Department of Production Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Superovulation and embryo transfer in dairy cattle – effect of management factors with emphasis on sex-sorted semen

by

Marja Mikkola

ACADEMIC DISSERTATION

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Director of studies	Professor Magnus Andersson DVM, PhD Department of Production Animal Medicine Faculty of Veterinary Medicine University of Helsinki Finland
Supervisor	Adjunct Professor Juhani Taponen DVM, PhD Department of Production Animal Medicine Faculty of Veterinary Medicine University of Helsinki Finland
Pre-examiners	Associate Professor Renée Båge DVM, PhD, Dipl ECAR Department of Clinical Sciences Swedish University of Agricultural Sciences Sweden
	Professor Peter J. Hansen MS, PhD Department of Animal Sciences University of Florida USA
Opponent	Associate Professor Peter L.A.M. Vos DVM, PhD, Dipl ECAR and ECBHM Department of Farm Animal Health Section Reproduction Faculty of Veterinary Medicine Utrecht University The Netherlands
Chairman	Professor Magnus Andersson DVM, PhD Department of Production Animal Medicine Faculty of Veterinary Medicine University of Helsinki Finland

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“There is a grandeur
in this view of life, with its several powers,
having been originally breathed into a few forms or into one;
and that, whilst this planet has gone cycling on
according to the fixed law of gravity,
from so simple a beginning endless forms
most beautiful and most wonderful
have been, and are being, evolved.”

- Charles Darwin
On the Origin of Species

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ABSTRACT

Multiple ovulation and embryo transfer (MOET) has been established in cattle breeding since the 1970s. It is an efficient means to increase the number of offspring from genetically superior females. Despite nearly 50 years of development, the average number of transferable embryos recovered in a single embryo collection has remained nearly constant at approximately six embryos per donor. Several factors contribute to the outcome of superovulation and embryo recovery. These comprise animal-related, environmental and management factors. The most prominent factor affecting the success of superovulation is an animal-related attribute: the ovarian follicular population responsive to exogenous gonadotropin stimulation. Environmental factors, such as heat stress or other external factors causing stress, can compromise the embryo yield after superovulation. However, such factors represent a management challenge. The superovulatory outcome is additionally affected by several factors that can conflict with management decisions, including nutritional management of the donor, gonadotropin treatment protocol and semen and technical performance of donor inseminations.

The purpose of the work presented in this thesis was to investigate management factors that affect the efficacy of MOET. First, the effect of nutritional protein in the form of rapeseed meal on superovulation of dairy heifers was studied. One-year old heifers were fed diets formulated to meet energy requirements for 800 g daily gain and crude protein either at 14% or 18%, which was higher than the feeding recommendations and the common practice on farms. There was no effect of the higher protein level on the ovulation rate, total number of embryos recovered or the number of transferable embryos. Feeding an energy-adequate diet containing moderate or high protein with respect to feeding recommendations resulted in comparable embryo yields.

The efficacy of two commercial FSH products was compared in a retrospective study on superovulations of heifers and cows on Finnish dairy farms and an embryo collection station. A highly purified porcine FSH with a low LH:FSH ratio, Folltropin, was used for 2592 superovulations, and Pluset, containing equal amounts of LH and FSH, was used for 1398 superovulations. Pluset-treated donors had a higher ovulation rate, yielding 1.1 recovered structures (embryos and ova) more than those receiving Folltropin. However, the difference was characterized by more unfertilized ova (UFO). For transferable embryos, the number, quality and

developmental stage were similar for both preparations. Therefore, it can be concluded that the efficacy of the preparations is comparable.

The effect of sex-sorted semen on efficacy of MOET was investigated from a dataset of commercial embryo collections and transfers. A total of 443 embryo collections produced with sex-sorted semen and 1528 produced with conventional semen were analyzed. Sex-sorted semen decreased the number of transferable embryos and increased proportions of UFO and degenerated embryos, compared with non-sorted semen. The decrease was more evident in cows than in heifers. The proportion of poor quality embryos was higher and there was a slight delay in the embryo developmental kinetics for sexed embryos. The risk of recovering no transferable embryos was increased when sex-sorted semen was used. Pregnancy rates after transfer of embryos produced with sex-sorted semen were 12% lower than for embryos originating from conventional semen. It can be concluded from these studies on sexed semen that the use of sex-sorted semen is profitable because more female calves can be produced from a donor heifer, wasting less recipient resources. For superovulated cows, equal numbers of female calves can be produced per embryo collection, but the need for only half the number of recipients compared with using conventional semen favors the use of sexed semen when female progeny are desired.

ORIGINAL ARTICLES

This dissertation is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Mikkola M., Mäntysaari P., Tammiranta N., Peippo J., Taponen J. Effect of dietary protein on embryo recovery rate and quality in superovulated heifers. Anim Reprod Sci. 2005, 87: 193-202.

II Mikkola M., Taponen J. Embryo yield in dairy cattle after superovulation with Folltropin or Pluset. Theriogenology 2017, 88: 84-88.

III Kaimio I.*, Mikkola M.*, Lindeberg H., Heikkinen J., Hasler J.F., Taponen J. Embryo production with sex-sorted semen in superovulated dairy heifers and cows. Theriogenology 2013, 80: 950-954. (*equal authorship)

IV Mikkola M., Taponen J. Quality and developmental rate of embryos produced with sex-sorted and conventional semen from superovulated dairy cattle. Theriogenology 2017, 87: 135-140.

V Mikkola M., Andersson M., Taponen J. Transfer of cattle embryos produced with sex-sorted semen results in impaired pregnancy rate and increased male calf mortality. Theriogenology 2015, 84: 1118-1122.

ABBREVIATIONS

AI	artificial insemination
AFC	antral follicular count
AMH	Anti-Müllerian hormone
BUN	blood urea nitrogen
CL	corpus luteum, corpora lutea
COC	cumulus-oocyte-complex
CP	crude protein
DUI	deep uterine insemination
eCG	equine chorionic gonadotropin
ET	embryo transfer
GnRH	gonadotropin-releasing hormone
FSH	follicle stimulating hormone
i.m.	intramuscular
IVF	*in vitro* fertilization
IVP	*in vitro* production
LH	luteinizing hormone
MOET	multiple ovulation and embryo transfer
NEB	negative energy balance
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
UFO	unfertilized oocyte, unfertilized ova

INTRODUCTION

Multiple ovulation and embryo transfer (MOET) entered into standard use in dairy cattle breeding since commercialization of the industry in the early 1970s, enhancing production of multiple offspring from genetically superior females. However, the efficiency of a superovulation and embryo collection procedure has not improved markedly during four decades of commercial embryo transfer (ET). The efficiency of a superovulation procedure is evaluated in terms of the numbers of viable embryos, pregnancies and live calves.

The number of viable embryos recovered from a donor in one embryo collection averages six, but varies considerably among animals, with approximately 15% of embryo collections resulting in no transferable embryos and a small proportion of donors producing 20-50 embryos. Among all donors in the Finnish breeding program, 35% at the embryo collection center produce 70% of the embryos. The unpredictability arising from individual variation in the superovulatory response is a major limiting factor determining the efficiency of MOET in breeding programs. There are numerous donor-related, environmental and management factors that affect the success of superovulation and embryo recovery in cattle.

The objective of this thesis was to study the principal management factors affecting the efficacy of MOET: nutrition, semen type and gonadotropin preparation. The initial reason for the studies included in this thesis originated from the daily work of the author as an ET-practitioner and the need to find solutions to very practical problems. The major nutritional factors influencing the success of superovulation are energy balance and protein intake and quality. Negative energy balance of high yielding dairy cows can compromise the superovulatory response. Today the majority of embryo production in Finland and other countries applying genomic selection in breeding programs relies on heifers, for which energy deficiency does not play a substantial role. However, a relatively common practice on Finnish dairy farms is to provide heifers low to moderate protein feed. Among nutritional factors, the effect of dietary protein content on superovulatory success of dairy heifers was studied in this dissertation.

Several studies comparing the effect of FSH preparations on superovulatory response have been published. However, large-scale data evaluation of the efficacy of Folltropin and Pluset, the two products available on the Finnish veterinary

market, has been lacking. A retrospective study of superovulation data investigated the effects of FSH treatment protocol.

Sex-sorted bovine semen became commercially available in the early 2000s. Insemination of superovulated donors with sex-sorted semen represents a means to produce progeny of the desired gender. However, the results are not generally comparable with those achieved with non-sorted semen. Embryo yield and quality, as well as the probability of a recipient conceiving, can be compromised. This has impeded large-scale use of sex-sorted semen in ET practice. Sex-sorted semen for superovulated donors and its effect on embryo yield and quality was investigated. Additionally, sex sorted semen was studied in relation to the calving rate of recipients after transfer of embryos produced with sexed semen and calf mortality.

REVIEW OF LITERATURE

1. Superovulation and embryo recovery in cattle

The basic principle of MOET is to produce embryos from a donor animal of superior genetic value following transfer of embryos into recipient animals of lower genetic value. The method aims to increase the number of progeny from the donor, simultaneously decelerating the spread of poor genes of the recipients in the herd. International embryo trade is a growing business in addition to operating at farm level and as a part of a national breeding program. Embryos are considered a safe way to share genetic material across countries because the risk of transmitting diseases is negligible for most pathogens compared with trading live animals. The fundamental steps of MOET are: stimulation of growth of ovarian follicles by administration of gonadotropins, induction of estrus with a luteolytic treatment, insemination and collection of embryos on day 7 post insemination, and fresh embryo transfer to synchronized recipients or freezing of embryos.

Superovulation is usually initiated during the mid-luteal phase of the estrous cycle, from eight to 12 days after estrus. The physiological principle for this approach is based on follicular waves of which there are usually two or three during an estrous cycle, the second emerging around day 10 in most cycles. The aim is to target the exogenous FSH towards a cohort of emerging, gonadotropin-responsive follicles, avoiding the phase of follicular growth when a dominant follicle is functional and the subordinate follicles regressing. An alternative to an approach relying on the stage of estrous cycle is to synchronize the follicular waves either by hormonal or mechanical manipulation. Hormonal control of follicular emergence is based on a progesterone-releasing intravaginal device or progestin implant, combined with GnRH (Deyo et al. 2001) or estradiol (Bo et al. 2006) in those countries where estradiol is approved for food-production animals. An effective approach for wave synchronization, but which is impractical under farm conditions, is to aspirate all follicles ≥5 mm, or even only the two largest follicles, a day or two before initiation (Baracaldo et al. 2000). Manipulation of follicular waves is not the practice in Finland where most superovulation treatments are initiated approximately ten days after the reference estrus. Despite this not being a standard procedure in the Nordic countries, most current superovulation protocols rely on exogenous control of follicular waves (Bo & Mapletoft 2014).

The routine practice is to inject FSH twice daily over four to five days. In a four-day standard protocol, prostaglandin F2α (PGF2α) is administered 60 to 72 hours after the initiation of the treatment. Estrus can be detected on average within 48 hours after induction of luteolysis with PGF2α and AI is performed 12 hours after the onset of estrus. An option for estrus-based insemination is a controlled induction of ovulation, following with a fixed-time AI, thereby avoiding the arduous estrus detection. This approach of timed AI is not utilized in Finland, instead it is recommended that the donor be inseminated 12 h or later after the onset of estrus. More than one insemination is the standard practice to provide sufficient numbers of viable sperm for each potential oocyte ovulating. The time interval from first to last ovulation in a superovulated donor is on average 8.3 h (range 4 to 14 h), the majority of ovulations occurring during the first four hours of the ovulation period (Purwantara et al. 1994). When conventional semen is used for insemination, a standard practice is to repeat the AI after approximately 12 hours. With sex-sorted semen, numerous protocols have been used, resulting in highly variable rates of success.

The embryos are recovered from the uterus by transcervical flushing on day 7 after fertilization (6 to 8 days). Several options are available for the further handling of embryos. They can be transferred fresh during the same day, or cooled and transferred the following day or even after several days. Diagnostics, such as determination of sex or genomic breeding value, are enabled via a biopsy consisting of only approximately ten cells because whole genome amplification technique can be used (Humblot et al. 2010). Currently there is increasing interest in developing alternatives to temporary storage other than freezing. Because genotyping of bovine embryos by profiling of single-nucleotide polymorphisms (SNP) is gradually being adopted in breeding programs, demand has been created for storing the embryos for up to one week until the results of genomic analysis are available. Recent research has demonstrated that embryos can maintain viability at +4 °C for as long as ten days (Ideta et al. 2015). Hypothermic storage of embryos is the current focus of research, but the technique is applied to only a small proportion of embryos. Regarding the numbers of embryos, the most frequent procedure is to freeze them with ethylene glycol as a cryoprotectant. Frozen embryos can subsequently be stored for decades in liquid nitrogen.

