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FERTILITY OF SEX-SORTED SPERM IN SEASONAL-CALVING PASTURE-BASED DAIRY HERDS

A thesis submitted to University College Dublin in fulfilment of the requirements for
the degree of Doctor of Philosophy (PhD) in the School of Agriculture and Food

Science by

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October 2019



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STATEMENT OF ORIGINAL AUTHORSHIP

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Clio Maicas

A handwritten signature in black ink, appearing to read 'Clio Maicas', with a stylized flourish at the end.

THESIS ABSTRACT

Use of X-sorted sperm increases the proportion of female dairy offspring from artificial insemination (AI); thus, it can be used to rapidly produce high numbers of dairy heifers, and has the potential to accelerate herd expansion and, through targeting elite females, increase profitability. However, sex-sorted sperm generally achieves poorer field fertility than conventional, unsorted, sperm. This limits its widespread adoption, particularly in pasture-based systems, which are highly dependent on reproductive efficiency to achieve compact calving at the beginning of the grass-growing season. During the last years, improvements in the sex-sorting technology have aimed to close the gap in field fertility between sex-sorted and conventional sperm. Given the potentially severe impact of poor fertility in a seasonal pasture-based system such as that operated in Ireland, there was a need to conduct field studies to evaluate the fertility of fresh and frozen X-sorted sperm under Irish conditions.

In the first study (Chapter 2), we investigated the phenotypic fertility performance of dairy heifers and lactating cows inseminated after spontaneous oestrus with fresh (1 or 2 million sperm dose) or frozen (2 million sperm dose) SexedULTRA™ X-sorted sperm compared with fresh conventional sperm (3 million sperm dose) in seasonal-calving pasture-based dairy herds. X-sorted sperm achieved relative pregnancy per AI (P/AI) ranging from 78.4 to 88.9% of those achieved with conventional sperm. P/AI achieved with frozen X-sorted sperm did not differ from that achieved with fresh X-sorted sperm. Doubling the concentration of sperm in fresh X-sorted straws did not improve P/AI. There was a significant bull effect on P/AI in both lactating cows and heifers. Additionally, P/AI of lactating cows inseminated with fresh sperm (conventional and X-sorted) varied between bulls as dispatch-to-AI interval increased. Heifers had greater P/AI than lactating cows, and P/AI in lactating cows decreased with increasing parity. Among young cows (parity ≤ 2), the following cow characteristics were associated with greater likelihood of pregnancy establishment after insemination with X-sorted sperm: BCS ≥ 3 , DIM > 60 d, and fertility sub-index of the EBI $> \text{€}100$.

In the second study (Chapter 3), we investigated the phenotypic fertility performance of lactating dairy cows inseminated with frozen SexedULTRA-4M™ X-sorted sperm (4 million sperm per straw) compared with frozen conventional sperm (15

million sperm per straw) in seasonal-calving pasture-based dairy herds. Overall, P/AI was greater for cows inseminated with frozen conventional sperm than for those inseminated with frozen X-sorted sperm (59.9% vs. 45.5%; 76.0% relative to frozen conventional sperm). The effect of sex-sorting on P/AI was highly variable between bulls, with greater variation amongst bulls whose ejaculates were shipped to the sorting centre compared with resident bulls. Frozen X-sorted sperm resulted in greater relative P/AI in cows with high and average fertility potential (defined by parity ≤ 2 , DIM at AI ≥ 70 , EBI fertility sub-index $> \text{€}60$) than in cows with low fertility potential. In 33.1% of the enrolled herds, the P/AI achieved with frozen X-sorted sperm was $\geq 90\%$ of the P/AI achieved with frozen conventional sperm; this was primarily a reflection of herds that had excellent performance with frozen X-sorted sperm, but performed relatively poorly with frozen conventional sperm.

