryo death before day 7. Its function was suggested to be related to lipid metabolism even though the cellular mechanism is poorly understood (Mignani et al., 2020). The higher expression of this gene in our HF embryos is indeed an interesting finding that could explain differences in bull fertility but requires more investigation.

BPI was the single gene showing the highest DE in the LF group. The gene product is a lipid-transfer protein with the capacity to neutralize endotoxin. In humans, it is produced by neutrophils and the epithelial lining of mucosa as part of an antimicrobial defense mechanism (Schultz and Weiss, 2007). Proteins encoded by *BPI* and the *BPI*-like PLUNC genes from the same superfamily, have been found in the seminal plasma of rams (Soleilhavoup et al., 2014; van Tilburg et al., 2020), and the spermatozoa membrane of mice (Zhou et al., 2014) and rodents (Yano et al., 2010), and are hypothesized to have a role in the sperm-oocyte fusion process (Li et al., 2013). Even if BPI were to be identified in the semen of bulls, the finding in the embryos of the present study is likely not a direct result of paternal transcripts deposited to the oocyte at fertilization, since these transcripts start

to degrade at EGA and should not be abundant in the analyses of embryonic gene expression at the blastocysts stage (Graf et al., 2014; Jiang et al., 2014). Neither *BPI* in cattle, nor its human orthologue has been assigned to any pathway, and to the authors knowledge, the role of *BPI* in embryo development has not been reported in earlier studies. However, one RNA-seq study that compared different stages of *in vivo* produced cow embryos found that *BPIAF1* (a *BPI*-like PLUNC gene) is a hub gene in blastocysts. This information was validated with literature of human and mice blastocysts (Jiang et al., 2014). Further research is necessary to study the role of *BPI* in embryo development.

Limitations of the Study

Defining the significance of our interpretations to our findings is challenging, given that cell signaling in embryo development is controlled by a vast number of processes with overlapping actions and shared receptors (Leese and Brison, 2015, p.180). Superovulation could have altered the gene expression of some genes in the current study compared to a normal *in vivo* produced embryo (Mundim et al., 2009), but this alteration applies to both LF and HF embryos and should not affect the differences between the groups. Moreover, it is not certain that the death of the conceptuses from LF bulls occurs at the blastocyst stage or before implantation, even if most embryo death probably occurs before day 8 after conception (Diskin et al., 2016). Equally, although fertilization failure is not the main problem of non-successful coupling (Sreenan and Diskin, 1986; Diskin et al., 2016), we cannot rule out that the LF bulls in our study might have had a weak fertilization capacity. However, two recent studies on early embryo development in high and low fertility bulls, showed no difference in fertilization rate (Kropp et al., 2017; O'Callaghan et al., 2021), while one showed a difference in the development until day 7 between the two groups (O'Callaghan et al., 2021). In the present study, it is uncertain whether embryos were lost in the *in vivo* collection process, or whether the embryos that did not develop to the blastocyst stage, had the potential to do so. Equally, although the maternal effect was reduced by using very similar heifers with equal living conditions, each embryo was inevitably affected to some extent by the individual differences in the genetics of the heifers. Another limitation of the study was that we had to choose a mix of embryos of IETS stages 5, 6 and 7, which could have affected the relative expression of some genes. However, despite of this and individual differences, the distribution in the PCA plots supports the argument that the selected embryo stages are sufficiently uniform to study the differences between the HF and LF bulls. Equally, it would have been interesting to include embryos of all qualities in the two groups, and not only the highest quality. However, it is well known that gene expression varies between individuals and if this variation is too large it may obscure potential differences between groups of individuals. Embryos of different quality are expected to differ in expression profiles. Hence, in order to keep the individual variation within the groups to a minimum, only embryos of the highest quality were used.

Significance and Future Perspective

To our knowledge, this is the first study to investigate the transcriptome of *in vivo* produced embryos for the influence of paternal field fertility. Comparing our results to a similar study that investigated bull field fertility and embryo transcriptomic profiles in *in vitro* produced blastocysts (Kropp et al., 2017), we found few evident similarities in the genes or pathways that were differentially expressed. This underlines the importance of studying *in vivo* produced embryos even though it is a challenging approach. To understand the sire's effect on the embryo, one needs to study a complex relationship between several factors such as aspects of the semen and spermatozoa, molecular genetics and epigenetics. The bull's effect on the embryo as reported in the present study, might be caused by either bull DNA, or regulations by proteins, transcriptome or epigenetic factors deposited in the oocyte at fertilization. Regardless of the type of contribution, it is certain that it originates from the spermatozoa. Therefore, further epigenetic investigations of both spermatozoa and the resulting embryos from the same bulls would be highly interesting.

The present study adds important information to the current understanding of the paternal influence on the genetic components in embryo development. Although the field of bull fertility has received clear attention and progress during the last decade, further research is needed to clarify this complex matter, with the goal to find biomarkers that aid the prediction of bull fertility.

CONCLUSION

There was a tendency of a higher blastocyst recovery rate from heifers inseminated with the HF bulls compared to the LF bulls. Sires with a high or low field fertility produced embryos with different transcriptomic profiles, represented by the expression of 62 transcripts, several of them known to be crucial for embryo survival and development potential. The LF embryos showed a higher activity in pathways related to sterol metabolism and terpenoid backbone synthesis, while HF embryos expressed genes linked to anti-apoptosis and the regulation of cytokine signaling. The leukocyte transendothelial migration and the insulin signaling pathways were associated with enrichments in both groups. Our results suggest that the sire's genetic contribution affects all these important processes, linked to pre-and peri implantation regulation in the developing embryo. The mechanism or contributing component in the spermatozoa that affects the embryo demand further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here:PRJNA762262.

ETHICS STATEMENT

The animal study was reviewed and approved by the Norwegian Food Safety Authority, approval ID 11732.

AUTHOR CONTRIBUTIONS

AK, IO, and PG planned the study and acquired funding. SD-L, AK, AS, IO, and GG designed the details of the study. SD-L and AK performed the embryo collection, while GG carried out the RNA-sequencing and AS the subsequent bioinformatics and statistical analyses. SD-L executed the pathway analyses and the initial draft of the manuscript. AS wrote sections of the manuscript. All authors contributed to the discussion and editing of the manuscript and approved to the submission of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.780113/full#supplementary-material

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Supplementary Material

Supplementary Table 1: Outcome from cDNA production and RNA-sequencing

Fertility group	Embryo ID_Bull ID	IETS stage	cDNA concentration (ng/μl)	Raw read pairs	Clean read pairs	Clean reads %	Overall alignment % (clean reads)	Assigned fragments (read pairs)
HF	6HF_F	6	1.92	14 452 521	14 272 329	98.75	94.14	12 035 422
	7HF_F	6	4.26	10 576 857	10 372 368	98.07	94.46	8 875 618
	9HF_F	7	2.10	10 727 374	10 570 662	98.54	94.77	9 053 036
	22HF_E	7	8.34	11 151 012	11 013 848	98.77	95.28	9 255 163
	23HF_E	7	1.79	11 220 469	11 055 601	98.53	90.38	8 489 011
	25HF_E	6	7.72	13 618 537	13 410 107	98.47	94.96	11 458 301
	27HF_E	6	2.68	12 007 766	11 859 178	98.76	95.02	10 080 841
	29HF_C	7	5.64	10 680 818	10 530 598	98.59	95.47	9 082 237
	31HF_C	6	5.78	10 824 828	10 663 433	98.51	94.96	9 113 239
	32HF_C	6	3.92	14 597 117	14 357 428	98.36	93.46	12 042 081
	35HF_C	5	17.1	13 272 288	13 093 999	98.66	95.58	11 396 884
	41HF_D	7	6.00	12 660 892	12 498 892	98.72	93.20	10 191 932
	42HF_D	6	4.86	11 989 521	11 780 644	98.26	94.26	9 871 632
LF	13LF_K	6	1.64	13 678 471	13 487 721	98.61	94.38	11 234 348
	14LF_K	6	2.00	12 423 286	12 245 907	98.57	94.66	10 409 460
	48LF_H*	6	0.926	13 898 832	13 648 931	98.20	80.94	8 527 018
	49LF_H	7	6.72	21 583 312	21 275 878	98.58	94.98	17 867 228
	50LF_H	7	8.40	9 165 231	8 995 376	98.15	93.84	7 504 194
	51LF_H	7	7.46	12 364 514	12 185 164	98.55	95.38	10 522 277
	52LF_H	7	9.14	10 978 344	10 798 704	98.36	95.27	9 303 217
	53LF_H	7	12.2	11 824 901	11 674 310	98.73	95.25	10 043 333
	57LF_H	7	7.04	11 433 681	11 245 667	98.36	94.14	9 455 553
	63LF_H	6	9.12	13 409 146	13 245 803	98.78	95.20	11 418 022
	66LF_I*	6	0.262	13 679 142	13 494 347	98.65	91.41	10 343 817