2. Major animal-related factors affecting the outcome of superovulation

2.1. Ovarian follicular waves

Several animal-related, environmental and management factors contribute to the outcome of superovulation treatment in cattle (Kafi & McGowan 1997, Stroud & Hasler 2006). Among the intrinsic, animal-related factors are those related to physiological condition, such as the age, breed and status of the ovarian follicular populations. During the early years of evolution of superovulation, it was agreed that success rate was determined by ovarian status at the time of gonadotropin treatment (Monniaux et al. 1983). In the 1980s and early 1990s research focused on defining the optimal phase of follicular waves for commencing superovulation. Numerous studies concluded that the superovulatory response was compromised in the presence of a dominant follicle at the time of treatment initiation (Guilbault et al. 1991, Wehrman et al. 1996, Brogliatti et al. 1997, Murphy et al. 1998, Kohram et al. 1998, Shaw & Good 2000, Kim et al. 2001). The increase in follicular recruitment and ovulation rate in animals not presenting a dominant follicle does not, however, necessarily lead to greater transferable embryo yield. Very few studies have demonstrated an improvement in embryo yield in the absence of the dominant follicle (Wehrman et al. 1996, Kim et al. 2001). There is inconsistency among results of studies, some not having established any effect of the presence of a dominant follicle (Bergfelt et al. 1997, Diaz et al. 2001). The criteria for the definition of a functionally dominant follicle and the time-interval between dominant follicle ablation and initiation of superovulatory treatment vary among studies. This discrepancy may explain the contrasting results. Diaz et al. (2001) concluded that the exogenous FSH overrides the potential inhibitory effects that a dominant follicle might exert on the pituitary and ovary.

The presence or absence of a dominant follicle is inherently connected to the status of subordinate follicles. During the phase of functional dominance, the subordinate follicles regress and the responsiveness to gonadotropin stimulation is poor. Taken together, the results of research focusing on the phase of dominance and number of potentially recruitable follicles, Kanitz et al. (2002) proposed that the number of ovulations depends on the number of follicles responding to the superovulatory treatment by proliferation, differentiation and ovulation within 120 hours. The number and quality of these responsive follicles are crucial for a superovulatory response.

2.2. Count of antral follicles

The above-mentioned follicular population is affected by the stage of a follicular wave. However, recent research has substantiated the claim that the main determinant of the follicular populations is the ovarian antral follicular count (AFC) of follicles ≥ 3 mm in diameter. It is now clearly established that the major factor influencing the response to ovarian stimulation is the ovarian reserve of gonadotropin-responsive follicles during the initiation of FSH treatment (Monniaux et al. 1983, Singh et al. 2004, Monniaux et al. 2010). This attribute is both genetically and epigenetically defined, and can be considered to be directly animal-dependent, whereas the initiation of super-stimulation at an optimal time of the follicular wave can be considered partly dependent on management decisions.

The number of follicles recruited in a follicular wave varies greatly among animals, but is highly repeatable for an individual (Ireland et al. 2007, Mossa et al. 2010, Mossa et al. 2012). The ovarian follicular reserves are formed during fetal life. The peak number of primordial follicles is reached during the first trimester of gestation, after which some start to differentiate within 90 to 140 days of gestation (Russe 1983). At birth, the number of ovarian follicles is approximately 130,000, varying markedly among individual female calves (Erickson 1966). There is increasing evidence that in addition to genetic determinants, the follicular development in utero is also epigenetically programmed. Factors such as maternal under-nutrition (Mossa et al. 2009) and disease (Evans et al. 2012) during gestation were demonstrated to decrease the number of ovarian follicles in the offspring. Species- and breed-related differences in the follicular populations have been reported. Bos Indicus, compared with Bos Taurus, has double the number of small antral follicles, detected in the ovaries at the emergence of a follicular wave (Evans et al. 2012).

The number of follicles potentially responding to gonadotropin treatment can be predicted through ultrasound scanning of ovaries before initiation of the ovarian stimulation (Singh et al. 2004). A promising method is the determination of Anti-Müllerian hormone (AMH). This hormone, which in female cattle, is expressed exclusively in the granulosa cells, reflects the size of the pool of gonadotropin-responsive follicles (Ireland et al. 2011). Thus AMH can be used as an endocrine marker for the size of the antral follicular population and prediction of superovulatory response in terms of numbers of FSH-responsive follicles and ovulations (Rico et al. 2009) as well as transferable embryos (Rico et al. 2009, Monniaux et al. 2010, Rico et al. 2012, Souza et al. 2015).

2.3. Age

Some variation in the superovulation success among donors can be explained by age. However, this applies mostly to old donors, greater than ten years of age, because the numbers of gonadotropin responsive follicles decrease with age (Malhi et al. 2006, Malhi et al. 2008). Considering the current increases in cattle breeding intensity employing genomic selection, there is rapidly declining interest in producing embryos from old donors. Thus the practical significance of ageing is insignificant. However, the other side of the coin is the growing interest in breeding programs towards the other extreme, very young heifers. For successful *in vivo* embryo production, the prerequisite is cyclicity of the donor, which sets a limit on the age of the female. There is anecdotal evidence that initiation of super-stimulation during the early cycles leads to inferior superovulatory success than initiation after later cycles. *In vitro* production (IVP) of embryos does not require cyclicity of the donor, therefore allowing oocyte retrieval from prepubertal calves. However, the blastocyst development declines when oocytes collected from young donors are subjected to IVP (Galli et al. 2001).

3. Environmental factors affecting the outcome of superovulation

Environmental factors, such as high ambient temperatures, can interfere with the oocyte and follicular quality and endocrine pathways of superovulation (for review see Hansen et al. 2001, Hansen 2007, Hansen 2009). Adverse effects of heat stress on reproductive performance represent a well-established problem, not only in subtropical and tropical climates, but also in temperate climates during hot summer periods. Because the relevance of this problem in Finland is markedly low – despite being globally important and even gaining in importance because of climatic change – this subject is discussed very briefly, without detailing the mechanisms involved.

Embryonic survival can be compromised in heat-stressed animals, whether they are single-ovulating or superovulated. The deleterious effects of heat stress manifest at the follicular, oocyte and embryonic levels. The fate of the ovulating follicle is determined days or weeks before ovulation; exposure to heat stress interferes with follicular recruitment and growth (Roth et al. 2000), granulosa cell steroid production (Wolfenson et al. 1997, Roth et al. 2001, Li et al. 2016), and oocyte maturation (Edwards et al. 2005, Schrock et al. 2007, Hooper et al. 2015). Studies of the effects of heat stress on fertilization show ambivalent results, many studies

reporting compromised fertilization as a consequence of heat stress. After fertilization, heat stress impairs early embryonic development. The vulnerability of the embryo is higher during the earlier stages, and resistance to heat increases after the embryonic genome activation at the late 8-cell stage. The gradual increase in heat resistance was initially shown in superovulated cows by Ealy et al. (1993): exposure to heat stress the day after estrus compromised embryonic development, but not if exposed later, on days 3, 5 or 7. Exposure of superovulated heifers to high ambient temperatures during the period from one to seven days after insemination increased the proportion of degenerated embryos (Putney et al. 1988). Benyei et al. (2003) demonstrated that embryo donors kept for a long time at high ambient temperature showed compromised performance in embryo production The superovulatory response was evaluated as the number of CL and live embryos. It can be concluded that heat stress compromises the success rate of superovulation and embryo collection. Conversely, transfer of embryos from donors not experiencing heat stress to exposed recipients can be used to bypass the deleterious effects of heat stress on pregnancy rates of lactating cows (reviewed by Rutledge 2001). In Finland, occasionally in June-August, heat stress is suspected to compromise superovulatory success.

Also other environmental stressors, such as transportation, can influence the success rate of superovulation. Transportation during superovulation treatment was demonstrated to decrease ovulation rate while increasing blood cortisol concentrations (Edwards et al. 1987). Stress-induced elevation of cortisol during the preovulatory period suppresses gonadotropin secretion, inhibiting the LH surge and thereby causing ovulation failure (for review see Dobson & Smith 2000). Despite this phenomenon being clearly documented to result from transportation, any comparable stress, such as regrouping of animals, can act similarly and should be avoided during the superovulatory treatment.

4. Management factors affecting the outcome of superovulation

Several management factors can influence the outcome of superovulation, including nutritional management and the protocol for gonadotropin treatment, in addition to artificial insemination (AI) timing, competence and semen quality. These are discussed further in the following sections with emphasis on the subjects covered in the original articles.

4.1. Superovulatory treatment

Factors associated with the superovulatory treatment have a marked influence on the outcome, including the type and purity of the gonadotropin and the frequency and route of administration.

4.1.1. Gonadotropin preparations

During the establishment of the modern ET industry in the 1970s, the most frequently used gonadotropin used for bovine superovulation was equine chorionic gonadotropin (eCG). In addition to eCG, FSH purified from porcine pituitaries soon became commercially available. The advantage of using eCG is the long half-life of 40 hours in cattle (Murphy & Martinuk 1991) and therefore fewer injections are needed in comparison with frequently administered FSH, which has a half-life after i.m. injection of only five hours (Demoustier et al. 1988). Disadvantages of a long half-life, however, are problems such as continuous ovarian stimulation, asynchronous ovulations and unovulated follicles in addition to abnormal endocrine profiles, which have helped FSH to become the standard approach to superovulation. A better superovulatory response after stimulation with FSH compared with eCG has been clearly established (Boland et al. 1991).

Currently the FSH preparations approved for veterinary use derive from porcine and ovine pituitary tissue. Despite being highly purified, LH varies in activity in the products, which can represent a source of variation in the superovulatory response. High LH contamination impairs the ovarian response to super-stimulation, resulting in decreased yield of viable embryos (Chupin et al. 1984, Donaldson et al. 1986). The origin of the deleterious effects of excess LH is considered to be an untimely maturation of the oocytes (Moor et al. 1984). Another disadvantage resulting from the nature of the origin of the products is inconsistency among batches. Considerable variation derives from the approximately 60 isoforms of FSH and LH that are produced in the pituitary. This variation in bioactivity affects embryo yield (Kanitz et al. 2002).

To bypass the adverse effects of LH contamination and variable bioactivity among FSH batches, bovine recombinant FSH has been applied for superovulation, with encouraging results (Carvalho et al. 2014). In the future, it can be expected that the animal-origin products currently used will be replaced by recombinant gonadotropins as their commercial development proceeds. The consistency of the gonadotropin will improve because contamination with other pituitary hormones is

avoided and less variation among batches will occur. The risk of disease transmission via gonadotropins of animal origin will be bypassed and ease of administration also enhanced because recombinant gonadotropins can be incorporated in long-acting formulas (Hesser et al. 2011).

4.1.2. Administration of gonadotropins

The relevance of the timing of superovulatory treatment in relation to the stage of follicular waves was discussed in Section 2.1. In addition to the initiation of the treatment, protocols can vary in frequency of gonadotropin administration. Less frequent administration is more convenient and improves protocol safety from both human and animal perspectives. Bo et al. (1994) reported a superovulatory response comparable to eight i.m. injections of FSH with only a single subcutaneous injection in beef cattle. However, site of injection and amount of subcutaneous fat affected the response. This regimen and similar results were not repeatable for Holstein cows (Lovie et al. 1994). Lovie et al. (1994) modified the approach by dividing the dose into two injections for Holstein cows. The first injection comprised 75% of the total dose and the remaining 25% was administered 48 h later. The response in terms of ovulations and embryos was numerically intermediate for the split-dose regimen compared with the standard protocol of multiple injections or a single injection.

The standard protocol is to dilute the lyophilized FSH into saline, but an alternative is dilution into hyaluronan, a natural polysaccharide. The resulting viscous hyaluronan-FSH solution is released slowly, enabling less frequent administration. A single subcutaneous injection of a hyaluronan-FSH solution resulted in a superovulatory response comparable with the standard protocol of eight injections in beef cows (Tribulo et al. 2011). Likewise, an approach of splitting the total dose into two doses administered 48 h apart was as successful as the standard protocol (Tribulo et al. 2012). Comparable results have been achieved in superovulating beef cows and heifers, but reports of high superovulatory success in high-yielding dairy cows are lacking. Biancucci et al. (2016) demonstrated a higher superovulatory response with slow-release FSH in beef heifers treated with two injections 48 h apart compared with the standard protocol of eight injections. They also investigated the concentrations of cortisol in the hair, indicating the level of stress caused by frequent handling of the animals. Animals in the control group that received eight injections had higher cortisol concentrations 14 and 21 days after the initiation of the treatment compared with the split-injection group. A question

arises: was the superior response of animals receiving only two gonadotropin injections partly influenced by the lower activation of the hypothalamic-pituitary-adrenal axis? Since stress-induced increase in cortisol level can interfere with LH secretion and inhibit ovulation, as covered in Section 3, development of protocols incorporating reduced handling of animals may have beneficial effects.