Although there is a growing number of calves derived from X-sorted sperm, relatively few follow-up studies have investigated whether sex-sorting had subsequent effects on calf and adult characteristics. A detrimental effect on health and productivity of offspring derived from X-sorted sperm, especially if female offspring are affected, could negate the advantages of a biased offspring sex ratio. In the third study (Chapter 4), we compared perinatal and postnatal mortality as well as productive and reproductive performance of offspring derived from AI with X-sorted or conventional sperm processed from the same ejaculates. Calf survival during the first year of life was not different between animals derived from AI with X-sorted or conventional sperm. Male calves had higher mortality rates during the first 2 months of age than female calves independent of sperm treatment. No differences existed due to sex-sorting in survival, and reproductive or lactation performance of female offspring. Carcass conformation and fat scores of steers, and carcass weight of young bulls were not affected by sperm treatment. Herd rearing factors, and not the sex-sorting process, seemed to be involved in the greater overall carcass weight of steers derived from conventional sperm, since the distribution of slaughter age did not differ between steers derived from conventional or sex-sorted sperm when the analysis was restricted to herds that reared steers of both types.

In conclusion, X-sorted sperm achieved lower P/AI than conventional sperm in seasonal-calving pasture-based heifers and lactating cows inseminated after spontaneous oestrus. However, P/AI achieved with X-sorted sperm was dependent on

the bull, fertility potential of the cow, and herd. Female offspring derived from AI with X-sorted or conventional sperm did not differ for any of the performance characteristics studied. Further research is needed to confirm that the sex-sorting process does not contribute to differences in carcass traits between steers derived from AI with X-sorted or conventional sperm.

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ABBREVIATIONS

AI	Artificial insemination
BCS	Body condition score
CASA	Computer-assisted sperm analysis
CI	Confidence interval
CONV	Conventional sperm
DAFM	Department of Agriculture, Food and the Marine
DIM	Days in milk
EBI	Economic Breeding Index
FTAI	Fixed-time artificial insemination
GnRH	Gonadotropin-releasing hormone
IVP	<i>In vitro</i> embryo production
LSM	Least square means
MOET	Multiple ovulation embryo transfer
MSD	Mating start date
NRR	Non-return rate
P/AI	Pregnancy per AI
PGF2 α	Prostaglandin F2 α
PTA	Predicted Transmitting Ability
SEM	Standard error of the mean
SS	Sex-sorted sperm

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CHAPTER 1: Literature review

1.1 Sperm journey through the female tract

In cattle, typically 4 to 12 billion sperm per ejaculate are deposited in the anterior vagina after natural mating. From there, sperm travel through the vagina, cervix and uterus to finally reach the oviduct, where fertilisation takes place. During this journey, sperm encounter a series of barriers that act as selection steps. In the case of AI, because sperm are directly deposited in the uterus avoiding the vagina and cervix, a reduced number of sperm is routinely deposited (e.g., 15 to 20 million frozen-thawed conventional sperm, 2 to 4 million sex-sorted sperm). Of the billions of sperm deposited in the anterior vagina during natural mating or the millions of sperm deposited in the uterus by AI, only a few hundred reach the oviduct, and only one fertilises the ovulated oocyte (Miller, 2018).