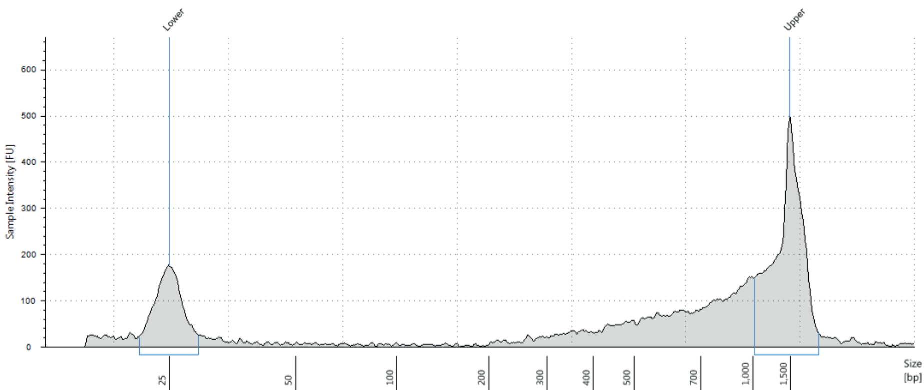
**These samples were considered outliers due to poor cDNA concentration and sequencing quality, and hence removed from further analyses.*

Supplementary Table 3: Human orthologues

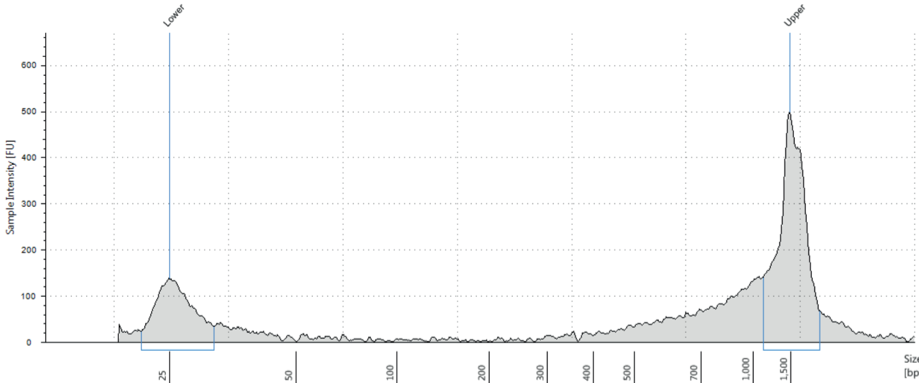
Bovine gene ID	Gene symbol	Log2 foldchange	Adj.p-value	Human orthologue
Highly expressed in LF embryos				
ENSBTAG00000014046	*BPI*	10.90	1.72E-03	ENSG00000101425
ENSBTAG00000003305	*NCF1*	5.81	6.69E-03	ENSG00000158517
ENSBTAG00000047563	*CLDN9*	3.69	6.76E-03	ENSG00000213937
ENSBTAG00000026893	*EXOC3L4*	3.26	1.70E-03	ENSG00000205436
ENSBTAG00000049434	(Non-annotated gene)	2.99	2.52E-03	ENSG00000155380
ENSBTAG00000051376	(Non-annotated gene)	2.43	2.52E-03	ENSG00000151729
ENSBTAG00000013854	*CALML5*	2.17	7.14E-10	ENSG00000178372
ENSBTAG00000011839	*HMGCS1*	2.05	6.13E-04	ENSG00000112972
ENSBTAG00000054516	*CYP17A1*	1.95	2.60E-02	ENSG00000148795
ENSBTAG00000017819	*PMVK*	1.74	1.21E-02	ENSG00000163344
ENSBTAG00000004905	*KRT19*	1.67	2.83E-02	ENSG00000171345
ENSBTAG00000003068	*MSMO1*	1.58	6.49E-04	ENSG00000052802
ENSBTAG00000006305	*AK1*	1.51	4.30E-03	ENSG00000106992
ENSBTAG00000017864	*PRPH*	1.47	2.52E-03	ENSG00000135406
ENSBTAG00000055207	*SCD*	1.47	4.39E-03	ENSG00000099194
ENSBTAG00000004075	*IDI1*	1.39	2.60E-04	ENSG00000067064
ENSBTAG00000012432	*FDFT1*	1.37	1.05E-02	ENSG00000079459
ENSBTAG00000007840	*HMGCR*	1.34	4.30E-03	ENSG00000113161
ENSBTAG00000004881	*MTHFD2*	1.30	2.84E-03	ENSG00000065911
ENSBTAG00000014127	*PTGS2*	1.26	4.30E-03	ENSG00000073756
ENSBTAG00000055124	(Non-annotated gene)	1.23	2.76E-03	No orthologue
ENSBTAG00000003948	*FDPS*	1.10	2.52E-03	ENSG00000160752
ENSBTAG00000003100	*SMTN*	1.05	2.76E-02	ENSG00000183963
ENSBTAG00000004982	*GPLD1*	1.04	1.53E-02	ENSG00000112293
ENSBTAG00000032914	*SLC11A2*	1.00	2.51E-02	ENSG00000110911
ENSBTAG00000006471	*OSBPL11*	0.96	2.75E-02	ENSG00000144909
ENSBTAG00000014227	*NDFIP2*	0.95	4.30E-03	ENSG00000102471
ENSBTAG00000019246	*SC5D*	0.92	4.56E-02	ENSG00000109929
ENSBTAG00000055014	*SH3BGRL2*	0.85	3.07E-02	ENSG00000198478
ENSBTAG00000044015	*RBM12*	0.73	2.73E-02	ENSG00000244462
ENSBTAG00000012317	*PNP*	0.71	4.78E-02	ENSG00000198805
ENSBTAG00000016896	*HERPUD1*	0.70	2.52E-03	ENSG00000051108
ENSBTAG00000017258	*ACSL3*	0.67	2.70E-02	ENSG00000123983
ENSBTAG00000011899	*USP4*	0.64	6.95E-03	ENSG00000114316

Highly expressed in HF embryos				
ENSBTAG00000031825	(Non-annotated gene)	8.02	2.60E-04	ENSG00000131943
ENSBTAG00000046257	*GIMAP4*	5.85	3.57E-02	ENSG00000133574
ENSBTAG00000014560	*HLX*	5.72	3.80E-02	ENSG00000136630
ENSBTAG00000030882	*hsd20b2*	3.48	1.85E-02	No orthologue
ENSBTAG00000015836	(Non-annotated gene)	2.82	1.91E-04	ENSG00000283632
ENSBTAG00000010123	*APOE*	2.65	2.36E-02	ENSG00000130203
ENSBTAG00000014596	*EFHD1*	2.43	1.70E-03	ENSG00000115468
ENSBTAG00000027444	*SVIL*	2.37	4.98E-02	ENSG00000197321
ENSBTAG00000054434	(Non-annotated gene)	2.08	3.33E-02	No orthologue
ENSBTAG00000033429	*FAM229B*	1.96	4.92E-02	ENSG00000203778
ENSBTAG00000049950	(Non-annotated gene)	1.86	4.92E-02	No orthologue
ENSBTAG00000026758	(Non-annotated gene)	1.83	2.60E-02	ENSG00000211454
ENSBTAG00000017094	*SHMT1*	1.72	2.73E-02	ENSG00000176974
ENSBTAG00000038384	*KRT5*	1.48	1.21E-02	ENSG00000186081
ENSBTAG00000054234	(Non-annotated gene)	1.47	2.86E-02	ENSG00000178934
ENSBTAG00000003568	*CLDN10*	1.43	4.44E-03	ENSG00000134873
ENSBTAG00000004386	*SOCS1*	1.39	4.30E-03	ENSG00000185338
ENSBTAG00000012511	*BAD*	1.36	1.72E-03	ENSG00000002330
ENSBTAG00000003043	*GNG2*	1.33	2.09E-02	ENSG00000186469
ENSBTAG00000006086	*MMP28*	1.33	1.72E-03	ENSG00000271447
ENSBTAG00000022028	*DERL3*	1.17	2.52E-03	ENSG00000099958
ENSBTAG00000013922	*MOSPD1*	1.15	4.47E-02	ENSG00000101928
ENSBTAG00000020528	*PCOLCE*	1.12	1.01E-02	ENSG00000106333
ENSBTAG00000003222	*ASNS*	1.00	4.18E-02	ENSG00000070669
ENSBTAG00000010740	*CLTB*	0.88	4.21E-02	ENSG00000175416
ENSBTAG00000052249	(Non-annotated gene)	0.80	2.60E-02	ENSG00000204531
ENSBTAG00000021111	*POU5F1*	0.77	2.26E-03	ENSG00000204531
ENSBTAG00000017932	*CCDC84*	0.65	3.75E-02	ENSG00000186166