In contrast to decreasing the number of injections, there is evidence of beneficial effects for prolonging the treatment period, at least for some types of donor. The hypothesis behind such an approach is that when gonadotropin pre-stimulation is initiated two to three days before the actual superstimulation, small subordinate follicles of ≥1 mm commence growth. During a standard four-day treatment the time would not be adequate for these to develop into ovulatory follicles. Pretreatment of beef cows with eCG (Caccia et al. 2000) or FSH (Bo et al. 2008) was suggested to be beneficial and could be applied, if not routinely, at least on an ad hoc basis for low-responding donors.

4.2. Sex-sorted semen

4.2.1. Technique of sperm sorting

A starting point for the commercialization of flow cytometric sorting of semen was the birth of live offspring from sorted rabbit semen in 1989 (Johnson et al. 1989). The first calves were produced using *in vitro* fertilization with sex-sorted semen by Cran et al. (1993). Subsequently sex-sorted semen was used for AI of cattle (Seidel et al. 1998) and the first commercial license was granted in 2002. Today, sexed semen is well established in cattle breeding, but it is not possible to determine the precise volume of sexed semen on the worldwide market. However, the estimate is that sex-sorted semen represents slightly less than five percent of the semen sales volume in the United States (Seidel 2014).

The biological basis of the technique rests on the 4.2% difference in the length of chromosomes in sperm bearing X- or Y-chromosomes (Moruzzi 1979). Detailed description of the procedure of sperm sorting is beyond the scope of this review, but was thoroughly described by Seidel and Garner (Seidel & Garner 2002, Garner & Seidel 2008, Garner et al. 2013). However, a brief description of the principles is essential to understand the effects of sorting procedure on semen. The initial step is the staining of sperm DNA with the nucleic acid-specific fluorophore Hoechst 33342. The fluorescently stained sperm are then sorted according to the intensity of the fluorescence using flow cytometry. Stained spermatozoa are directed as a

stream, one by one, to pass through two fluorescence detectors, positioned at a 90 degrees angle to each other. Each detector measures the intensity of fluorescence resulting from laser excitation in the DNA-bound dye molecules of X- and Y-sperm. The stream of sperm is broken into droplets using a crystal vibrator. Opposing charges are placed for droplets containing X- or Y-sperm. These then fall through positive and negative electrical fields. Electrically charged sperm are separated into oppositely charged fields, forming two distinct streams, one containing X- and the other Y-sperm. In the case that the fluorescence cannot be accurately measured, the droplet is left uncharged and sorted into a fraction to be discarded. Such droplets contain sperm positioned incorrectly or not definably for other reasons, including dead sperm, two sperm or no sperm. After sorting, the X- and Y-chromosome containing sperm populations are collected into a biologically supportive media prior to concentration and cryopreservation. The industrial standard is to package 2 million sperm per insemination dose, which represents only 13% of the sperm count of standard conventional semen doses. Sperm sorting can provide subpopulations of X- or Y-bearing bovine sperm at a rate of 8,000 sperm per second, while maintaining a commercially accepted purity of 90%. The sacrifice for this is that the sperm have to tolerate staining, mechanical forces, electrical charging, high speed, and exposure to a laser. Since the introduction of the technique, technical improvements have reduced the mechanical stress and increased the sorting rate and purity of the end product. The reduction of hydromechanical pressure from 50 psi to 40 psi has improved fertility (Schenk et al. 2009). However, the technical challenges, as well as the low number of sperm in sexed semen doses, contribute to the impaired pregnancy rates achieved with sex-sorted semen compared with the conventional approach.

4.2.2. AI of single-ovulating females

Pregnancy rates after use of sex-sorted semen are generally lower than for unsexed semen, despite the technical improvements of the sexing procedure, such as lowering the pressure during the process, having bridged the gap between sorted and conventional semen. Both the effects of the sorting procedure and a low number of sperm in a standard dose of sorted semen are responsible for the reduction in fertility. It has been estimated that two thirds of the reduction in the pregnancy rate is because of the low number of spermatozoa and one third because of the damage caused by the sorting (Frijters et al. 2009). A rule of thumb is that in well-managed herds, fertility remains approximately ten percentage points lower using sorted semen than using conventional semen (Seidel 2014).

The main application of sex-sorted semen is in breeding dairy heifers, for which there are several advantages. Firstly, the fertility of heifers is superior to that of lactating cows. Secondly, an advantage in the use of sex-sorted semen for heifers is the ease of calving smaller and lighter female calves and thereby the occurrence of less dystocia compared with giving birth to male calves (Tubman et al. 2004). Thirdly, producing the replacements from heifers shortens the generation interval. Since the best genes in a herd are among the youngest animals, the genetic progress is accelerated when they are used to produce the next generation. Furthermore, one interesting perspective should be added to the advantages of a heifer calving a heifer by means of sexed semen. Hinde et al. (2014) demonstrated programming of the mammary function by offspring *in utero*. Analysis of 2.39 million lactation records revealed that the sex of the fetus of the first parity dam affects her milk production. Heifers carrying a heifer calf produce approximately 445 kg more milk over the first two lactations than when carrying a bull calf. In addition to the milk production of the dam, epigenetic programming of the milk production of the offspring has been demonstrated. Female calves gestated and born in the absence of maternal lactation produce more milk than heifers born to lactating mothers (Gonzalez-Recio et al. 2012). Overall, a female fetus is beneficial for future milk production or its heifer dam, and having a heifer as a dam is beneficial for future milk production of a heifer calf. Despite relatively small effects from the farm perspective, the effects should be considered from the broader perspective of the dairy industry.

4.2.3. In vivo embryo production

Sex-sorted semen is used for superovulated donors to enhance production of heifer calves from genetically superior females. Genomic breeding has facilitated the selection of breeding females, favoring young heifers as embryo donors rather than cows, skewing the proportions of cows and heifers. In Finland more than two thirds of embryo collections are from heifers and currently most breeding companies invest solely in heifers for embryo production.

Embryo yield is generally compromised in superovulated animals inseminated with sex-sorted semen compared with those inseminated with conventional semen. The reduction is more apparent in cows than in heifers. Experimental designs comparing the two semen types generally apply two approaches: from the biological point of view, comparison of equal sperm numbers per insemination is the rational approach, revealing the nature of the reduction – if it is due to the impaired quality

or reduced dose. A study by Sartori et al. (2004) compared deep uterine horn AI of equal doses of sexed or conventional semen (10 million sperm/dose) in superovulated Holstein heifers. There was a reduction in the fertilization rate when sexed semen was used. The number and proportion of viable embryos collected from heifers inseminated with sexed semen was compromised compared with unsexed semen, suggesting that sexing per se reduces the fertilization potential of semen. Another study utilizing equal – relatively low – numbers of sperm (five million/ dose) did not reveal a difference in the embryo production between sexed and conventional semen (Hayakawa et al. 2009). In a study by Schenk et al. (2006), inseminating large numbers of sex-sorted sperm (10 million) compromised embryo yield. However, in this study the control group received 40 million non-sorted sperm. For non-sorted sperm in single-ovulating females, maximum fertilization is achieved with 10 million sperm, and increasing the dose unlikely improves fertility appreciably (Foote & Kaproth 1997).

However, from the economic point of view, it is not reasonable to deposit sexed semen of similar sperm numbers as conventional semen. Therefore, in studies comparing the two semen types, utilization of doses generally used in commercial practice are justified. Most studies of sexed semen have compared a low dose of sorted semen with a traditional AI-dose of conventional semen. Several have clearly demonstrated a decrease in the numbers or proportions of transferable or good quality embryos and higher proportions of unfertilized ova for cows inseminated with low numbers of sex-sorted semen (Baruselli et al. 1997, Schenk et al. 2006, Peippo et al. 2009, Hayakawa et al. 2009, Soares et al. 2011). For heifers, the adverse effect of sexed semen on embryo production has been moderate or negligible (Hayakawa et al. 2009, Peippo et al. 2009, An et al. 2010).

Deep uterine insemination (DUI) is a common approach that aims at ensuring sufficient numbers of sorted spermatozoa at the fertilization site of the donor. However, there is no clear evidence of the superiority of this technique compared with insemination in the uterine body. Some have inseminated both semen types in the uterine body (Schenk et al. 2006, Hayakawa et al. 2009, Larson et al. 2010, Soares et al. 2011), some have used deep uterine horn insemination for both semen types (Sartori et al. 2004) and others have deposited sex-sorted semen deep in uterine horns and conventional semen in the body (Peippo et al. 2010, An et al. 2010). Comparison among studies is not valid because of numerous confounding factors in the protocols, such as control of ovulation, timing of insemination, type of donor, number of bulls and numbers of sperm, which cannot be controlled. DUI

in superovulated cows using conventional semen was not preferable compared with insemination in the uterine body (Carvalho et al. 2013). In heifers (n=17) however, DUI resulted in a higher number of fertilized structures recovered, and an increased fertilization rate, compared with insemination in the uterine body. However, the authors stated that the experiment should be repeated on more animals to confirm the effect of insemination site. Moreover, whether this outcome of a dose of 20 million unsorted sperm is similar to a low dose of sorted semen remains unanswered. Some studies applying conventional semen on superovulated donors did not report an advantage in depositing the semen in the uterine horn compared with in the body (Pallares et al. 1986, Gatius et al. 1988), while others established a higher fertilization rate in heifers inseminated in the uterine horns compared with in body, but there was no effect on cows, neither superovulated (Carvalho et al. 2013) nor single-ovulating (Andersson et al. 2004). Overall, there exists more evidence suggesting no benefits of DUI in superovulated animals than for recommending the technique. However, the research on conventional semen cannot be extrapolated to sex-sorted semen. In the absence of complete evidence, the practice for AI of superovulated donors with sex-sorted semen in Finland currently employs the DUI technique.

As with the dose and site of insemination, the timing of AI with sexed semen on superovulated animals affects the fertilization rate and subsequent embryo yield. There is evidence in single-ovulating animals that delaying the insemination with sexed semen close to ovulation, i.e. 24 hours after the onset of estrus, increases pregnancy rates (Schenk et al. 2009, Sales et al. 2011). The fertilization rate in superovulated animals is lower than in single-ovulating animals (Hawk 1988, Hyttel et al. 1991, Saacke et al. 1998). The superovulated female genital tract poses challenges for spermatozoa transport. Disturbances in the transport of spermatozoa are demonstrated by the lower number of accessory spermatozoa on the *zona pellucida* of ova/embryos collected from superovulated compared with single-ovulating animals (Saacke et al. 1998). This suggests that fewer spermatozoa are available at the fertilization site in superovulated animals, leading to increased fertilization failure. In a study comparing sexed and unsexed semen for superovulated heifers, the group inseminated only once with sexed semen had fewer accessory sperm compared with an equal dose of conventional or sexed semen inseminated twice (Sartori et al. 2004). However, it is not known if this was because of suboptimal timing, or the combination of semen type and timing.

Another attribute of sexed semen, requiring accurately timed AI in relation to ovulation, is the shortened life span of sperm caused by the sorting process (Maxwell et al. 2004). It has been suggested that the sorting process causes "pre-capacitation" of sperm, shortening the time required for capacitation in the genital tract and subsequent life span (Lu & Seidel 2004, Schenk et al. 2009).

4.2.4. Calves produced with sex-sorted semen

The sex ratio of calves born from AI with X-semen has generally been reported to be 86% to 91% females (DeJarnette et al. 2009, Borchersen & Peacock 2009, Norman et al. 2010, Healy et al. 2013). Concerns about the effects of the sexing procedure, particularly exposure of sperm to Hoechst 33342 dye and UV laser, have facilitated several studies on offspring resulting from sexed semen. A study by Pozzi et al. (2014) revealed no mutagenic risk for *in vitro* embryos produced with sex-sorted semen. Most large field studies on calves born from AI with sexed semen indicate no difference in the overall rate of stillbirths attributable to sorted and non-sorted semen when the data are adjusted for age at calving (Norman et al. 2010, DeJarnette et al. 2011). The incidence of dystocia is lower, especially in heifer calvings, as referred to in Section 4.2.2. However, several studies revealed an increased incidence of male calf mortality (DeJarnette et al. 2009, Norman et al. 2010, Healy et al. 2013) when the calf originated from Y-sperm incorrectly sorted to the X-fraction. The presumption is that such Y-sperm included in the fraction of X-sperm have a higher DNA content than normal Y-sperm. This aneuploidy could account for compromised viability of the offspring.