In the vagina, sperm are briefly exposed to vaginal fluid, whose ultrastructural and rheological properties may influence sperm motility (Rutllant et al., 2005). The low pH of the vaginal fluid, useful as antimicrobial defence but potentially deleterious for sperm motility (Carr et al., 1985), is likely neutralized by the pH of the seminal plasma, which ranges from 6.7 to 7.4 in domestic species (Roberts, 1986). Then, sperm transverse the cervix, which contains multiple folds and grooves filled with cervical mucus (Mullins and Saacke, 1989) that limits the progression of sperm with abnormal motility and morphology (Hanson and Overstreet, 1981; Barros et al., 1984; Katz et al., 1990). In the uterus, sperm swim in the direction of the oviduct by active flagellar movement, aided by endometrial contractions under endocrine control (Hawk, 1987; Abramowicz and Archer, 1990). The utero-tubal junction, composed of mucosal folds forming cul-de-sacs that face back towards the uterus and with a particularly tortuous and narrow lumen (Yániz et al., 2000), acts as an anatomical, physiological and mucous barrier that regulates the entry of sperm to the oviduct (Suarez, 2004). There, sperm are largely sequestered before ovulation in the caudal region of the isthmus forming an oviductal storage reservoir by binding to the epithelial surface (Hunter and Wilmut, 1984). Binding to the oviduct epithelium occurs between the sperm heads and glycans of the oviductal epithelial cells in a species-specific manner (Miller, 2018), which in case of the cow involves fucose recognition (Lefebvre et al., 1997). The bovine sperm reservoir preferentially retains sperm with intact acrosomes (Gualtieri and Talevi, 2000) and at a non-capacitated status (Lefebvre and Suarez, 1996). Capacitation and motility of sperm stored in the reservoir are suppressed, and

sperm viability and fertilising capacity is preserved while awaiting ovulation (Pollard et al., 1991; Rodriguez-Martinez, 2007). Closer to ovulation, sperm are gradually released from the reservoir in the isthmus towards the ampulla (Rodriguez-Martinez, 2007), which may serve to prevent the incidence of polyspermic fertilisation (Hunter and Leglise, 1971). It is still debated whether sperm release is due to a change in sperm behaviour, in oviduct cell function or in the composition of oviduct fluid, as well as if a signal stimulates the release of sperm or whether small fractions of sperm are released continuously (Miller, 2018). During their transit through the oviduct, sperm attain two of the main physiological endpoints of capacitation: the ability to undergo the acrosome reaction and the change in motility pattern known as hyperactivation (Stival et al., 2016). Once in the ampulla, sperm meet the oocyte after crossing the cumulus cells surrounding it, bind to the zona pellucida, and undergo the acrosome reaction; the precise site where sperm undergo the acrosome reaction is still under debate (Yanagimachi, 2011; Sutovsky, 2018). The release of acrosomal contents enables sperm-zona penetration and prepares sperm for adhesion and fusion with the oolemma (Cuasnicú et al., 2016). After successfully crossing the zona pellucida, sperm enter the perivitelline space, and adhesion and fusion between oolemma and sperm plasma membranes occur. Bull sperm contributes chromosomes, centriole, perinuclear theca proteins and regulatory RNAs to the zygote (Sutovsky, 2018).

1.2 Compensable and noncompensable sperm deficiencies

The maximum fertility that a bull can achieve differs among bulls independently of sperm dose (Sullivan and Elliott, 1968). The minimum number of motile sperm required for achieving that maximum fertility is independent of the maximum fertility achievable (Den Daas et al., 1998) and differs among bulls, with low-fertility bulls requiring more sperm than high-fertility bulls to reach their maximum fertility (Sullivan and Elliott, 1968). These observations led to the classification of sperm deficiencies into compensable or noncompensable. Bulls requiring more sperm to reach the maximum conception rate achievable in the relevant female population are considered to have compensable seminal deficiencies, while those having lower fertility independent of sperm dosage are considered to have noncompensable sperm deficiencies. Compensable deficiencies are considered to be those important to sperm transport and function in the female reproductive tract, from semen deposition to

initiation of fertilisation and blockage of polyspermy, while noncompensable deficiencies are those important to complete fertilisation and sustain embryo development (Saacke, 2008).