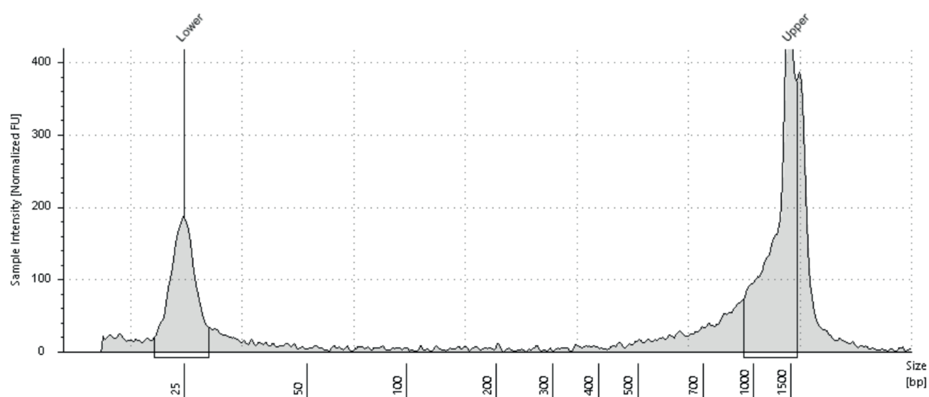
(A) Embryo ID 6HF



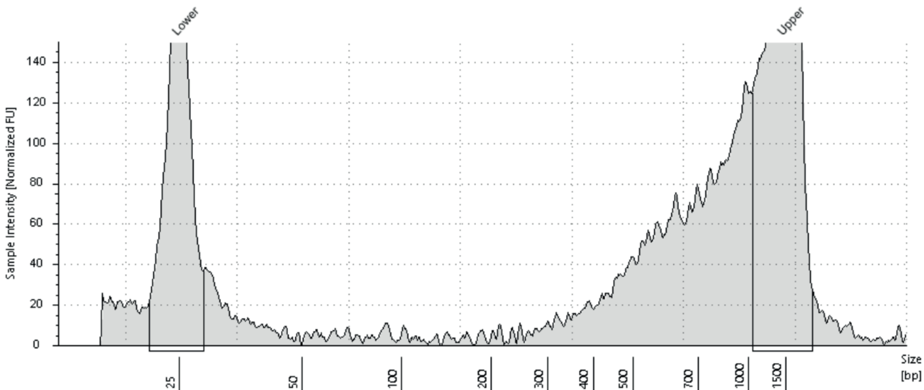
(B) Embryo ID 7HF



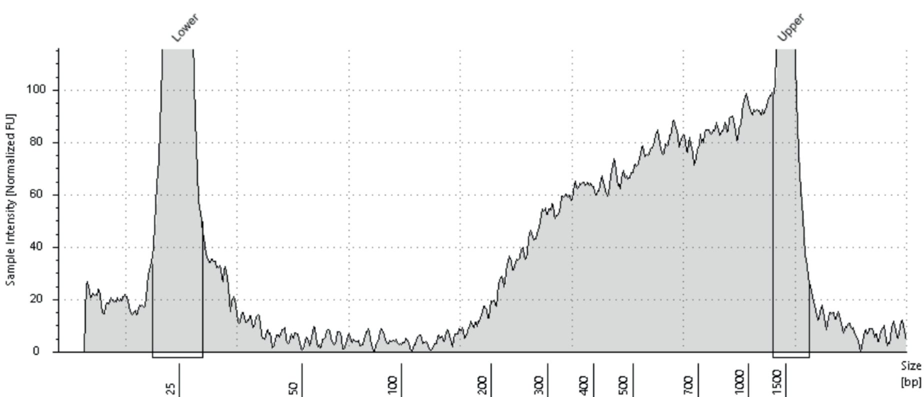
(C) Embryo ID 9HF



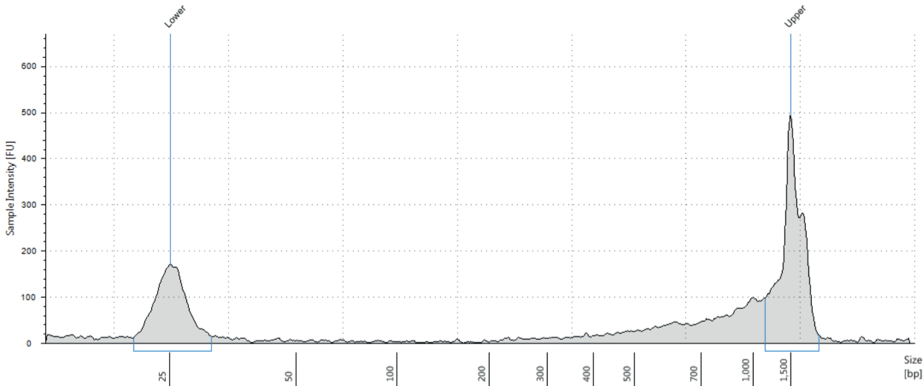
(D) Embryo ID 22HF



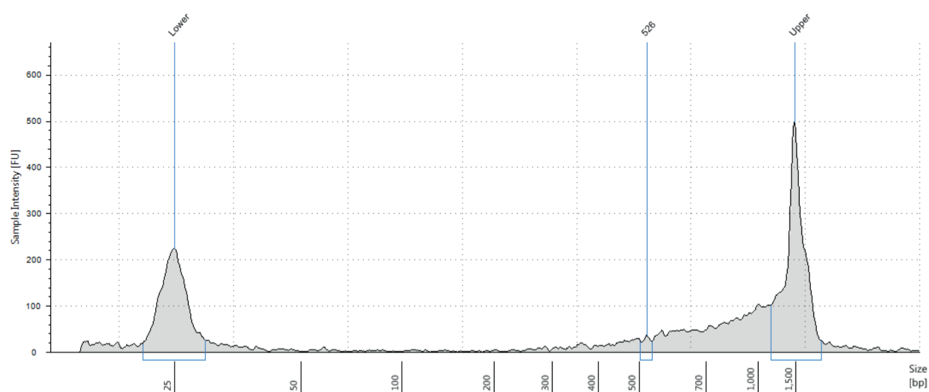
(E) Embryo ID 23HF



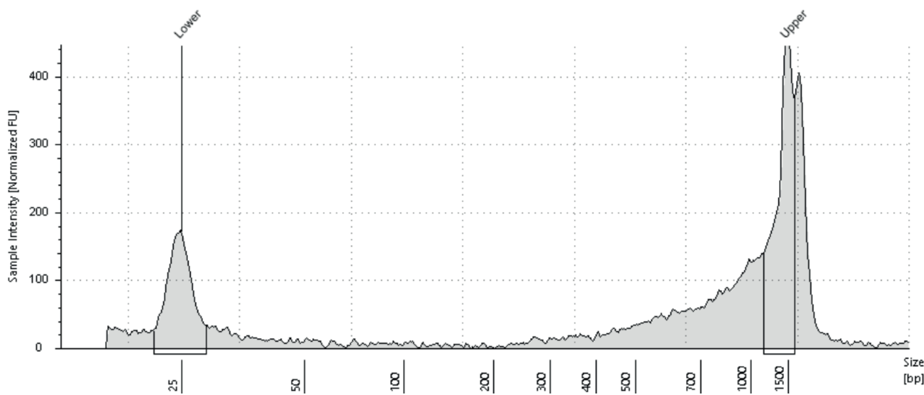
(F) Embryo ID 25HF



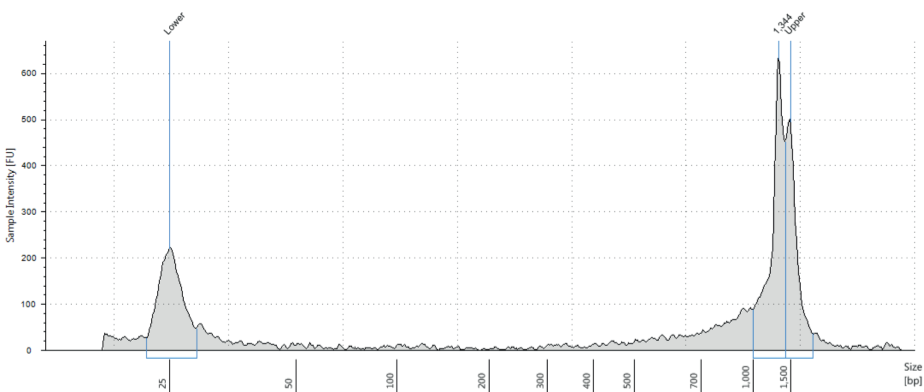
(G) Embryo ID 27HF



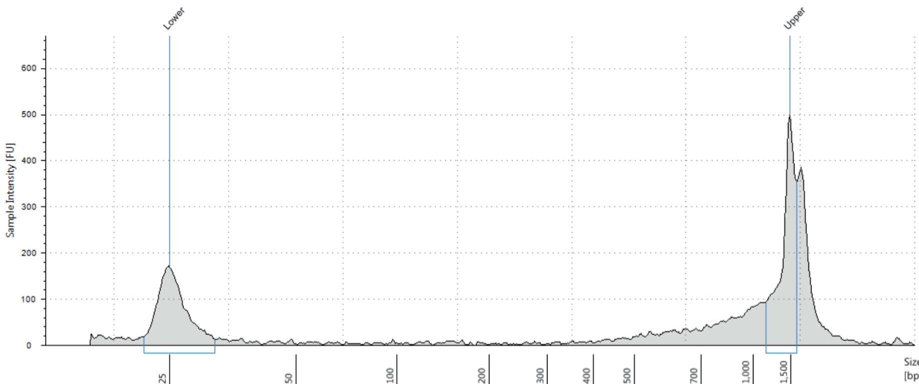
(H) Embryo ID 29HF



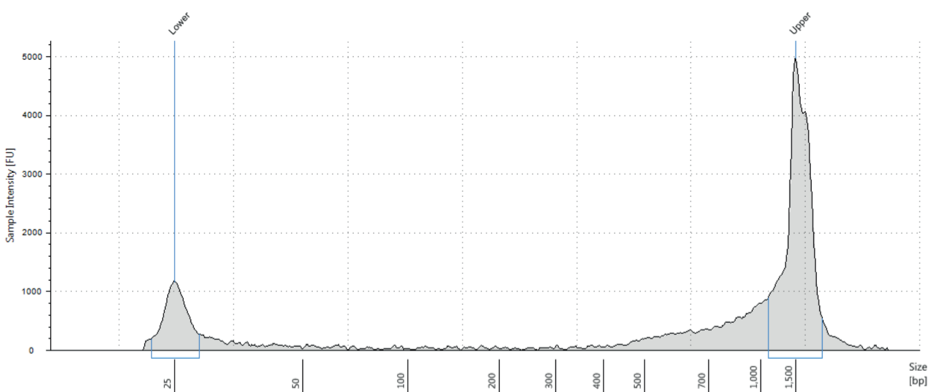
(I) Embryo ID 31HF



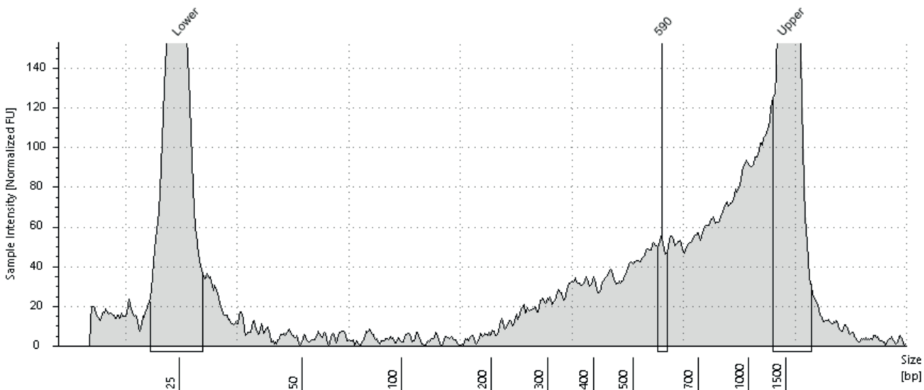
(J) Embryo ID 32HF



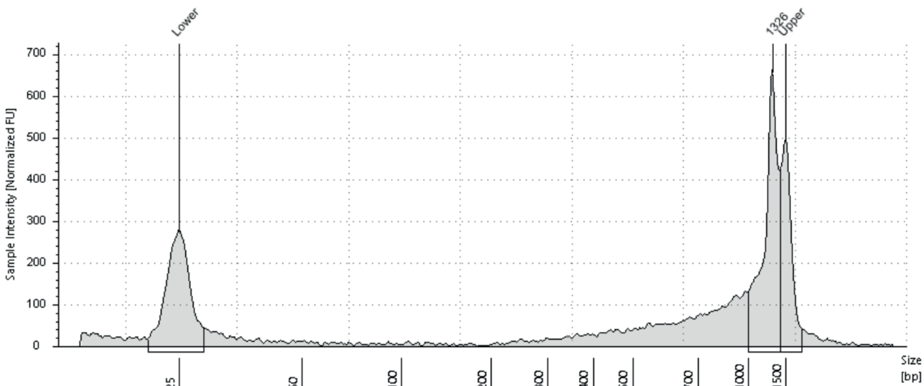
(K) Embryo ID 35HF



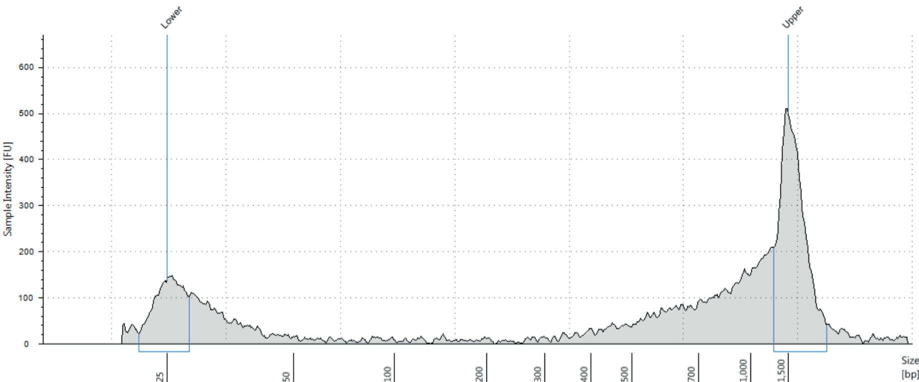
(L) Embryo ID 41HF