4.3. Nutrition

The effects of nutritional factors such as energy intake, protein source and level, and supplementation of fatty acids, vitamins and minerals on embryo production have been studied extensively. Of all nutritional factors, energy and protein intake were reported to have the greatest impact on fertility in single- and superovulated animals. Because one of the original articles included in this dissertation investigated the effects of nutritional protein level on embryo production, emphasis is placed on protein, and other nutritional factors are covered in less detail.

4.3.1. Energy balance

The deleterious effects of negative energy balance (NEB) postpartum on fertility are well recognized. The most apparent consequence of the NEB during early lactation is the postponed resumption of estrous cyclicity. The delay in first ovulation postpartum is a direct consequence of reduced LH pulse frequency, inadequate to support follicle maturation and ovulation. Metabolic changes occurring during energy deprivation include elevated concentrations of blood non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), triacylglycerol and urea as well as decreased concentrations of insulin, glucose and insulin-like growth factor-I (IGF-I). The low levels of glucose, insulin and IGF-I restrain the estrogen production of dominant follicles and consequently inhibit the LH surge and ovulation (Butler 2000).

In superovulated cattle both energy deficit during early lactation and overfeeding (in cases of non-lactating animals) exert adverse effects on embryo production. For lactating cows, the most important nutritional factor affecting the embryo production is energy balance. Since cyclicity is a prerequisite for initiating superovulatory treatments, the donor has typically bypassed the postpartum anestrus. From the practical and economic point of view, there are two consequences of severe NEB. The embryo production procedures are delayed until regular estrous cycles have resumed, thereby increasing the subsequent calving interval of the donor, likewise the risk of an unsatisfactory embryo yield increases. The imbalance of milk production and energy intake in high-yielding cows compromises early embryonic development, most likely by effects mediated by changes in oocyte quality (Santos et al. 2004). Embryo quality in superovulated lactating cows is inferior compared with dairy heifers or beef cows (Leroy et al. 2005a).

Systemic changes in several metabolites and metabolic hormones are reflected in the follicular fluid during the period of NEB. Several *in vitro* studies investigated the mechanisms by which the altered follicular environment interferes with oocyte competence. High concentrations of NEFA together with low glucose during oocyte maturation *in vitro* are detrimental to the oocyte, impairing developmental competence and early embryonic development (Leroy et al. 2005b). Similarly, *in vitro* maturation of oocytes in concentrations of elevated BHB combined with low glucose, mimicking subclinical and clinical ketosis, compromised the developmental competence of the oocyte (Leroy et al. 2006). From a practical perspective, evaluation of the effects of NEB is more complex than can be

concluded from studies on the effects of metabolites and metabolic hormones on oocyte quality. Because the time of follicular development from primordial to tertiary follicle is estimated to take 80 to 100 days, the effects of adverse conditions during initial follicular development can be carried over this long period until ovulation. These follicles, which initiated their growth during the negative energy balance peripartum, can have altered gene expression, resulting in formation of dysfunctional follicles ovulating poor quality oocytes (Britt 1992).

In addition to the effects of metabolites and metabolic hormones, changes in blood concentrations of steroid hormones can impair oocyte quality, fertilization and embryonic development. In the postpartum dairy cow, high dry matter intake results in increased blood flow in the intestinal tract and liver and an increase in the metabolic clearance of progesterone and estradiol, leading to decreased concentrations in the blood (Sangsritavong et al. 2002, Wiltbank et al. 2006). The effect of dry matter intake on steroid hormone levels has been demonstrated also in non-lactating animals. Nolan et al. (1998) reported that superovulated heifers on an unrestricted diet had lower progesterone concentrations than heifers on restricted feeding. The superovulatory response in terms of ovulation rate and number of transferable embryos was similar for high and low energy groups but after culture, blastocyst development was superior in embryos from heifers fed a low energy diet (Nolan et al. 1998). In this study, as also in a study by Yaakub et al. (1999), there was a tendency for a higher ovulation rate in heifers on a restricted-energy diet. Conversely, Gong et al. (2002) reported an increased superovulatory response in number of ovulations – without evaluation of the embryo yield – for heifers fed a high-energy diet compared with those fed a maintenance diet. Animals on a high-energy diet expressed elevated levels of insulin and progesterone, compared with those on a maintenance diet. Also several other authors showed increased follicular recruitment in animals fed high-energy diets (Gutierrez et al. 1997, Armstrong et al. 2001), in conjunction with elevated insulin and progesterone levels.

The key parameter used to evaluate the outcome of superovulation is the transferable embryo yield rather than follicular recruitment or ovulation rate. Yaakub et al. (1999) reported that more transferable embryos were recovered from heifers fed restricted concentrates compared with those fed *ad libitum*. There is little consistency in the literature, but most authors have concluded that feeding excess energy is detrimental to embryo quality (Santos et al. 2008, Sartori et al. 2010).

Fatty-acid supplementation for superovulated donors has been investigated by several authors, but there are discrepancies among the studies and diverse results have been reported (Velazquez 2011). The varying effects are explained partly by differences in superovulation protocols, fatty acid composition and intake, and energy status and lipid metabolism of the donors, in addition to environmental factors such as heat stress. The majority reported either no beneficial effect (Ryan et al. 1992, Thomas & Williams 1996, Thangavelu et al. 2007) or a detrimental effect (Coscioni et al. 2002, Petit et al. 2008) of fatty acid supplementation for superovulatory success. Supplementation of fatty acids can increase cholesterol concentrations in single-ovulating (Staples et al. 1998) and superovulated cattle (Ryan et al. 1992). Excess fatty acids in the follicular fluid can impair oocyte developmental competence *in vitro* (Adamiak et al. 2006) and has been associated with subfertility (Båge et al. 2007). Lipid accumulation in the embryo impairs viability by increasing the sensitivity to cryopreservation (Abe et al. 2002). There seems not to be adequate evidence supporting nutritional fatty acid supplementation for embryo donors.

Interpreting of the results of various nutritional management protocols for superovulated donors is complex, but some conclusions can be drawn. For lactating cows, the key factor is to avoid NEB by ensuring adequate and balanced energy and protein intake. For heifers, there is more risk of overfeeding than underfeeding energy, and therefore the energy intake should be calculated for a desired daily gain and body condition score.

4.3.2. Dietary protein

Dietary crude protein (CP) is mostly hydrolyzed in the rumen to ammonia, which is used as a substrate for production of microbial protein by the rumen microbiota. Excess ammonia is transported to the liver and converted into urea in the urea cycle. Urea, being a relatively small molecule with a molar mass of 60.06 g/mol, is able to move freely across cell membranes. Thus when blood urea levels are high it diffuses from the bloodstream into organs such as the reproductive tract. Excess protein intake results in elevated blood urea nitrogen (BUN) and ammonia concentrations that have been associated with compromised fertility, although there is little consensus among reports. Several mechanisms involved in the toxic effects of urea have been proposed. The mechanisms of fertility reduction are manifested in the ovary, oviduct and uterine environment rather than at the hypothalamic-pituitary axis. Initially, it was demonstrated that as a consequence of excess rumen-

degradable protein intake the uterine lumen pH decreases (Jordan et al. 1983, Elrod et al. 1993) and this was linked to decreased fertility (Elrod & Butler 1993). Later studies, however, are inconsistent and the significance of decreased uterine pH remains uncertain. It was proposed that the deleterious effects of high protein intake are more likely to act at the ovarian level rather than in the uterine environment. This hypothesis is supported by experiments where embryo transfer was performed using donors and recipients differing in urea levels. Embryos recovered from high BUN donors resulted in a compromised pregnancy rate when transferred to recipients of high or low BUN, regardless of recipients' BUN concentration (Rhoads et al. 2006). Similar conclusions were reported (Gath et al. 1999, Gath et al. 2012) when transferring good quality embryos to recipients on high urea diets. There was no effect of recipient diet urea supplementation on the pregnancy rate in animals on a high or on a low energy diet.

The hypothesis that high urea and/or ammonia in circulation affects the follicle and oocyte has been tested by several groups in *in vitro* and *in vivo* designs. In a study by Sinclair et al. (2000) heifers were fed a high plasma ammonia generating diet for 18 days during the late antral follicle development stage. Cleavage rate and blastocyst production rates were lower in oocytes from heifers on a high ammonia diet than from those on a low ammonia diet. Similarly, impaired oocyte quality and *in vitro* production of embryos under elevated blood urea concentrations were demonstrated in dairy cows (Armstrong et al. 2001, Santos et al. 2009). Blanchard et al. (1990), feeding two groups of dairy cows isocaloric and isonitrogenous diets (16% CP) differing in the proportion of rumen degradable protein, reported a higher fertilization rate in superovulated donors fed a diet with less rumen-degradable protein (64% vs. 73%). The proportion of embryo collections yielding no transferable embryos was higher when more rumen-degradable protein was fed, suggesting a fertilization failure or early degeneration of embryos.

In contrast, O'Callaghan et al. (1997) did not suggest any deleterious effects on oocyte quality, expressed *in vitro* as the cleavage rate and blastocyst yield, in heifers fed excess urea for a period of nine days. When the fertilization and early embryonic development until three days post insemination were studied *in vivo*, high urea did not compromise these in superovulated heifers with high blood urea levels (Gath et al. 1999, Gath et al. 2012). Similarly, cows fed 12.3% or 27.4% crude protein produced equal numbers and quality of embryos after superovulation (Garcia-Bojalil et al. 1994).

It has been proposed that not only the dose but also the duration of a high dietary nitrogen intake is crucial to the effects on the reproductive functions. Dawuda et al. (2004) compared groups of superovulated cows fed excess quickly-degradable urea nitrogen for a short or long period before embryo recovery. Cows receiving the diet for ten days before insemination yielded similar numbers of equal quality embryos as the control cows. However, cows fed the experimental diet only for the period from insemination to embryo recovery yielded fewer embryos of poorer quality than in the long duration or control group. They concluded that the excess of quickly degradable urea nitrogen was toxic to embryos, but the effect was bypassed when cows were allowed to adjust for ten days. The feeding period in the aforementioned experiments varied from nine days to four weeks or even more before oocyte and/or embryo quality was evaluated. The observations of Dawuda et al. (2004) are therefore not completely supported by all, and mechanisms other than increased urea and/or ammonia might be involved. Also, the magnitude of the elevation of urea in blood, as well as ammonia concentration, should be considered. In the study by Dawuda et al. (2004) the mean plasma urea concentration in the high nitrogen group was higher than in other studies.

It appears that data on the effects of nutritional factors on superovulation and embryo yield are inconsistent. Nevertheless, dietary-induced changes in superovulatory response are demonstrated. The multifactorial interactions of the metabolic status of the donor animal (lactating/non-lactating), body condition, composition of the ration, type of protein/fatty acid supplement, duration of energy restriction/overfeeding, as well as interactions of dietary components, make the results difficult to interpret and to take into practice. The deleterious effects of energy deficiency on oocyte quality and subsequent embryonic development are clear, but the effects of high protein intake are erratic, and it seems that excess protein is well tolerated in non-lactating animals, i.e. when the donor is not simultaneously undergoing energy deprivation. The combined effects of energy deficiency and excess protein should be avoided for embryo donors.

AIMS

The aim of this thesis was to investigate management factors affecting the efficacy of MOET. The specific aims of the studies were to examine:

- embryo yield in superovulated dairy cattle inseminated with sex-sorted and conventional semen
- the quality and developmental stages of embryos produced with sex-sorted semen
- pregnancy rate of embryos produced with sexed and conventional semen
- the effects of nutritional protein level, higher than the standard practices on Finnish farms, on embryo yield and quality in superovulated dairy heifers
- embryo yield and quality after superovulation using two commercially available FSH products

MATERIALS AND METHODS

Two types of studies were included in this thesis. Study I was carried out in a research farm at the Natural Resources Institute (at the time of the experiment Agrifood Research Centre Finland), Jokioinen, Finland. Other experiments were retrospective analyses of embryo transfer data registered in the national database of Agricultural Data Processing Center Ltd., Vantaa, Finland, and data provided by the embryo transfer teams of Faba coop and VikingGenetics Finland.

1. Donors, recipients and embryos

In Study I, animals (n=37) were regularly cycling Ayrshire heifers, on average one year of age and 310 kg live weight. Animals in Studies II–III and V were Holstein and Ayrshire cows and heifers on Finnish dairy farms and at an ET station. Animals in Study IV were only on farms, excluding those on the ET station. Numbers of donor animals in Studies II–IV are presented in Table 1. Number and type of embryos and recipients in Study V are presented in Table 2.