1.3 Assessment of sperm quality in AI centres

After sperm processing and freezing, AI centres apply quality controls to ensure that sperm contained in dispatched straws pass minimum post-thaw standards of sperm quality and, therefore, will have the potential to achieve normal fertility potential when used in well managed herds. Quality control parameters typically include ejaculate volume and sperm concentration, both important to determine the number of straws that can be produced from an ejaculate, as well as pre- and post-thaw sperm motility and sperm morphology. Sperm motility and morphology are usually assessed under a phase contrast microscope by trained technicians. However, this visual assessment of sperm motility and morphology is prone to be inaccurate and biased due to technician subjectivity (Vincent et al., 2012); therefore, computer-assisted sperm analysis (CASA) systems have been developed to obtain more complete and accurate measures and have been progressively adopted by AI centres. The capabilities and limitations of CASA systems have been extensively review by Amann and Waberski (2014). Sperm motility parameters assessed by CASA have been correlated with *in vivo* and *in vitro* bull fertility (Budworth et al., 1988; Farrell et al., 1998; Kasimanickam et al., 2006; Kathiravan et al., 2008; Sellem et al., 2015). The presence of morphology abnormalities in bull sperm has been correlated with low *in vivo* fertility (Saacke, 1970; Al-Makhzoomi et al., 2008) and is considered an indicator of the presence of noncompensable deficiencies (Saacke, 2008). Motility and morphology tests routinely conducted in AI centres are generally sufficient to discard ejaculates with very poor sperm quality, but they fail to detect sperm differences between approved ejaculates of variable fertility (Hossain et al., 2011; Fair and Lonergan, 2018).

Fluorescence microscopy can be used to assess sperm attributes important for fertilisation, such as acrosome, membrane or DNA integrity. However, it is time-consuming, and the number of sperm analysed per sample is limited to few hundred. Thus, it has been substituted by flow cytometry, which allows the analysis of thousands

of cells within seconds in combination with a multiparametric approach, and has become a popular tool to evaluate sperm attributes (Vincent et al., 2012; Harstine et al., 2018). Plasma membrane integrity is commonly assessed using propidium iodide in combination with SYBR-14 (or Hoechst 33342) (Garner and Johnson, 1995). Although both fluorochromes bind to the DNA, propidium iodide only penetrates sperm with a disrupted plasma membrane; thus, membrane-intact sperm (defined as “viable” sperm) fluoresce green because of SYBR-14, while those with disrupted membrane fluoresce red (“dead”), including a moribund cell subpopulation (green–red). Early changes in membrane stability and intactness similar to those occurring during capacitation (Flesch and Gadella, 2000) can be flow-cytometrically assessed by the triple fluorochrome combination of YO-PRO-1 (a membrane-impermeable DNA-binding probe) with Hoechst 33342 and Merocyanine 540 (Hallap et al., 2006). Merocyanine 540 monitors the level of scrambling of the phospholipids of the plasma membrane lipid bilayer, increasing the intensity of its fluorescence with an increase in the membrane lipid disorder. Acrosomal integrity is a prerequisite for fertilisation; during the acrosome reaction, the plasma membrane situated on the sperm head fuses with the outer membrane of the acrosome and the enzymatic content of the acrosome is released (Kierszenbaum, 2000). Acrosomal integrity is assessed using plant lectins labelled with a fluorescent agent (usually FITC), such as *Pisum sativum agglutinin* (PSA) or *Arachis hypogaea agglutinin* (PNA). Because PSA has a nonspecific binding affinity to egg yolk, PNA is the lectin of choice when evaluating sperm extended in egg yolk-containing media (Thomas et al., 1997). PNA binds to β -galactose moieties of the outer acrosomal membrane (Flesch et al., 1998). Sperm DNA fragmentation can be assessed with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay, which identify DNA strand breaks with fluorescent-labelled nucleotides (Gorczyca et al., 1993). However, TUNEL assay requires multiple steps, and the degree of DNA damage within a cell cannot be quantified, only the number of cells with DNA damage. A simpler and more reliable assay to evaluate the level of sperm DNA fragmentation by flow cytometry is the sperm chromatin structure assay (SCSA) (Evenson and Jost, 2000; Evenson, 2016). In the SCSA, the ratio between single- and double-stranded DNA in each spermatozoon following acid treatment is determined by measuring the metachromatic shift of acridine orange from green (single-stranded DNA) to red (double-stranded DNA) fluorescence.