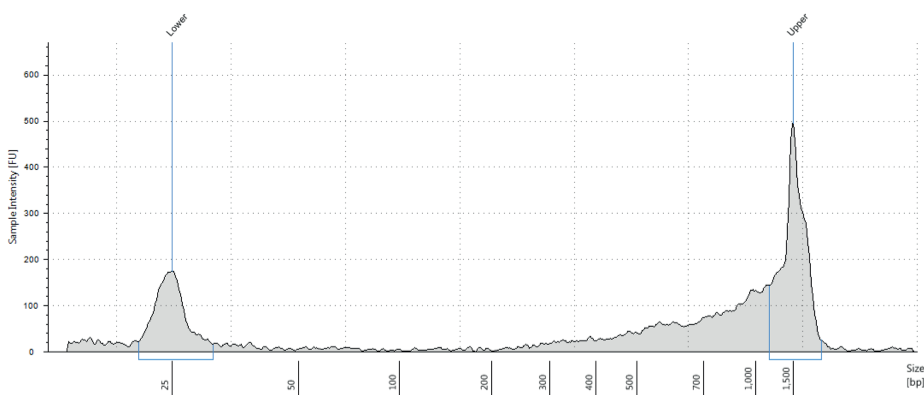
(M) Embryo ID 42HF



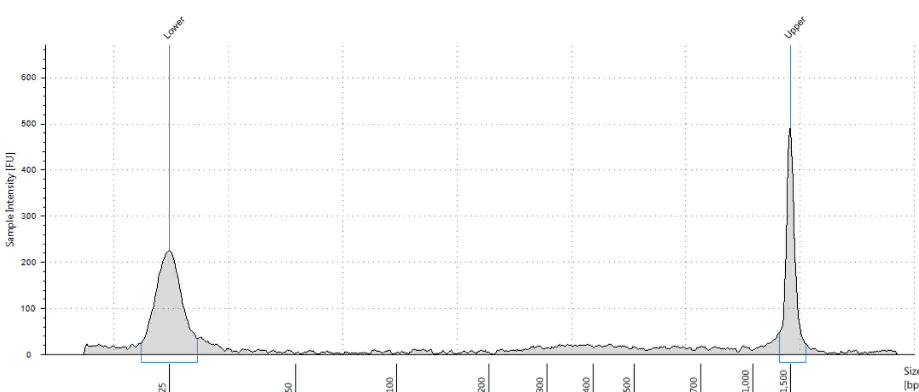
(N) Embryo ID 13LF



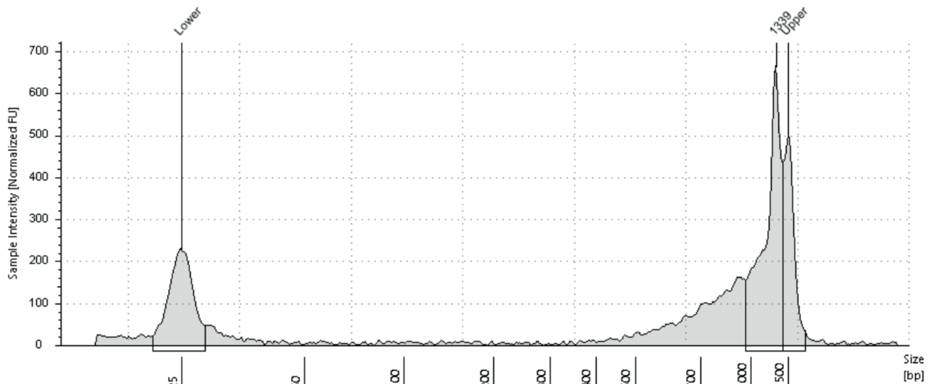
(O) Embryo ID 14LF



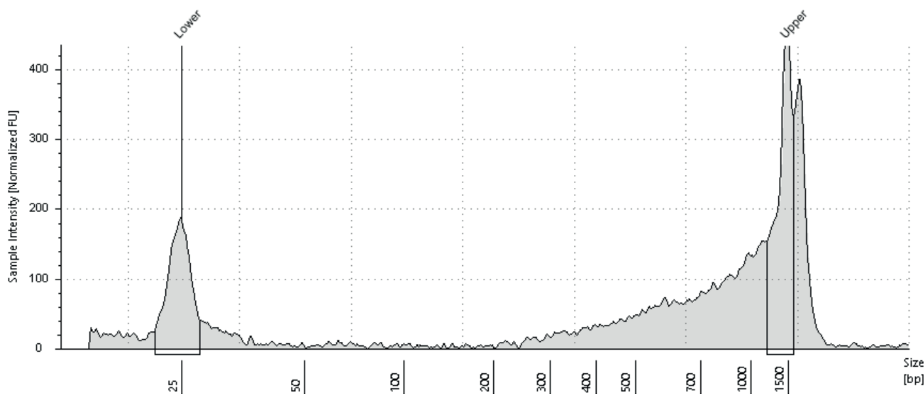
(P) Embryo ID 48LF*



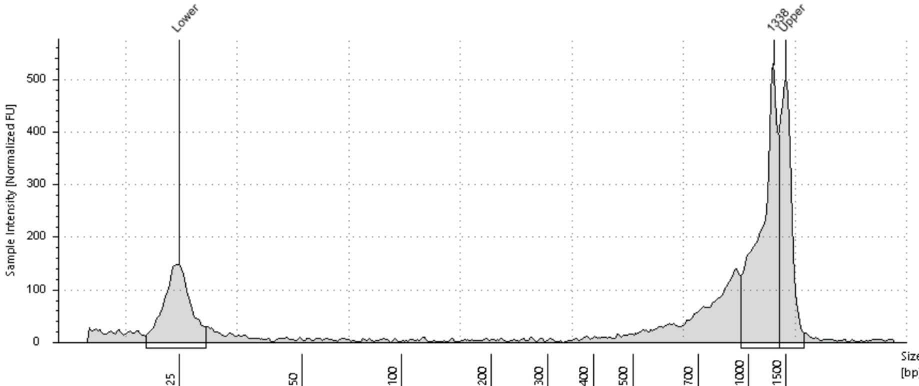
(Q) Embryo ID 49LF



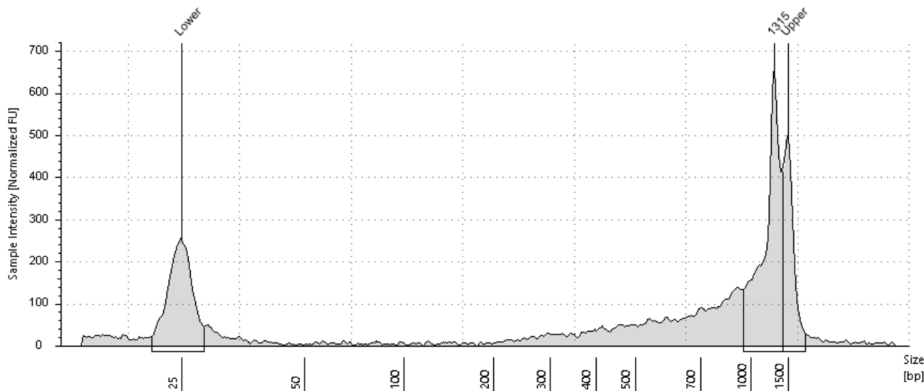
(R) Embryo ID 50LF



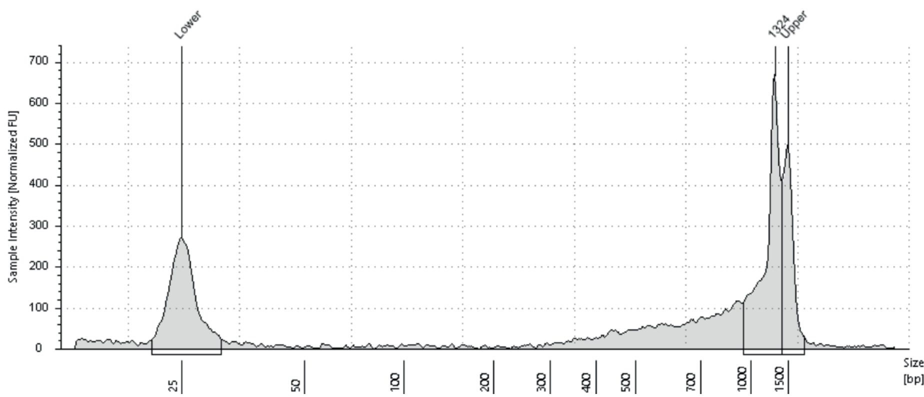
(S) Embryo ID 51LF



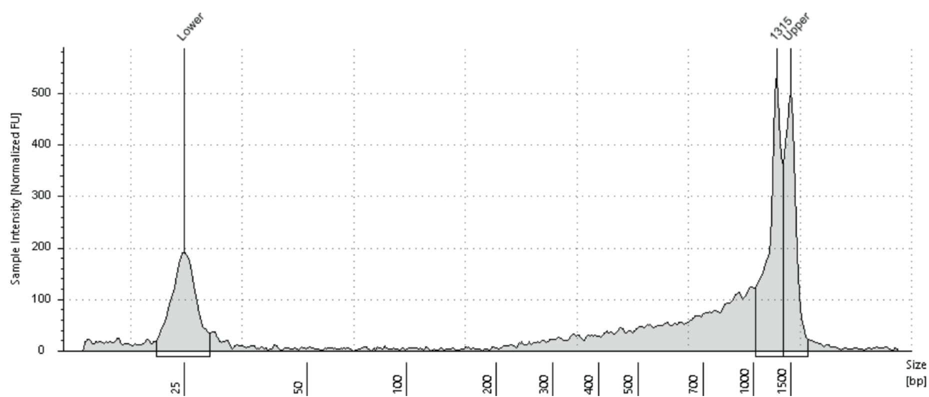
(T) Embryo ID 52LF



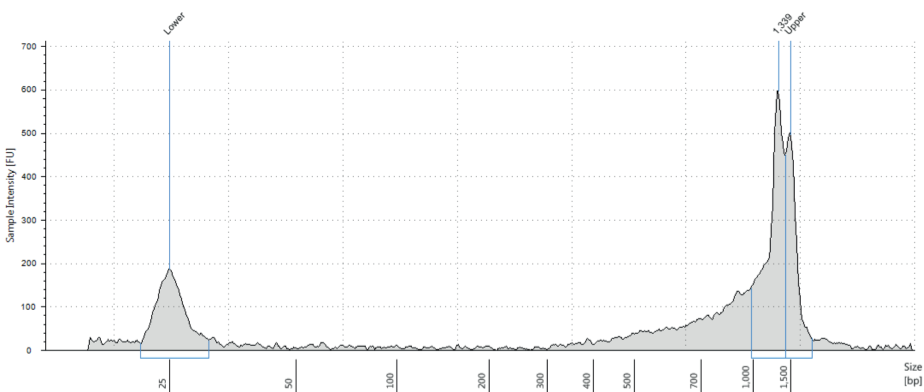
(U) Embryo ID 53LF



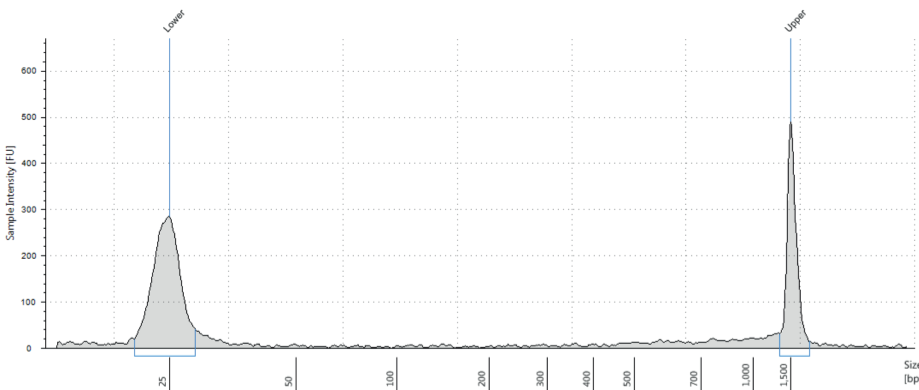
(V) Embryo ID 57LF



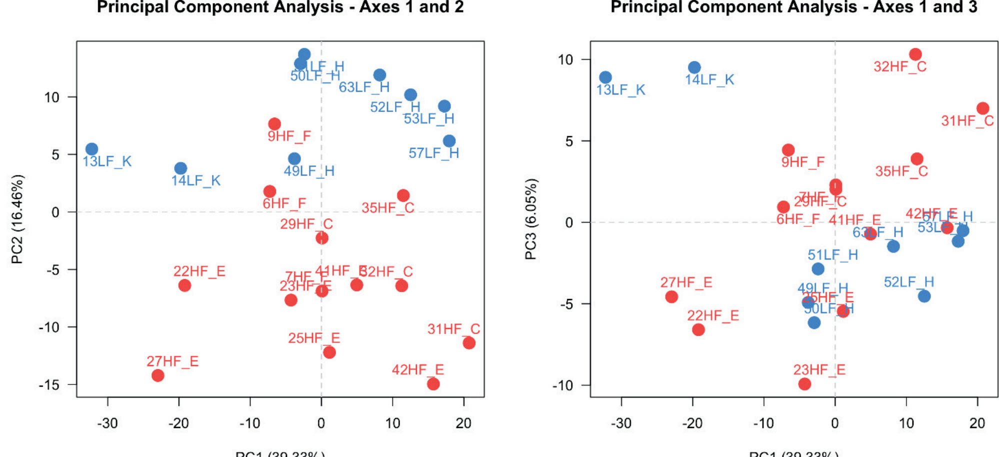
(W) Embryo ID 63LF



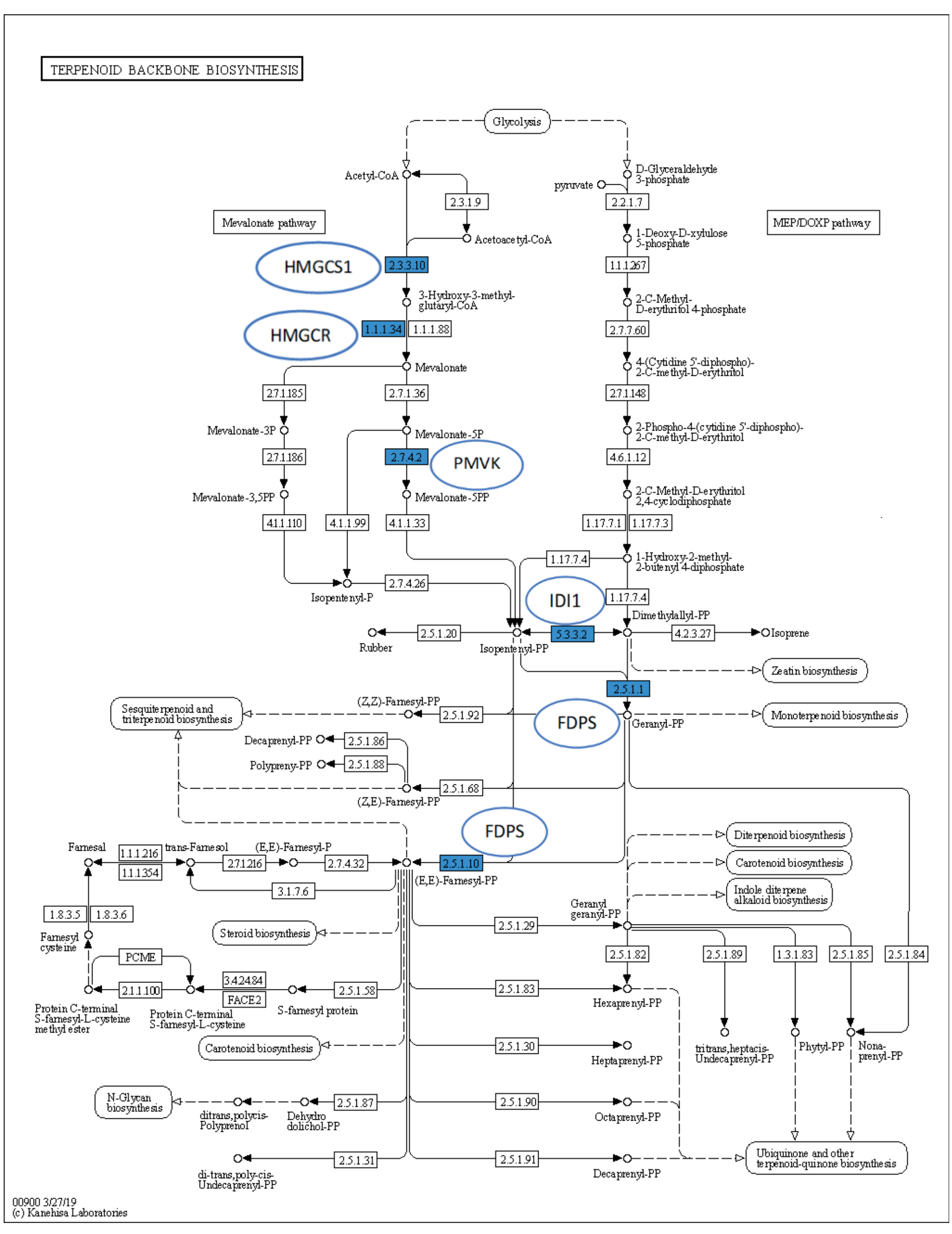
(X) Embryo ID 66LF*