Table 1. Number of donors in Studies II-IV by breed, parity (heifer/cow) and treatment (Folltropin/Pluset, sex-sorted (SEX)/conventional (CONV) semen).

Study		Heifers		Cows		All
		Ayrshire	Holstein	Ayrshire	Holstein	
II	Folltropin	1220	867	309	196	2592
	Pluset	808	120	280	190	1398
III	SEX	28	105	65	23	218
	CONV	607	338	155	169	1269
IV	SEX	66	256	46	75	443
	CONV	348	659	169	352	1528

Table 2. Number of embryos transferred in Study V by semen (SEX=produced with sex-sorted semen, CONV=produced with conventional semen), type (fresh/frozen) and recipient parity (cow/heifer).

Recipient	SEX Embryo		CONV Embryo	
	Fresh	Frozen	Fresh	Frozen
Heifer	470	484	1533	4645
Cow	500	287	1402	3117
All	970	771	2935	7762

2. Experimental design

2.1. *Nutritional protein (I)*

The experiment was conducted in three separate trials (Trials 1-3) during one year. Animals were allocated to two groups, receiving either 14% (n=18) or 18% (n=19) crude protein in isocaloric diets formulated to meet the energy requirements (Diet 14 and Diet 18, respectively). The diet consisted of dry hay and a commercial concentrate mix. The protein source of the concentrate was rapeseed meal. Additional rapeseed meal was added to Diet 18 to reach the level of 18% crude protein. Feeding was continued during 71-72 days preceding the embryo collection. After four weeks of feeding, blood samples were collected to monitor serum urea concentrations and confirm a nutritionally induced difference between the two groups. The first sample was collected in the morning before feeding and a postprandial sample was taken 3.5 h after feeding.

Estruses were synchronized with a 9 to 11-day treatment with an intravaginal progesterone-releasing device (CIDR Plus, Inter Ag, Hamilton, New Zealand) combined with an i.m. injection of dexcloprostenol (0.15 mg, Genestran, Vetcare, Salo, Finland) one day before the CIDR removal. Superovulatory treatments were initiated 10 days after the induced estrus. The treatment consisted of eight intramuscular injections of ovine pituitary FSH, a total dose of 212 IU of NIH-FSH-S1 (Ovagen, Immuno-Chemical Products Ltd., Auckland, New Zealand) in decreasing doses over four days, and an i.m. injection of dexcloprostenol with the

sixth FSH-injection. Heifers were inseminated 48 and 60 h after the induction of luteolysis with frozen-thawed semen of bulls of proven fertility.

Embryos were recovered by transcervical uterine flushing on day 7 after AI. Flushing was performed using commercial flushing and holding media as well as commercial disposables. Embryos were evaluated under a stereomicroscope and classified according to the IETS guidelines (Robertson & Nelson 2010) into three quality grades (1=excellent or good, 2=fair, 3=poor) and six developmental stages (4=compact morula, 5=early blastocyst, 6=blastocyst, 7=expanded blastocyst, 8= hatched blastocyst and 9=expanding hatched blastocyst) by experienced ET personnel.

2.2. *Superovulation using two commercial FSH-preparations (II)*

In Study II, data for 3,990 superovulations and subsequent embryo recoveries during a 16-year period on Ayrshire and Holstein cows (n=975) and heifers (n=3,015) were analyzed to evaluate the superovulation success using two commercial FSH products (Folltropin, Vetoquinol S.A., Lure cedex, France, and Pluset (Laboratorios Calier, S.A., Barcelona, Spain) differing in their LH:FSH ratio. The LH:FSH ratio is 0.12 in Folltropin, whereas in Pluset it is 1.0. The group superovulated with Folltropin comprised 2,592 superovulations, 80% of which were performed on heifers and 20% on cows. The group stimulated with Pluset consisted of 1,398 superovulations, 66% and 34% performed on heifers and cows, respectively. The superovulation and insemination protocols were as follows. Animals were superovulated with twice daily intramuscular injections of either Folltropin or Pluset during four days, initiated on day 9 to 12 after the onset of estrus. Declining doses of 630 IU (Folltropin) or 800 to 900 IU (Pluset) of FSH in total were administered to cows, heifers receiving 420-490 IU (Folltropin) or 500-600 IU (Pluset). The selection of dose was based on the manufacturers' recommendation: the dose for cows was 90% and 85% of manufacturers' recommendations for F and P, respectively. For heifers, the dose was adjusted, taking into account age and size of the donor, ranging from 67% to 77% of the cow doses of Folltropin and 59% to 70% of the Pluset doses. A luteolytic dose of PGF2α or synthetic agonist was administered simultaneously with the sixth (cows) or seventh (heifers) FSH injection. Inseminations were initiated 12 h after the onset of estrus. The insemination protocol for conventional semen consisted of two inseminations 9-15 h apart. When sex-sorted semen was used, usually three inseminations were performed using the DUI technique. Two straws of sex-sorted

semen, containing 2 million sperm in each, were deposited deep in uterine horns at both insemination events. Only one straw was used for the third insemination 9-12 h later by dividing it between the uterine horns.

Embryos were collected by transcervical uterine flushing on day 7 after AI. Commercial flushing and holding media and commercial disposable equipment were used in all steps of the procedure. Embryos were evaluated under a stereomicroscope (60 x magnification) and classified according to the IETS guidelines (Robertson & Nelson 2010). Transferable embryos were washed according to the IETS approved protocol (Stringfellow 2010) and further processed for either fresh transfer or freezing. Embryos were frozen in 1.5 M ethylene glycol using standard procedures for a controlled rate freezing.

Embryo yield, quality and developmental stage of embryos, as well as the proportion of collections yielding no transferable embryos and proportion of donors having a low response to ovarian stimulation, were analyzed for each preparation. The 56-day non-return rate was applied as an indicator of embryo viability. Non-return and calving rates of 19,400 embryo transfers produced with Folltropin (n= 12,228) or Pluset (7,172) were analyzed. Calving rate was calculated as the proportion of recipients that were not artificially inseminated, or did not undergo a new embryo transfer after the initial embryo transfer, and that had calved in fewer than 290 days after the transfer. Of the fresh F-embryos, proportions of grade 1, 2, and 3 were 53%, 33% and 14%, respectively. Of the fresh P-embryos, the proportions were 46%, 38% and 16%, respectively.

2.3. *Effect of sex-sorted semen on embryo production (III, IV)*

In Study III, data for 1,487 embryo collections performed on Finnish dairy farms was analyzed to compare sex-sorted and conventional semen in embryo production. Sex-sorted semen was used for 218 donors (SEX) and conventional for 1,269 donors (CONV). Superovulation, insemination and embryo recovery protocols were as described in Section 2.2. The number of all recovered structures, transferable embryos, UFO and degenerated embryos were studied. Furthermore, the proportions of collections yielding no transferable embryos were determined.

In Study IV, embryo yield was further analyzed in a dataset of 1,971 commercial embryo collections, including material from Study III complemented with more recent embryo collections. The material that originated from the breeding station was excluded from Study IV because no sex-sorted semen was used and therefore

it would have included only the conventional group, possibly biasing the data. Donors were inseminated with sex-sorted (n=443) or conventional semen (n=1,528). Superovulation, insemination and embryo recovery protocols were as described in Section 2.5. The main focus of this study was the quality and developmental stage of embryos produced with sex-sorted or conventional semen. The distribution of embryos into quality grades and developmental stages and the average quality grade and developmental stage of collections in SEX and CONV, were analyzed. The average quality grade and developmental stage were analyzed by calculating a mean quality grade and developmental stage for all transferable embryos recovered in a single collection. These means served as quality and developmental stage indices for recovery. In addition, the number of all recovered structures and transferable embryos and proportions of UFO and degenerated embryos were studied. Furthermore, the proportions of collections yielding no transferable embryos were scrutinized.

2.4. Effect of sex-sorted semen on calf production (V)

In Study V, data for 12,438 embryo transfers were analyzed, of which 1,741 embryos were produced with sexed and 10,697 with conventional semen. Embryo transfers were performed on farms both in heifer and cow recipients. Pregnancy rates were determined as the proportion of recipients not having a subsequent insemination or embryo transfer, and calving in fewer than 290 days after the transfer. Calf mortality was analyzed among calves born from sexed or conventional semen. Calf mortality was calculated as the proportion of abortions at a stage of 180 or more days of gestation and neonatal death of calves younger than one week of age for all births.

3. Statistical analyses

All statistical analyses, except for the data on dietary treatments, were performed with IBM SPSS Statistics version 10 (I) (SPSS Inc., 1983), version 19 (III) (IBM Corp., 2010), version 21 (V) (IBM Corp., 2012) and version 22 (II, IV) (IBM Corp., 2013). The nonparametric Mann-Whitney U-tests were used to analyze differences in numbers of all recovered structures, transferable embryos and proportions of UFO and degenerated embryos between feeding groups (I), semen types (III, IV) and FSH-products (II).

Chi-square tests, or whenever necessary Fisher's exact tests, were used to analyze the proportion of embryo collections yielding no transferable embryos (II-IV) and calf mortality (V) and the proportions of low-responders (II).

Binary logistic regression was used to analyze the effect of sex-sorted semen (V) and FSH-preparation in embryo production (II) on the recipient pregnancy rate.

Effect of dietary treatments in Study I was analyzed with the GLM procedure of Statistical Analysis System software (SAS Institute, 1987). Two-way analysis of variance was used to test the differences in daily gains and serum urea concentrations between the dietary treatments and among the three trials.

RESULTS

1. Effect of nutritional protein supplementation on embryo yield (I)

A nutritional protein level of 18% crude protein was adequate to elevate blood urea concentration compared with one of 14%. The diets were isocaloric, resulting in similar average daily gain in both groups (821±245 and 808±171 g/day for Diet 14 and Diet 18, respectively). Serum urea concentration prior feeding was 4.26±0.79 and 5.26±0.58 mmol/l for Diet 14 and Diet 18, respectively. In both groups, 3.5 h after feeding the urea concentrations were elevated to 4.39±0.86 and 5.80±0.79 mmol/l for Diets 14 and 18, respectively. There was a difference in the urea levels between the groups at both sampling times ($P<0.001$), confirming that the protein intake differed between the groups.

The superovulatory response, evaluated as the number of CL, did not differ between the groups. The average number of CL was 13.3±4.0 and 12.7±4.2 in Diet 14 and Diet 18, respectively. The protein feeding did not affect the embryo yield, evaluated as the total number of embryos or ova, the number of transferable embryos or the number of degenerated embryos and UFO. The recovery rate of embryos (number of embryos per number of CL) was 72% and 87% in Trials 2 and 3, respectively. However, in Trial 1 it was exceptionally low, only 45%. Because Trial 1 differed from Trials 2 and 3 with respect to the recovery rate and also the increased proportions of degenerated embryos and UFO, the results were analyzed using two approaches: including all Trials 1-3 and also for only Trials 2 and 3, excluding Trial 1. The number of transferable embryos for all trials was 4.9±5.1 and 5.4±6.5 in Diet 14 and Diet 18, respectively. When Trial 1 was omitted, the numbers were 6.9±4.8 and 6.4±7.4, respectively. The higher crude protein content in Diet 18 seemed to improve the quality grades of embryos: more grade 3 embryos were recovered for Diet 14 than for Diet 18 (20.2% and 13.2%, respectively, $P=0.053$). When Trial 1 was excluded, the proportion of grade 3 embryos was 20.2% and 11.9% ($P<0.05$) for Diets 14 and 18, respectively.

2. Effect of FSH preparation on embryo yield (II)

Embryo yield after superovulation with Folltropin or Pluset was analyzed collectively for all donors, and also separately for cows and heifers. There was no difference in embryo yield among the breeds, and the results are therefore presented without specifying the breed. Pluset yielded 1.1 recovered structures more than Folltropin (11.9 vs. 10.8, $P<0.001$). Consequently however, the number of transferable embryos did not differ among the groups (7.0 and 7.1 for Folltropin and Pluset, respectively), neither did the number of degenerated embryos (1.8 and 1.9 for Folltropin and Pluset, respectively); rather there was an increase in the number of UFO in Pluset (2.0 and 3.0 for Folltropin and Pluset, respectively, $P<0.001$).

The quality of embryos did not differ between the groups. The distribution of embryos into quality grades 1 to 3 was similar between Folltropin and Pluset groups. Mean quality grades, expressed as indices, were 1.35±0.42 in the Folltropin group and 1.36±0.43 in the Pluset group. Likewise, the developmental kinetics of embryos was similar, i.e. no difference was detected in the distribution of embryos at developmental stages from morula to expanding hatched blastocyst. The mean developmental stage of embryos was 5.20±0.78 and 5.17±0.75 for Folltropin and Pluset groups, respectively.