1.4 History of artificial insemination in cattle

The history of artificial insemination (AI) in cattle has been extensively reviewed by, among others, Foote (2002), Lonergan (2018) and Moore and Hasler (2017).

The first successful AI was carried out in a dog in 1784 by Lazzaro Spallanzani (1729–1799), who had previously reported that sperm frozen in snow became motionless but remained viable after thawing. Other early reports include the first documented application of AI in humans around 1790 by John Hunter (1728–1793), and the use of AI in rabbits, dogs and horses in 1897 by Walter Heape (1855–1919). Ilya Ivanovich Ivanoff (1870–1932) developed practical methods of AI in domestic farm animals, dogs, rabbits and poultry, and was the first to successfully conduct AI in cattle. His work was essential for the establishment of an AI laboratory by the Russian Ministry of Agriculture in the early 1900s where veterinarians were trained in the technique of AI, and for the accomplishment of the first large-scale AI breeding of cows in 1931. By 1938, AI of farm animals had been practiced and developed in Russia more than in other country, with around 1.2 million cows bred by AI that year (Herman, 1981). Victor Milovanov (1904–1992) continued the work of Ivanoff and was the first to design artificial vaginas for collection of bull semen (previously, the semen was collected from sponges placed in the vagina). He published a book on “Artificial Insemination of Farm Animals” in 1938. Danish veterinarians founded the first cooperative dairy AI organization, in 1936, which established the method of rectovaginal fixation of the cervix still used nowadays, and also developed the first straws, paraffined cellophane tubes in which the semen could be packaged (Sørensen, 1940). The first US farmer-owned AI cooperative was founded in 1938 by Enos J. Perry (1891–1983).

The distribution of sperm doses was restricted by the short lifespan until the development of “extender” media. The first extender was an egg-yolk media (Phillips and Lardy, 1940), and was later improved by the addition of sodium citrate (Salisbury et al., 1941), and antibiotics (Almquist et al., 1949; Foote and Bratton, 1950). The Cornell extender (Foote and Bratton, 1950), which contained the antibiotic mixture of penicillin, streptomycin and polymyxin B, was used for many years as the standard, helping to eradicate many venereal diseases, and was later replaced by the Cornell University Extender (CUE, Foote et al., 1960). These media extended the longevity of sperm and made it possible to reduce (from > 100 million to 4 million sperm) the number of sperm needed per insemination, which helped to meet the increasing

demand for semen of popular bulls in a quickly expanding AI industry. However, it was noted that increasing dilution reduced sperm survival when semen was diluted in egg-yolk-citrate diluents. Pat Shannon (1928–) added caproic acid and catalase and reduced the volume of egg yolk from 20 to 5% to create “Caprogen”; the adverse effect of dilution was alleviated such that the number of sperm required to maintain fertility could be further reduce to 1 to 2 million per insemination and sperm could be maintained between 15 to 27°C rather than at 5°C (Shannon et al., 1984). Currently, the use of fresh sperm is mainly limited to countries such as New Zealand and Ireland, where it helps to overcome the high demand of sperm from particular sires during the peak of the breeding season. Although Carpogen is the industry standard for fresh sperm extension, other extenders are being used (e.g., INRA96, Murphy et al., 2017).