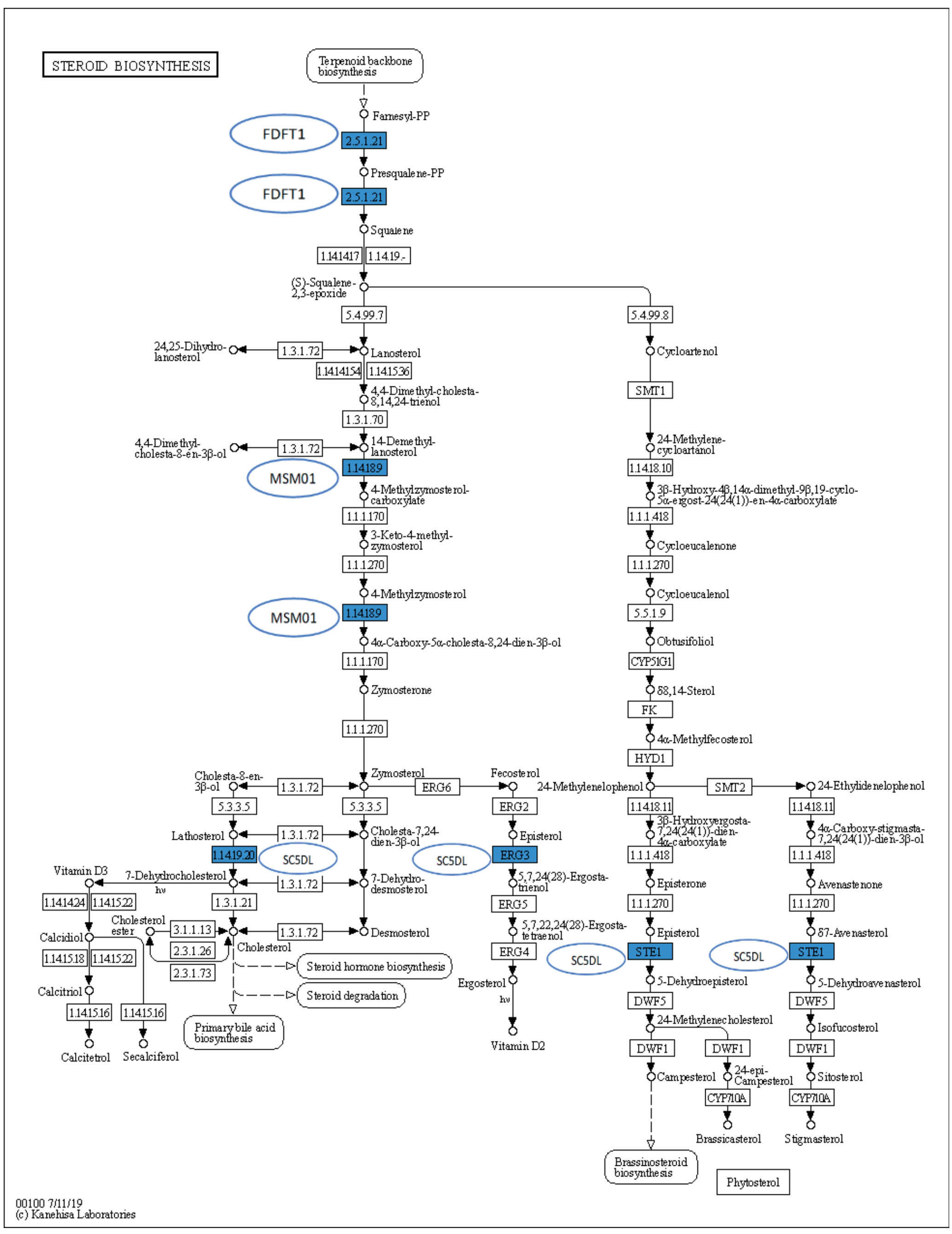
Supplementary figure 1: TapeStation profiles for all sequenced embryos. *These samples were not included in the DE-analysis.



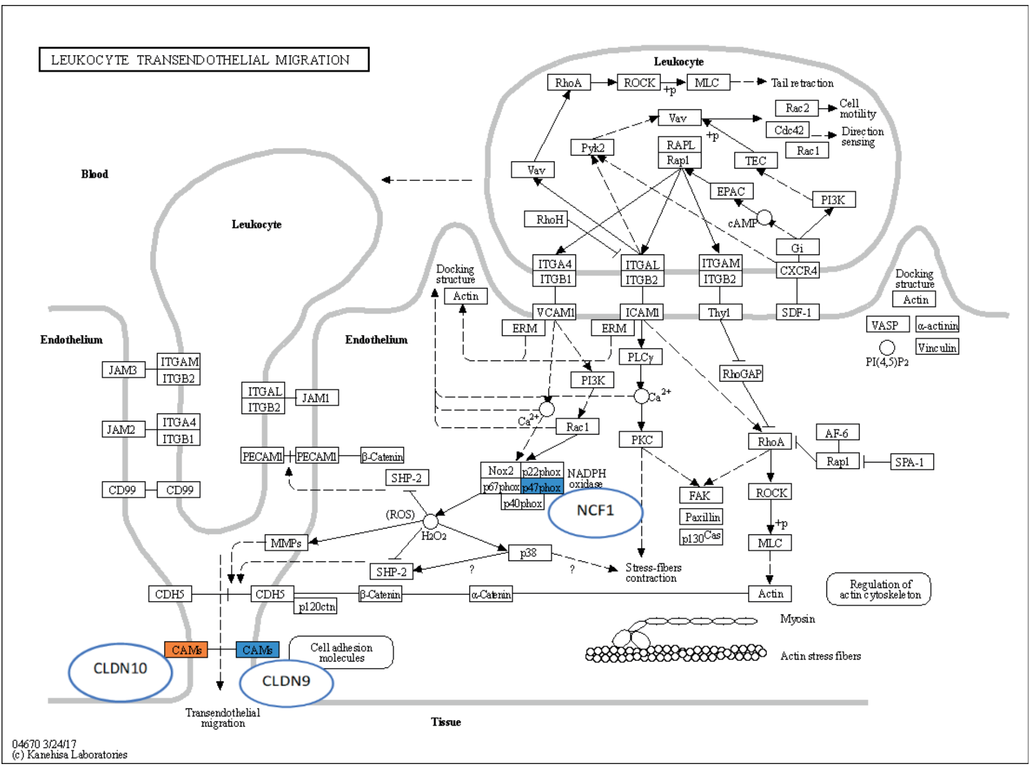
Supplementary Figure 2: Distribution of individual embryos, without embryo 48 and 66 which were considered outliers.


TERPENOID BACKBONE BIOSYNTHESIS
Glycolysis
Acetyl-CoA
D-Glyceraldehyde 3-phosphate
pyruvate
2.3.1.9
2.2.1.7
Mevalonate pathway
Acetoacetyl-CoA
1-Deoxy-D-xylulose 5-phosphate
MEP/DOXP pathway
HMGCS1
2.3.3.10
1.1.1.267
3-Hydroxy-3-methyl-glutaryl-CoA
2-C-Methyl-D-erythritol 4-phosphate
HMGCR
1.1.1.34
1.1.1.88
2.7.7.60
Mevalonate
4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol
2.7.1.185
2.7.1.36
2.7.1.148