The success of superovulation was evaluated moreover by determining the proportion of collections yielding no transferable embryos. For heifers, 11.5% of collections in the Folltropin group and 14.8% in the Pluset group were unsuccessful ($P=0.14$). For cows, these represented 15.6% and 15.7% of collections in Folltropin and Pluset groups, respectively (=0.965).

In addition to the collections producing no transferable embryos, donors were investigated that had an exceptionally low ovarian response, i.e. less than five CL, and recovery of a maximum of four embryos or ova. The proportion of low-responder heifers was 6.4% in the Folltropin group and 5.2% in the Pluset group, there being no statistically significant difference ($P=0.184$). Neither did the proportion of low-responders differ ($P=0.117$) between cows in the Folltropin (11.3%) and Pluset groups (8.3%).

The viability of embryos, expressed as the 56-day non-return rate of recipients, was similar among the groups. Embryos originating from animals treated with Folltropin resulted in a non-return rate of 64.0% and for Pluset-treated donor

embryos the non-return rate was 62.4% (P=0.145). There was a reduction of 22.4 percentage points between the NRR and calving rate after transfer of F-embryos and correspondingly 22.0 percentage points for P-embryos. NRR did not differ between same quality grades of F and P embryos; NRR of grade 1 F-embryos was 65.0% and of P-embryos 63.6% (P=0.074). Respectively, NRR for grade 2 embryos was 60.3% and 57.0% (P=0.115) and for grade 3 embryos 58.9% and 52.3% (P=0.085).

3. Effect of sex-sorted semen on embryo yield and quality (III, IV)

In Studies III and IV, embryo yield after insemination with sex-sorted or conventional semen was characterized. There was no difference between Ayrshire and Holstein breeds for any of the attributes, and all results are therefore presented for heifers and cows combined, irrespective of breed. In Study III, the number of transferable embryos decreased with the use of sexed semen. In SEX cows, there was a decline of 4.2 transferable embryos (P<0.001). In heifers, the decline was not as evident. Sex-sorted semen resulted in only 1.1 embryos less, the reduction not reaching statistical significance. The average number of transferable embryos in heifers was 6.1 vs. 7.2 and in cows 4.9 vs. 9.1 for SEX and CONV, respectively.

Both in heifers (P<0.05) and cows (P<0.01), the mean number of UFO was higher for SEX. Additionally, in cows the number of degenerated embryos, 2.4 on average, was higher for SEX compared with 1.5 for CONV (P<0.01). The increased proportions of non-viable embryos resulted in only 45% of embryos being transferable in the SEX cows group, compared with 70% transferable embryos in the CONV cows group.

The embryo collections analyzed in Study III were included in a larger dataset of Study IV. As the number of SEX embryo collections doubled from 218 in Study III to 443 in Study IV, the statistical power increased, resulting in statistical significance in the reduction of transferable embryos and in the proportion of unsuccessful collections, unlike in Study III. The average numbers and proportions of all structures recovered, transferable embryos, UFO and degenerated embryos for SEX and CONV are presented in Table 3. The reduction in transferable embryo yield was 1.4 embryos in heifers and 3.2 in cows. The proportions of UFO and degenerated embryos increased for SEX, both in heifers and cows.

Table 3. Mean numbers of all structures recovered, transferable embryos, UFO and degenerated embryos from superovulated heifers and cows inseminated with sex-sorted (SEX) or conventional semen (CONV) (% of all recovered structures). (From paper IV)

	Heifers			Cows		
	SEX (n=322)	CONV (n=1007)	P	SEX (n=121)	CONV (n=521)	P
All Structures	10.7	10.9	ns	12.5	12.3	ns
Transferable	6.2 (58)	7.6 (70)	<0.001	5.4 (44)	8.6 (70)	<0.001
UFO	2.9 (27)	1.9 (18)	<0.001	4.8 (38)	2.3 (19)	<0.001
Degenerated	1.6 (15)	1.3 (12)	0.011	2.3 (18)	1.4 (11)	0.003

ns= non-significant

The effect of sexed semen on the quality of transferable embryos was analyzed using quality grade indices, representing the means of quality grades in each collection, which are presented in Table 4. There was a statistically significant difference in the indices for SEX and CONV groups, indicating that the use of sexed semen compromises the quality of embryos. This was also apparent when the distribution of quality grades for SEX and CONV embryos was analyzed. The proportion of grade 1 embryos decreased in SEX compared with CONV, 6.5 percentage points for heifers ($P<0.001$) and 11.9 percentage points for cows ($P<0.001$). Correspondingly, proportions of grade 2 and 3 embryos increased for SEX.

Table 4. Means (±SD) for the quality grade indices (1-3 according to the IETS classification recommendations) of embryo recoveries in heifers and cows inseminated with sex-sorted or conventional semen. (From paper IV)

	SEX		CONV		
	n	Grade	n	Grade	P
Heifer	322	1.43 ± 0.48	1007	1.28 ± 0.37	<0.001
Cow	121	1.41 ± 0.45	521	1.23 ± 0.33	<0.001
All	443	1.43 ± 0.47	1528	1.27 ± 0.36	<0.001

The effect of sexing on the developmental kinetics of transferable embryos was analyzed using the developmental stage indices, which are presented in Table 5. In heifers, there was a statistically significant difference in the indices between SEX and CONV groups. In cows, the difference approached statistical significance. The proportions of morulae among viable embryos were similar in SEX and CONV groups for both heifers and cows. The proportion of blastocysts was smaller in the SEX than in the CONV group for both heifers ($P<0.001$) and cows ($P=0.036$). Later developmental stages, expanded blastocyst and higher stages were more frequent in cows inseminated with conventional semen than those receiving sex-sorted semen ($P=0.015$).

In Study III, the use of sex-sorted semen did not result in more failed embryo collections, i.e. collections that yielded no transferable embryos. In cows, however, the risk of recovering no viable embryos with sexed semen was higher than in heifers ($P<0.05$). The proportion of failed recoveries in heifers was 12.2% (115/945) vs. 7.7% (10/130) and in cows 13.0% (42/324) vs. 18.2% (16/88) for conventional and sexed semen, respectively. In Study IV however, containing data from Study III (without collections on the ET station, where only conventional semen was used) supplemented with more data, the frequency of unsuccessful collections yielding no transferable embryos was higher for SEX, both in heifers and cows. The proportion of unsuccessful recoveries in heifers was 7.2% (73/1007) and 11.2% (36/322) for CONV and SEX, respectively. Thus there was a four percentage points increase in the proportion for SEX ($P=0.025$). In cows, the difference among the groups was greater, 11.7 percentage points ($P<0.001$), as the proportion of unsuccessful collections increased from 9.0% (47/521) in CONV to 20.7% (25/121) in SEX. Thus, the greater number of collections compared with Study III revealed that sex-sorted semen increases the risk of yielding no transferable embryos.

Table 5. Means (±SD) for the developmental stage indices (4-9 according to the IETS classification recommendations) of embryo recovery in heifers and cows inseminated with sex-sorted or conventional semen. (From paper IV)

	SEX		CONV		
	n	Developmental stage	n	Developmental stage	P
Heifer	322	5.09 ± 0.72	1007	5.25 ± 0.77	0.001
Cow	121	4.91 ± 0.73	521	5.07 ± 0.79	0.067
All	443	5.04 ± 0.73	1528	5.19 ± 0.78	0.001

4. Effect of sex-sorted semen in embryo production on pregnancy rate after ET and calf mortality (V)

Pregnancy rate after embryo transfer was affected by type of semen used for embryo production, type of embryo (fresh or frozen), quality grade and developmental stage of embryo and the parity of the recipient (heifer or cow). Neither the breed of an embryo nor the breed of the recipient had an effect on conception.

The pregnancy rate in recipients receiving an embryo produced with sex-sorted semen was lower than for embryos produced with conventional semen. The decrease was 12% (5.3% points) when a SEX embryo was transferred compared with a CONV embryo (38.8% vs. 44.1% in SEX and CONV, respectively, $P<0.001$). Pregnancy rates associated with fresh and frozen embryos, respectively, in CONV were 48.7% and 42.3%, and in SEX 40.0% and 37.3%.

The proportion of female calves was 49.6% and 92.3% in CONV and SEX groups, respectively. Overall calf mortality was similar in both groups, 9.0% in CONV and 8.9% in SEX groups. However, calf mortality analyzed according to gender revealed a higher mortality for male calves in the SEX group compared with males in the CONV group. Mortality of male calves in the SEX group was 16.0% whereas in the CONV group it was 9.2% ($P<0.005$).

DISCUSSION

1. Effect of nutritional protein on embryo production

In Study I, feeding heifers with rapeseed meal to reach a CP content of 18% in the diet was sufficient to result in elevated serum urea concentrations compared feeding a CP content of 14%. This feeding regimen did not, however, affect the superovulatory response of the heifers in terms of number of ovulations, total number of embryos and transferable embryos. These findings are in accordance with several others showing no effect of excess protein supplementation. Gath et al. (1999, 2012), feeding beef heifers with high or low energy diets supplemented with 250 g feed grade urea, did not establish any effect of high protein, nor a combined effect of protein and energy intake on ovulation rate. However, fertilization rate was compromised in the control group compared with groups fed urea. In this study, the serum urea levels were 7.1 mmol/l in the highest group, compared with 5.8 mmol/l in our study. Also, urea as a nitrogen source is more soluble than the rapeseed meal fed in our study, resulting in higher diurnal peak concentrations than with rapeseed meal, but despite that, no deleterious effects of urea were recorded.

Another study on non-lactating Holstein cows (Garcia-Bojalil et al. 1994) established no difference in follicular growth, ovulation rate, embryo number and quality and proportions of transferable embryos, degenerated embryos and UFO. The high protein group was fed 27.4% CP in the form of urea and soybean meal, whereas the diet of the control group contained 12.3% CP. These diets resulted in plasma urea concentrations of 7.6 mmol/l in the high group, i.e. higher than in our study or the studies of Gath et al. (1999, 2012). The plasma urea concentration was higher than the threshold value suggested by Butler at al. (1996) for lactating dairy cows (PUN >19 mg/dl, corresponding urea 6.77 mmol/l). Despite this, no deleterious effects were found, and it appears that the threshold value for cows in lactation cannot be adapted to non-lactating animals.

The protein feeding period was initiated 71-72 days before embryo collections. Because the superovulatory treatment took 22 days, the animals had an approximately 50-day period of acclimatization to the diet before the initiation of the FSH treatment. The long duration of the feeding period might have been advantageous. In lactating cows, Dawuda et al. (2002) reported no detrimental effect of feeding high levels of quickly degradable urea nitrogen (elevating plasma

urea concentration to approximately 9 mmol/l) from ten days before insemination on the total number of embryos, proportions of quality grades and embryo metabolism *in vitro*. However, the same diet initiated during the periovulatory period and continued until embryo recovery, possessed deleterious effects on embryos: the total number of embryos was reduced and proportionally fewer good quality embryos were recovered. However, the plasma urea concentrations were markedly higher than in Study I, and it is moot whether this relatively moderate urea concentration achieved in this study would have resulted in a different outcome had the duration been shorter.

Oocyte competence and embryo production *in vitro* were studied by Sinclair et al. (2000) feeding heifers diets containing 15% CP, but differing in their nitrogen release (low and high ammonia generating mixtures). These resulted in plasma urea levels of 5.7 and 7.0 mmol/l for low and high ammonia treatments, respectively. Oocyte competence was compromised in the high ammonia treatment group as fewer oocytes cleaved and developed into blastocysts. Possible causal factors for these divergent results can lie partly in the composition of the diets. Diets generating elevated blood urea concentrations do not necessarily cause a similar pattern in ammonia levels. Ammonia and urea, albeit both products of nitrogen metabolism, may have different actions on the reproductive system. Also, the overall metabolism of the animal involves interactions: it seems that animals fed energy-adequate diets can tolerate high nitrogen intake, but high nitrogen, together with energy deficiency, is more likely to affect adversely the reproductive performance. Moreover, for these complex interactions, there exists a question as to the extent to which the effects of urea *in vitro* are adaptable to oocytes and embryos developing *in vivo*.