Despite being stored in adequate extenders, fresh sperm has a limited shelf life and its fertility is only maintained stable when used within 3 days post-collection (Vishwanath and Shannon, 2000). Therefore, a more “permanent” preservation technique was needed. In 1949, Chris Polge (1926–2006) and colleagues discovered that the addition of glycerol to yolk-citrate extenders made it possible to successfully freeze sperm, and, soon, the first calf after AI with frozen sperm was born (Stewart, 1951). Since then, numerous extenders containing glycerol have been investigated and adopted by the AI industry. While milk-glycerol media (O'Dell and Almquist, 1957) are still used by some AI companies, the most commonly used media worldwide for cryopreservation of bull sperm are tris-buffered egg yolk-glycerol media (Foote, 2002). Based on the design of Sørensen's straw, Robert Cassou (1914–2015), the founder of IMV Technologies, created plastic straws that could be sealed (the “French straw”) and a gun for insemination (Pickett and Berndtson, 1974), which were quickly adopted, being more practical than the glass ampules previously used. Liquid nitrogen (–196°C) started to be used in the 1950s to preserve frozen sperm following demonstration that some biologic changes occurred within solid carbon dioxide (–79°C). At that time, insulation of the liquid nitrogen containers was inefficient and frequent refilling was required; private funding allowed the development of liquid nitrogen containers with improved insulation (Foote, 2002). Since around 40 to 50% of the sperm do not survive the freezing process, doses of frozen sperm contain more sperm (typically 15-20 million) than fresh doses. Over the years, different strategies have been evaluated to optimize the cooling curve and reduce its impact on sperm survival; currently, programmable units that permit the selection of the desired cooling and freezing curve

during sperm cryopreservation can be purchased (Dias et al., 2018). The development of techniques of sperm cryopreservation was fundamental for the expansion of the AI industry. The last published world survey on AI in cattle (Thibier and Wagner, 2002), conducted in 1998, indicated that approximately a fifth of the breedable female population in the world was bred by AI, and that almost 264 million of sperm doses were produced annually worldwide, with 95% of them being processed as a frozen doses.

1.5 Measurements of fertility

In broad terms, fertility is defined as the ability to produce viable offspring. Therefore, the most accurate measurement of success after insemination is calving rate. However, there are other fertility-related endpoints between the time of insemination and birth that can be used to define rates of success after insemination, such as fertilisation rate, non-return (to oestrus) rate (NRR), and conception rate (or pregnancy rate per AI).

Fertilisation rate infers something about the ability of sperm to fertilise the oocyte and the consequent formation of a zygote. Fertilisation rates can be measured *in vitro* within 48 h post insemination by evaluating extrusion of the second polar body, pronuclei formation, and/or the first mitotic cell division. The recovery of unfertilised oocytes and embryos after uterine flushing on Day 6 or 7 post insemination can be used to measure *in vivo* fertilisation rates. When high-fertility semen is used, fertilisation rates are generally > 90% in heifers, beef cows and moderate yielding dairy cows (Diskin and Morris, 2008), and > 80% in high yielding dairy cows (Sartori et al., 2009). However, calving rates are markedly lower (between 40% and 55%; Diskin and Morris, 2008) than fertilisation rates due to pregnancy loss after a successful fertilisation. Pregnancy loss generally decreases as pregnancy progresses and is much lower after Day 60 of pregnancy (Wiltbank et al., 2016). Most embryonic deaths (~80%) occur within the first three weeks following insemination (Santos et al., 2004; Berg et al., 2010), and are classified as early embryonic loss. In many such cases, the cow may never have even realised she was pregnant as there is little evidence of a difference in the endometrial transcriptome due to the presence of the conceptus prior to Day 15/16 (Forde et al., 2011; Bauersachs et al., 2012). Moreover,

20%–50% of high-producing lactating dairy cows have already experienced pregnancy loss by Day 6-7 of gestation (Sartori et al., 2010; Wiltbank et al., 2016). Since data regarding *in vivo* fertilisation rates are difficult to collect on a large scale, and they do not account for pregnancy losses that occur subsequently, later fertility endpoints such as NRR and conception rates are preferred and more commonly used.