Mevalonate-3P
Mevalonate-5P
2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
2.7.1.186
2.7.4.2
PMVK
4.6.1.12
Mevalonate-3,5PP
Mevalonate-5PP
2-C-Methyl-D-erythritol 2,4-cyclodiphosphate
4.1.1.110
4.1.1.99
4.1.1.33
1.17.7.1
1.17.7.3
1.17.7.4
1-Hydroxy-2-methyl-2-butenyl 4-diphosphate
2.7.4.26
IDI1
1.17.7.4
Isopentenyl-P
Dimethylallyl-PP
2.5.1.20
5.3.3.2
4.2.3.27
Isoprene
Rubber
Isopentenyl-PP
Zeatin biosynthesis
2.5.1.1
(Z,Z)-Farnesyl-PP
Sesquiterpenoid and triterpenoid biosynthesis
2.5.1.92
FDPS
Geranyl-PP
Monoterpenoid biosynthesis
Decaprenyl-PP
2.5.1.86
Polypreny-PP
2.5.1.88
2.5.1.68
(Z,E)-Farnesyl-PP
FDPS
Farnesal
1.1.1.216
trans-Farnesol
(E,E)-Farnesyl-P
2.7.1.216
2.7.4.32
2.5.1.10
Diterpenoid biosynthesis
1.1.1.354
(E,E)-Farnesyl-PP
Carotenoid biosynthesis
3.1.7.6
Indole diterpene alkaloid biosynthesis
1.8.3.5
1.8.3.6
Geranyl geranyl-PP
Farnesyl cysteine
Steroid biosynthesis
2.5.1.29
PCME
2.5.1.82
2.5.1.89
1.3.1.83
2.5.1.85
2.5.1.84
3.4.24.84
2.1.1.100
FACE2
2.5.1.58
2.5.1.83
Protein C-terminal S-farnesyl-L-cysteine methyl ester
Protein C-terminal S-farnesyl-L-cysteine
S-farnesyl protein
Hexaprenyl-PP
Carotenoid biosynthesis
2.5.1.30
tritrans,heptacis-Undecaprenyl-PP
Phytyl-PP
Nona-prenyl-PP
Heptaprenyl-PP
N-Glycan biosynthesis
2.5.1.87
2.5.1.90
ditrans,polycis-Polyprenol
Dehydro dolichol-PP
Octaprenyl-PP
Ubiquinone and other terpenoid-quinone biosynthesis
2.5.1.31
2.5.1.91
di-trans,poly-cis-Undecaprenyl-PP
Decaprenyl-PP
00900 3/27/19
(c) Kanehisa Laboratories