The results of our study indicated a tendency of heifers on a high protein diet to produce embryos of better quality because the proportion of poor embryos was higher in the group receiving 14% CP. This was not supported by others and the relevance of the finding remains ambiguous. The study did not investigate the embryos further, i.e. the pregnancy rate. In a study by Rhoads et al. (2006), transfer of embryos produced from lactating dairy cows with plasma urea concentrations of 8.7 mmol/l led to impaired pregnancy rate in the recipients. The design of our study did not allow the question to be answered of whether the dietary treatment would have affected the pregnancy rate after embryo transfer.

The background of this study was based on practical considerations. At the time of the study the feeding practices in Finland were based on a relatively low protein feed for dairy heifers. The aim of the study was to evaluate the effects of increasing the protein level of the diet above the official feeding recommendations. It can be concluded that feeding diets of 14% and 18% CP to dairy heifers did not affect the superovulatory response and the number of transferable embryos. A moderate increase in the CP content to 18% might be advantageous for embryo quality as evaluated by morphological classification.

2. Effect of FSH preparation on embryo yield and quality

There was no difference in the success rate of superovulation with Folltropin or Pluset when evaluated as the number of transferable embryos or the quality and development of embryos recovered. The total number of recovered structures was higher for Pluset. This increase was a result of a higher proportion of UFO. Our findings are supported by the results of an earlier experiment by Kelly et al. (1997) where a higher ovulation rate led to higher number of recovered structures and an increased proportion of UFO from animals treated with eight injections of Pluset compared with treatment with Folltropin. Their fairly small dataset compared multiple injections of Folltropin (n=23) and Pluset (n=22) as well as a single injection of each preparation. Martens et al. (2005) also found no difference in the efficacy of Folltropin or Pluset when superovulating Simmental cattle.

In our study, the quality of embryos was similar for Pluset and Folltropin treatments, determined as the morphological quality and viability of embryos after transfer, i.e. subsequent non-return rate. However, unlike in our study, Kelly et al. (1997) recovered more freezable embryos from animals treated with Folltropin. The divergent quality of embryos in our studies might originate from the protocols used. They administered heifers a higher dose of Pluset (1000 IU) during five days compared with our four-day treatment with a lower dose (500-600 IU). Also in the study of Martens et al. (2005), moderate doses of both preparations were used. The higher dose of Pluset, containing a FSH:LH ratio of 1:1, could account for impaired oocyte quality, resulting in fertilization failure. It was suggested for humans (Loumaye 2002) and cattle (Kanitz et al. 2002) that limited concentrations of LH are tolerated during the follicular growth phase of ovarian stimulation, but exceeding a certain threshold may cause adverse effects on the follicle and oocyte. The limitations of retrospective analysis of field data are obvious, and our approach does not enable investigation of dose-dependent effects of the preparations. The

amount of FSH administered was based on the manufacturers' recommendations and long-established practice. Thereby, a comparison of these two products, administrating equal doses of FSH, was not feasible, albeit this approach would have been reasonable if it had been possible to establish a prospective analysis.

Low doses of Folltropin or Pluset were used by Ferré et al. (2016) to stimulate follicular growth prior ovum pick-up (OPU). In this study, the FSH preparation with reduced LH, i.e. Folltropin, was superior to Pluset or eCG, both having LH activity, in terms of collected and viable oocytes, cleavage and embryonic development. Sendag et al. (2008), comparing Pluset and eCG prior to OPU concluded that Pluset was superior, yielding more oocytes of better quality and less variation in the ovarian response. The results, suggesting a deleterious effect of excess LH in the gonadotropin, are in line with the findings initially published in 1980s (Chupin et al. 1984, Donaldson et al. 1986) and subsequently confirmed by numerous research groups.

In the study by Kelly et al. (1997), animals treated with Pluset received prostaglandin later during the treatment period than those treated with Folltropin. The time interval from induction of luteolysis with prostaglandin until the first insemination was 12 h longer for Folltropin-treated than Pluset-treated donors. A comparable approach was the study of Ferré et al. (2016), delaying OPU in Folltropin-treated cows later than Pluset-treated with regard to the last FSH injection. Presumably the reason for selecting such an approach was because oocyte maturation is supposed to be accelerated in the presence of more LH. In our protocol, timing of the first insemination was 48 and 60 hours after the prostaglandin treatment for heifers and cows, respectively, regardless of the gonadotropin used, but adjusted if needed according to the onset and duration of estrus. It remains unanswered as to whether the 12 to 24 hours delay before insemination or oocyte collection for Folltropin, as others have applied, would have altered our results in favor of either preparation.

The morphological evaluation of embryos revealed no effect of FSH preparation. In addition to morphological analysis, the developmental potential of embryos was evaluated according to their ability to conceive. Equal 56-day non-return rates were achieved after transfer of embryos produced with Folltropin or Pluset.

3. Effect of sexed sperm on embryo yield, quality and developmental kinetics

3.1. Transferable embryo yield

It was clearly shown in our studies that the numbers and proportions of transferable embryos were decreased when superovulated donors were inseminated with a dose of 8 to 10 million sex-sorted sperm compared with 30 million unsorted sperm. Correspondingly, the proportions of non-viable embryos and UFO were increased. These issues were studied initially in Study III. In Study IV, a larger dataset was available because more embryo collections with sex-sorted semen had been performed by that time. The main goal of Study IV was to examine further the subset of transferable embryos from the data that were not accessible during Study III.

In Study III, the decrease in the number of transferable embryos by 1.1 when sex-sorted semen was used for heifers did not reach statistical significance. However, in Study IV, containing more than twice as many collections with sexed semen compared with Study III, the difference was 1.4 embryos and the increased statistical power was sufficient to show significance. This was also the case with degenerated embryos: in Study III no difference was detected in the number and proportion of degenerated embryos for heifers, but in Study IV the proportion of degenerated embryos was increased.

Comparisons of embryo recovery results among different experiments are challenging. For example, semen sorting protocols differ and not all papers mention the sorting pressure, which was reported to be important for semen viability (Schenk et al. 2009, Barcelo-Fimbres et al. 2011). All papers do mention however other influencing factors such as semen dosage, insemination protocols and insemination sites for sex-sorted and conventional semen, but the protocols differ considerably. It has been demonstrated that the timing of inseminations of superovulated donors is of utmost importance and that the amount of semen deposited 24 h after the detection of standing estrus is the key factor for successful embryo recovery in superovulated donors (Sartori et al. 2004). Soares et al. (2011) concluded that delaying inseminations with sexed semen from 12 and 24 h after estrus to 18 and 30 h improves the embryo yield. This is supported by the fact that ovulation occurs about 30 h after the onset of estrus, and the shorter capacitation time required for sorted semen (Lu & Seidel 2004, Schenk et al. 2009). In the earlier studies, semen dose at the time point of 24 h after detection of estrus varied from

approximately 2 million sperm (Schenk et al. 2006, Hayakawa et al. 2009, Peippo et al. 2009, Larson et al. 2010) to 4-5 million (Hayakawa et al. 2009, An et al. 2010, Soares et al. 2011) up to 10 million sperm (Sartori et al. 2004, Schenk et al. 2006, Hayakawa et al. 2009).

Utilizing a small number of sex-sorted sperm (approximately 2 million) 24 h after the detection of estrus in cows, the mean number of transferable embryos per donor cow remained relatively low in previous studies: 3.8 (Larson et al. 2010) and 3.1 (Schenk et al. 2009) when inseminating beef cows in the uterine body, or 2.5 (Hayakawa et al. 2009) and 2.1 (Peippo et al. 2009) when using DUI for dairy cows. Therefore, the insemination site did not seem to be of great importance when using low numbers of sperm.

Heifers inseminated with 4 million sex-sorted sperm 24 h after the detection of estrus produced a mean of 4.8 transferable embryos in a study by An et al. (2010). Hayakawa et al. (2009) inseminated 5 million sex-sorted sperm 24 h after the onset of estrus and recovered means of 5.6 and 5.2 transferable embryos from heifers and cows, respectively. These numbers are very similar to the results of Study IV, where 4 million sex-sorted sperm inseminated 24 h after the detected standing estrus resulted in a mean of 6.2 transferable embryos from heifers and 5.4 from cows. Hayakawa et al. (2009) inseminated into the uterine body, while in the study by An et al. (2010) and Studies III and IV semen was deposited in the uterine horns. Therefore, as with cows, the numbers of sperm seem to be more important than the insemination site.

While 4 to 5 million sex-sorted sperm for heifers 24 h after the detected estrus resulted in similar or moderately decreased numbers of embryos, compared with non-sorted, the question remains as to whether or not using higher sperm numbers would improve the results for cows. Schenk et al. (2006) did not reveal a difference in transferable embryo yield when inseminating 10 million sex-sorted sperm (4.6 transferable embryos) compared with 2 million (3.1 transferable embryos) into the uterine body 24 h after the detected standing estrus. Hayakawa et al. (2009) inseminated 10 million sex-sorted sperm at the same time point into the uterine body and recovered 5.04 transferable embryos from cows. In these studies, however, the number of superovulated donors was relatively low. Furthermore, the technique of semen sorting has been modified and improved since the earlier studies conducted at the initial decade of the 2000s. This may have contributed to the variable results of some of the earlier studies. In view of these results, inseminating 10 million sperm, instead of 4 to 5 million, 24 h after the onset of

standing estrus, did not increase the number of transferable embryos in superovulated cows.

3.2. Fertilization rate

In Study IV, when inseminated with sex-sorted semen, the proportion of UFO increased 9 and 19 percentage points in heifers and cows, respectively. Similarly, the proportion of degenerated embryos increased, by 3 and 7 percentage points in heifers and cows, respectively. The same pattern was reported in other publications. Utilization of a very small number (2 million) of sex-sorted sperm 24 h after the detected standing estrus seemed to result in a greater proportion of UFO, especially in cows: 39% (Hayakawa et al. 2009), 53% (Larson et al. 2010) and 70% (Sartori et al. 2004). However, increasing the number to 10 million sex-sorted sperm at 24 h did not apparently result in a greater proportion of transferable embryos, but increased the proportion of degenerated embryos to 44.6% (Hayakawa et al. 2009). The highest proportions of transferable embryos in cows inseminated with sex-sorted semen were 44% in Study IV, with 4 million sperm 24 h after the detected standing estrus, and in the study by Hayakawa et al. (2009) with 5 million sperm at 24 h. However, those figures are far from the 70% proportion of transferable embryos recovered from cows inseminated with conventional semen in Studies III and IV.

3.3. Embryo quality

The proportion of grade 1 embryos decreased when sexed semen was used. Correspondingly, the proportions of grade 2 and 3 embryos increased for sexed semen. Some reported a decrease in the proportion of grade 1 embryos when inseminations were performed with sex-sorted semen while others did not. Peippo et al. (2009) found no effect of sexed semen on the proportion of grade 1 embryos among transferable embryos in superovulated heifers and cows inseminated with sexed semen. On the other hand, results in agreement with ours were reported for superovulated beef cows producing a decreased proportion of grade 1 embryos (Larson et al. 2010). Also Soares et al. (2011) superovulating both Nelore and Holstein cows and Baruselli et al. (2008) superovulating Nelore cows, reported that the proportion of freezable embryos (grades 1 and 2) was lower when sexed semen was used. The number of animals in the sex-sorted sperm group ranged among the above-mentioned experiments from four to 59. The dataset for the present experiment differs in the numbers of animals and the nature of the data because all except for Peippo et al. (2009) and one trial of Hayakawa et al. (2009) were from

designed experiments rather than field data. Nevertheless, it is convincing that in general the observations are consistent, and the minor differences among the studies are most likely due to factors such as limited numbers of bulls, diverse insemination protocols regarding timing and insemination site and variable sperm numbers.

3.4. Developmental kinetics of embryos

Study IV revealed a difference in the developmental stage indices between heifers inseminated with sexed and conventional semen. The lower indices for the sex-sorted group indicated a slight delay in development when sexed semen was used. In cows, however, when the indices were used to describe developmental rates, there was a tendency for delayed embryonic development in the sexed embryos, but the difference did not reach statistical significance. This might be because the indices are based on means, which are not sensitive in detecting differences in all types of distributions. The distribution of developmental stages, despite the statistical significance, does not follow a regular pattern, and from the practical point of view the difference is negligible.

The developmental kinetics of embryos after fertilization with sex-sorted semen for *in vitro* produced embryos were studied by several authors. It has been concluded widely that IVF with sex-sorted sperm results in deviations in the developmental rate of embryos, manifesting in delayed cleavage and/or blastocyst formation rate (Wilson et al. 2006, Morton et al. 2007, Palma et al. 2008, Bermejo-Alvarez et al. 2008, Trigal et al. 2012). The mechanisms responsible for these alterations remain partly unexplained. Considering the deviations in *in vitro* embryos produced with sex-sorted semen, it would have been reasonable to establish more marked differences in the developmental kinetics of *in vivo* embryos. For *in vivo* embryos, investigation of developmental kinetics is very unrefined because transcervical uterine flushing in commercial embryo production setups enables only evaluation of day six to eight embryos. In the present data, the embryonic development seemed to be slightly delayed when sexed semen was used. Nevertheless, the difference in average developmental stage was small, and its biological relevance remains questionable.