Non-return rates are based on the lack of a detected or recorded return to oestrus (i.e., heat) following some specified interval. Frequently studied intervals are 28 days (28-d NRR), 56 days (56-d NRR) or 90 days (90-d NRR). Therefore, it is assumed that inseminated animals will not express oestrus again if they conceive and that they will remain pregnant through at least the previous period (i.e., 28, 56 or 90 days). Although NRR data are relatively easy to collect on a large scale, their accuracy can be significantly influenced by factors not associated with conception, such as poor oestrus detection, culling, death, and breeding management decisions. Thus, NRR data are highly prone to over-estimation and are always greater than conception rates.

Conception rates are defined by the percentage of females diagnosed as pregnant by rectal palpation, ultrasonography, or hormonal assay at some specified interval after insemination (e.g., 30 or 60 days). Although the cattle industry adopted the term “conception rate”, which, by definition, would be the percentage of zygotes formed per available oocyte, the more appropriate term “pregnancy rate per insemination (P/AI)” is currently preferred. Its main limitation is that it cannot account for embryo or foetal death that might subsequently occur.

The accuracy of fertility measurements such as NRR and P/AI (i.e., their proximity to the true calving rate) increases the closer the selected time-point is to the time of calving. Hence, fertility rates at early time-points (e.g., 28-d NRR, 30-d P/AI) are less accurate than those measured at later time-points (e.g., 56-d NRR, 60-d P/AI). Consequently, differences between treatments, if they exist, may become more evident as time from conception increases. A clear example of this phenomenon is illustrated by the results of Heyman et al. (2002), where differences in pregnancy rates after transfer of bovine adult or foetal somatic clones, embryo clones, or in-vitro produced (IVP) embryos, were increasingly more apparent as pregnancy progressed (Figure 1.1). Similarly, increased differences in NRR with time post AI have been documented during the analysis of differences in fertility between bulls (Humblot et al., 1991).

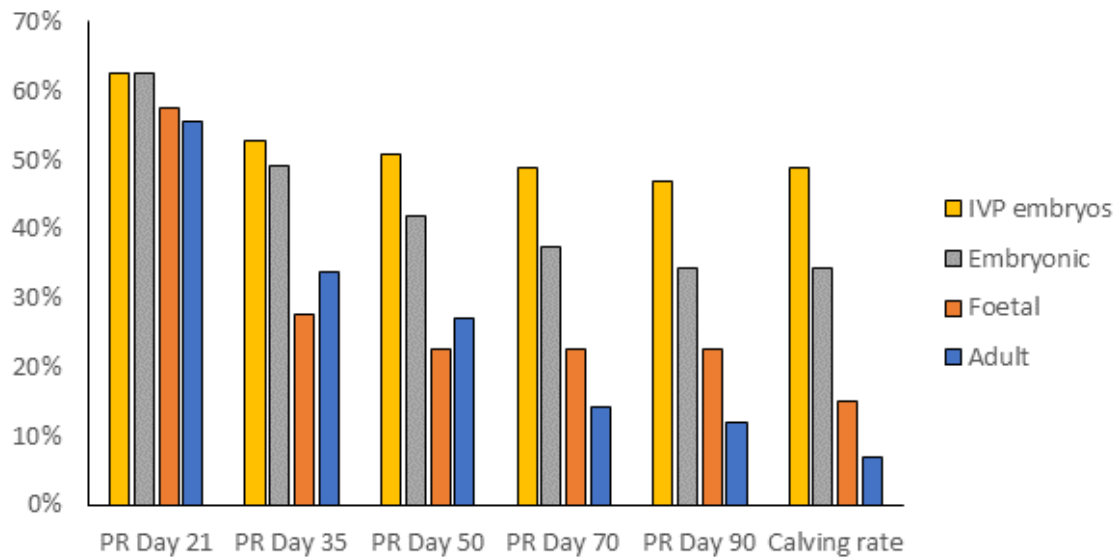


Figure 1.1. Pregnancy rates (PR) of heifers after transfer of adult somatic clones, foetal somatic clones, embryo clones, or in-vitro produced (IVP) embryos. Adapted from Heyman et al. (2002).