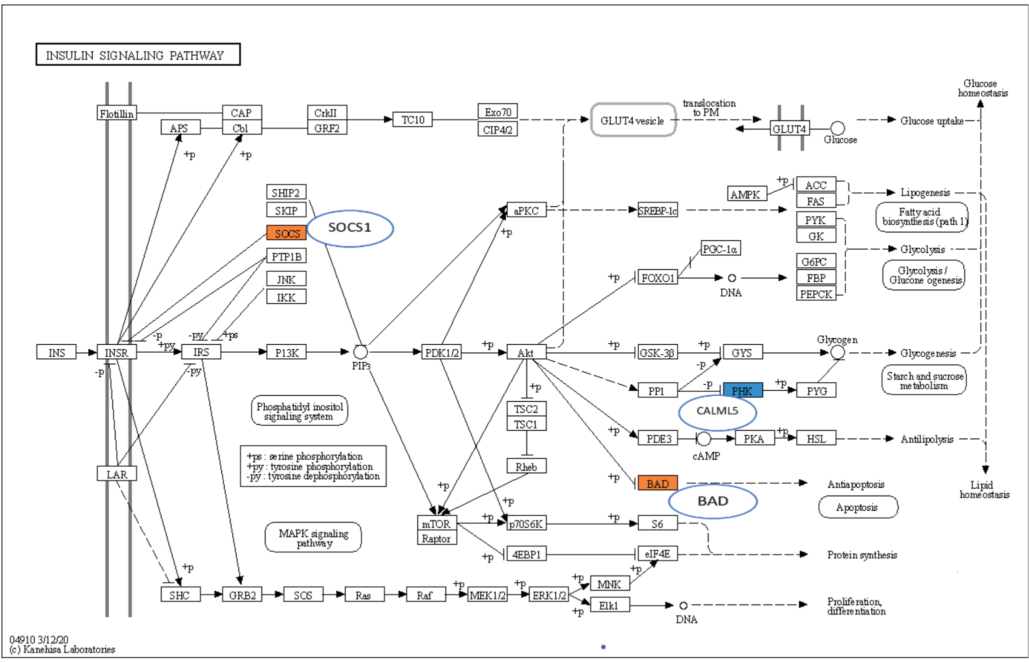
Supplementary Figure 3: Terpenoid backbone biosynthesis. Gene products that were highly expressed in the LF embryos are marked in blue. Gene symbols are marked in circles. Modified from KEGG chart bta00900, 3/27/19 Kaneshia Laboratories, URL: kegg.jp


STEROID BIOSYNTHESIS
Terpenoid backbone biosynthesis
Farnesyl-PP
FDFT1
2.5.1.21
Presqualene-PP
FDFT1
2.5.1.21
Squalene
1.14.14.17
1.14.19.-
(S)-Squalene-2,3-epoxide
5.4.99.7
5.4.99.8
24,25-Dihydro-lanosterol
1.3.1.72
Lanosterol
Cycloartenol
1.14.14.154
1.14.15.36
4,4-Dimethyl-cholesta-8,14,24-trienol
SMT1
1.3.1.70
4,4-Dimethyl-cholesta-8-en-3β-ol
1.3.1.72
14-Demethyl-lanosterol
24-Methylene-cycloartanol
MSMO1
1.14.18.9
1.14.18.10
4-Methylzymosterol-carboxylate
3β-Hydroxy-4β,14α-dimethyl-9β,19-cyclo-5α-ergost-24(24(1))-en-4α-carboxylate
1.1.1.170
1.1.1.418
3-Keto-4-methyl-zymosterol
Cycloeucalenone
1.1.1.270
1.1.1.270
4-Methylzymosterol
Cycloeucalenol
MSMO1
1.14.18.9
5.5.1.9
4α-Carboxy-5α-cholesta-8,24-dien-3β-ol
Obtusifoliol
1.1.1.170
CYP51G1
Zymosterone
δ8,14-Sterol
FK
1.1.1.270
4α-Methylfecosterol
HYD1
Cholesta-8-en-3β-ol
1.3.1.72
Zymosterol
ERG6
Fecosterol
24-Methylenelophenol
SMT2
24-Ethylidenelophenol
5.3.3.5
5.3.3.5
ERG2
1.14.18.11
1.14.18.11
Lathosterol
1.3.1.72
Cholesta-7,24-dien-3β-ol
Episterol
3β-Hydroxyergosta-7,24(24(1))-dien-4α-carboxylate
4α-Carboxy-stigmasta-7,24(24(1))-dien-3β-ol
1.14.19.20
SC5DL
SC5DL
ERG3
1.1.1.418
1.1.1.418
Vitamin D3
7-Dehydrocholesterol
1.3.1.72
7-Dehydro-desmosterol
5,7,24(28)-Ergosta-trienol
Episterone
Avenastenone
hν
1.14.14.24
1.14.15.22
1.3.1.21
ERG5
1.1.1.270
1.1.1.270
Cholesterol ester
3.1.1.13
1.3.1.72
Desmosterol
5,7,22,24(28)-Ergosta-tetraenol
Episterol
δ7-Avenasterol
Calcidiol
2.3.1.26
Cholesterol
ERG4
STE1
STE1
1.14.15.18
1.14.15.22
2.3.1.73
Steroid hormone biosynthesis
SC5DL
SC5DL
Ergosterol
5-Dehydroepisterol
5-Dehydroavenasterol
Calcitriol
Steroid degradation
hν
DWF5
DWF5
1.14.15.16
1.14.15.16
24-Methylenecholesterol
Isofucosterol
Primary bile acid biosynthesis
Vitamin D2
DWF1
DWF1
DWF1
Calcitetrol
Secalciferol
Campesterol
24-epi-Campesterol
Sitosterol
CYP710A
CYP710A
Brassicasterol
Stigmasterol
Brassinosteroid biosynthesis
Phytosterol
00100 7/11/19
(c) Kanehisa Laboratories

Supplementary Figure 4: Steroid biosynthesis. Gene products that were highly expressed in the LF embryos are marked in blue. Gene symbols are marked in circles. Modified from KEGG chart bta00100, 7/11/19 Kaneshia Laboratories, URL: kegg.jp



Supplementary Figure 5: Leukocyte transendothelial migration. Gene products that were highly expressed in the LF embryos are marked in blue, while gene products that were highly expressed in the HF embryos are marked in orange. Gene symbols are marked in circles. Modified from KEGG chart bta04670, 3/24/17 Kaneshia Laboratories, URL: kegg.jp



Supplementary Figure 6: Insulin signaling pathway. Gene products that were highly expressed in the LF embryos are marked in blue, while gene products that were highly expressed in the HF embryos are marked in orange. Gene symbols are marked in circles. Modified from KEGG chart bta04910, 3/12/20 Kaneshia Laboratories, URL: kegg.jp

The supplementary material for paper IV contains a datasheet (DataSheet3.xlsx) which can be found online:

https://www.frontiersin.org/articles/10.3389/fgene.2021.780113/full#supplementary-material

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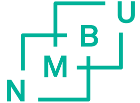

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