The decreased fertilization potential and impairment of embryonic development of sex-sorted semen are consequences of both a reduced number of sperm in most commercially produced doses and the damage caused to sperm by the sorting procedure (Frijters et al. 2009). However, the sorting process has undergone several improvements during recent years, reducing the stress on the sperm cells. This has

led to increased pregnancy rates after AI with sexed semen (Seidel 2014). Most of our data originate from sorted semen produced before the most recent improvements were applied. It remains to be clarified how the technical improvements will affect the efficacy of embryo production.

3.5. Unsuccessful collections

It appeared, according to Study III, that when sex-sorted semen was used, the probability of recovering no transferable embryos was similar to when using conventional semen. This would have been the conclusion without the complementary data of Study IV. However, Study IV highlights the importance of statistical power. As more data were analyzed, Study IV revealed that the probability of recovering no transferable embryos increased when sexed semen was used, both for heifers and cows.

From the practical point of view, taking account of the results of Studies III and IV, it can be concluded that sex-sorted semen decreases the number of transferable embryos, but more female embryos can be produced in a single embryo collection. The percentages of females in our data (Study V) were 49.6% and 92.3% for conventional and sex-sorted semen. Thereby the numbers of female embryos recovered from heifers inseminated with sex-sorted and conventional semen were 6.2 and 3.8, respectively. For cows, the numbers of female embryos for sexed and conventional semen were 5.4 and 4.3, respectively.

4. Effect of sexed semen on pregnancy rate and calf mortality after embryo transfer

Pregnancy rates after transfer of *in vivo* embryos produced with sex-sorted semen were lower than after transfer of embryos produced using conventional semen. The difference was five percentage points when frozen embryos were used (conventional 42.3% vs. sexed 37.3%, corresponding to a total decrease in pregnancy rate of 12%). The difference in pregnancy rate was even more (8.7% points) when fresh embryos were transferred. The distribution of quality grades was similar between fresh sexed and conventional embryos in this dataset. This irrational finding of a larger decrease in pregnancy rate of fresh embryos might be due to management decisions. Farmers are more motivated to transfer sexed embryos than conventional ones. Thus the selection criteria for recipients of sexed embryos can be less accurate because farmers want to improve the chances of

transferring as many embryos as possible. Recipients synchronized for conventional embryo recovery can be more easily discarded in the case of asynchrony, and extra embryos are frozen.

Studies on the effects of sex-sorted semen on fertilization rate and early embryonic development in superovulated donors are consistent, revealing a decreased fertilization rate and impaired embryonic development during the 7-day period from insemination to embryo recovery (Sartori et al. 2004, Baruselli et al. 2008, Hayakawa et al. 2009, Peippo et al. 2009, An et al. 2010, Larson et al. 2010, Soares et al. 2011, Kaimio et al. 2013). However, further monitoring of the *in vivo* embryos appears to be infrequent, and reports of pregnancy results are scarce. Baruselli et al. (2008) and Hayakawa et al. (2009), transferring *in vivo* embryos produced with sex-sorted semen (n=42 and n=27, respectively), reported equal pregnancy rates for sexed and conventional embryos. However, the number of sexed embryos was relatively low in these studies.

For *in vitro* embryos, in contrast, several reports were published on pregnancy rates of embryos fertilized with sex-sorted semen. Reasonable pregnancy rates can be achieved with sex-sorted IVP embryos (Wheeler et al. 2006, Pontes et al. 2010, Trigal et al. 2012). However, the effect of sex sorting is difficult to evaluate because the results are inconsistent, and often only a limited number of bulls are used for fertilization. In some studies, the bulls were pre-selected for fertility under laboratory conditions. Differences in the sperm handling, as well as in maturation, fertilization and culture procedures complicate the interpretation of results. Wilson et al. (2005, 2006) reported compromised conception rates after transfer of *in vitro* produced embryos fertilized with sexed semen. On the other hand, pregnancy rates comparable with non-sorted embryos were achieved with vitrified sex-sorted IVP embryos in a large-scale transfer experiment (Xu et al. 2006) and in a smaller study with fresh IVP embryos (Rasmussen et al. 2013).

Beilby et al. (2011) reported on indicators of embryonic viability, other than pregnancy rate, for ovine *in vivo* embryos. The authors demonstrated deviations in the expression of genes involved in general cellular function and maintenance of ovine embryos produced with sex-sorted semen. Correspondingly, in production of *in vitro* embryos, other parameters, aside from pregnancy rates, have been studied frequently. Sexed semen can compromise the cleavage rate (Bermejo-Alvarez et al. 2008, Palma et al. 2008, Trigal et al. 2012) and blastocyst formation rate is frequently lower with sexed semen (Wilson et al. 2006, Morton et al. 2007,

Bermejo-Alvarez et al. 2008, Palma et al. 2008, Trigal et al. 2012). A delay in embryonic development and ultrastructural changes in the embryo, such as an elevated proportion of immature mitochondria and deviations in the structure of rough endoplasmic reticulum and the nuclear envelope (Palma et al. 2008), were reported after *in vitro* fertilization with sex-sorted semen. Moreover, epigenetic changes were demonstrated in IVP embryos produced with sex-sorted semen, as the relative abundance of Glut-3 and G6PD, both developmentally important genes in day-7 embryos, was higher in IVP embryos produced with unsorted semen (Morton et al. 2007). Such epigenetic variations could be involved in the compromised viability of embryos and the resulting offspring.

In addition to the finding of an impaired pregnancy rate, we established that calf mortality was higher for male calves born from sexed semen than for male calves born from conventional semen. Previous results on the mortality of calves born from sexed semen are ambiguous. Several authors reported similar rates of mortality in AI or ET calves born from sex-sorted semen compared with conventional semen (Tubman et al. 2004, Borchersen & Peacock 2009, Rasmussen et al. 2013). However, the higher calf mortality rate of the incorrectly sexed males in this study parallels that reported by DeJarnette et al. (2009), who reported an increase of stillbirths among male calves born from X-sorted sperm. Similar findings were subsequently reported by others (Norman et al. 2010, Healy et al. 2013). This phenomenon is probably caused by aneuploidy, mainly trisomic, or large DNA translocations containing Y-sperm concentrated in the X-fraction due to higher DNA content than normal Y-sperm (DeJarnette et al. 2009). The higher mortality rate of these male calves does not influence the overall calf mortality of sex-sorted embryos because those embryos fertilized with Y-sperm, which have been sorted to the X-fraction, represent only a minor proportion, less than 10%, of the embryos.

Insemination of superovulated embryo donors with sex-sorted semen can be profitable, regardless of the reduction in embryo recovery rate. One of the major advantages of sexed semen in embryo production is that recipient management can be optimized when transferring fewer embryos of the undesired gender.

Taking the findings of Studies III, IV and V together, it can be concluded that the number of transferable embryos decreases when sex-sorted semen is used for superovulated donors. Despite the decrease in embryo production, more female embryos can be recovered per donor. However, the final outcome of a

superovulation and embryo recovery should be evaluated after transfer of embryos and birth of calves. Our finding that transfer of sex-sorted embryos resulted in a decline of five percentage points in the pregnancy rate compared with the result of using conventional semen (equal to a decrease in pregnancy rate of about 12%), should be considered when evaluating the outcome of embryo production with sorted semen. For a precise calculation of the final efficiency of MOET utilizing sex-sorted semen, the distribution of sexed and conventional embryos into quality grades 1-3 and the calving rate of each quality grade should be taken into account. Because the mortality of female calves born from embryos produced with sex-sorted semen did not differ from that for conventional semen, only live calves were counted in the following calculations.

The calving rates for fresh and frozen embryos of different quality grades in Study V can be used to calculate the female calf production for each semen and donor type on Finnish farms. Assuming all embryos were transferred fresh, the calving rates of grade 1, 2 and 3 fresh sexed embryos were 49.6, 31.3 and 22.2%, respectively (based on unpublished data from Study V). For fresh conventional embryos, the calving rates were 57.5, 42.9 and 29.0%, respectively. Considering the distribution of embryos into quality grades 1–3 in Study IV, the number of female calves born after an embryo collection on a heifer inseminated with sex sorted semen was 2.5, and 2.0 when conventional semen is used. For cows, the number of female calves was 2.2 and 2.4 for sexed and conventional embryos, respectively. Concluding, with fresh embryo transfers, more female calves (0.5 calves) can be produced when donor heifers are inseminated with sex-sorted semen compared with conventional semen. For cows, slightly more female calves (0.2 calves) can be produced with conventional semen.

However, the number of available recipients is often limited on Finnish farms, due to small herd size. Relatively seldom is it possible to transfer all embryos fresh on the day of collection. The data of Study V revealed a calving rate of 37.9% for grade 1 frozen sexed embryos and 42.7% for conventional embryos (unpublished). If grade 2 and 3 embryos were transferred fresh and grade 1 embryos frozen – as often is practical when there is a dearth of recipients –an embryo collection from a heifer inseminated with sexed semen would yield 2.0 female calves and 1.6 when conventional semen was used. For cows, the number of female calves would be 1.8 for both semen types. Thereby, transferring grade 1 embryos frozen and grade 2 and 3 embryos fresh would result in more female calves (0.4 calves) from heifers

inseminated with sex-sorted semen compared with conventional semen. For cows, equal numbers of female calves result from the use of both semen types.

The calculation that would most reliably represent the practice would utilize the overall calving rate of fresh and frozen embryos, as presented in Study V. The transfers of Study V represent a cross-section of all embryo transfers of Finnish farms during a five-year period. Thereby, the distribution of embryos into fresh and frozen transfers for each semen type can be considered more realistic than the above-mentioned simulations. The calving rates for sexed grade 1, 2 and 3 embryos were 42.8, 30.3 and 22.2%, respectively. For conventional embryos, the calving rates were 45.2, 40.7 and 29.2%, for grades 1, 2 and 3, respectively (unpublished data from Study V). When these calving rates are included in the calculation, the number of female calves would be 2.2 per embryo recovery from a heifer inseminated with sexed semen and 1.6 from one inseminated with conventional semen. For cows, the number of female calves would be 1.9 for each semen type. Taking into consideration that limited recipient resources represents a major bottleneck in efficient implementation of genomic breeding programs, sex-sorted semen can increase the efficacy of breeding schemes by facilitating the production of equal numbers or more calves of the desired gender than conventional semen, and furthermore economizing on the use of recipients.

CONCLUSIONS

- Feeding dairy heifers a diet containing 18% CP for a period of about 50 days before initiation of superovulatory treatment resulted in comparable superovulatory response and number of transferable embryos as one with 14% CP. Feeding 14% or less CP is common practice, and the feeding recommendations in Finland do not advise feeding animals over 200 kg live weight with additional protein, other than that gained from roughages. According to our results, the higher protein content in the form of rapeseed meal may be advantageous for embryo quality. Embryo donor heifers on dairy farms could be fed more protein than is common practice without adverse effects on embryo production.

- Superovulation of dairy heifers and cows with an FSH preparation low in LH, Folltropin, or a preparation with equal amounts of FSH and LH, Pluset, resulted in equal embryo yields in terms of numbers and quality of transferable embryos. The total number of recovered structures associated with Pluset was higher. This was a consequence of larger numbers of unfertilized ova.

- Fertilization rate was decreased when inseminating a superovulated donor with sex-sorted semen compared with using conventional semen. Sex-sorted semen compromised the number and quality of transferable embryos recovered from cows and heifers. There was also an indication of a slight delay in embryo developmental kinetics, but the biological relevance of this finding remains equivocal.

- The risk of recovering no transferable embryos was higher when sex-sorted semen was used compared with conventional semen, both in heifers and cows.

- Pregnancy rate after transfer of embryos produced with sex-sorted semen was reduced by 12% compared with embryos produced using conventional semen.

- Mortality of female calves born from embryos produced with sorted semen was comparable with their unsorted counterparts, but mortality of male calves was higher for sexed compared with conventional embryos.

- Despite the lower number of embryos produced with X-sorted semen and their being of inferior quality and producing fewer pregnancies, use of sexed semen is profitable when female progeny is demanded. The benefits are more pronounced in heifers, which are the most important group of donors in modern breeding schemes utilizing genomic selection. More female calves can be produced in heifers with sex-sorted semen and the need for recipient resources is smaller than that required for conventional embryos.

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