1.6 Time of insemination in relation to oestrus and ovulation

The objective of an insemination is to provide an adequate reservoir of competent, capacitated, motile sperm in the oviductal isthmus at the time of ovulation to ensure the highest possible fertilisation rate (Diskin, 2018). In cows, the mean interval from oestrus onset to ovulation time is estimated to be approximately 28 h, with a considerable variation (standard deviations of 5-8 h) around this mean value: 27.6 ± 5.4 h (Walker et al., 1996), 26.4 ± 5.3 h (Roelofs et al., 2005), 28.7 ± 8.1 h (Valenza et al., 2012), and 27.9 ± 7.7 h (Randi et al., 2018). The estimated mean lifespan of the sperm in the bovine female tract is ~30 h (Trimberger and Davis, 1943; Laing, 1945). However, significant variation around this average is expected because an inseminate contains a heterogenous population of sperm, and sperm lifespan can also be influenced by the uterine environment (Diskin, 2018). Although ovulated bovine oocytes have been shown to be fertilised up to 24 h after ovulation (i.e., fertilisable lifespan) (Thibault et al., 1967), their lifespan during which fertilisation results in a viable embryo (i.e., functional lifespan) is estimated to be between 8 to 12 h after ovulation (Laing, 1945; Brackett et al., 1980; Hunter and Wilmot, 1983).

The time of AI relative to ovulation has an effect on P/AI. Early studies found that maximum P/AI were achieved when cows were inseminated from midway to the end of standing oestrus (Werner et al., 1939; Trimberger and Davis, 1943; Trimberger, 1944; Barrett and Casida, 1946; Aschbacher et al., 1956). The results of these studies led to the “a.m./p.m.” rule, which dictates that cows seen in oestrus during the morning (*ante meridiem*; a.m.) should be inseminated that afternoon (*post meridiem*; p.m.), and cows showing oestrus behaviour during the afternoon (p.m.) should be inseminated the following morning (a.m.). Since then, some studies have reported similar P/AI when AI was conducted at the onset of oestrus vs. after 12 h (Foote, 1979; Gwazdauskas et al., 1986), and others have questioned the benefit of the a.m./p.m. rule compared to a once-daily AI protocol (Foote, 1979; Gwazdauskas et al., 1981; Gonzalez et al., 1985; Nebel et al., 1994; Lynch et al., 2010). Dransfield et al. (1998) used a 24-h surveillance system to monitor behavioural events associated with oestrus, and observed that the highest P/AI occurred between 4 and 12 h after the onset of standing activity. Pursley et al. (1998) observed similar P/AI and calving rates in lactating cows inseminated from 0 to 24 h after the second GnRH injection of an Ovosynch protocol (administration of GnRH followed 7 d later with PGF2 α followed 2 d later with a second GnRH; ovulation occurs 24 to 32 h after that second GnRH), but lower P/AI and calving rates, and greater pregnancy losses with AI after ovulation. Pursley et al. (1998) suggested that aging of the ovulated oocyte was probably the major contributor to the lower fertility rates and higher pregnancy loss when AI was conducted after ovulation. Dalton et al. (2001) evaluated the effect of the interval between oestrus onset and AI (0, 12, or 24 h) on the number of accessory sperm per oocyte, fertilisation rates and embryo quality in single ovulating dairy cows. Accessory sperm are those sperm trapped in the zona pellucida by the zona reaction after penetration of the fertilising spermatozoon, reflecting the number of sperm competing for fertilisation (Saacke et al., 1998). Dalton et al. (2001) observed that, although greatest median accessory sperm numbers and fertilisation rates were achieved when AI was conducted 24 h after oestrus onset, embryo quality declined with increasing intervals after onset of oestrus. They concluded that conducting AI 12 h after onset of oestrus provided a compromise in terms of maximising fertilisation and embryo survival rates (Dalton et al., 2001). However, determination of the exact time of oestrus onset is rarely conducted outside research studies. Moreover, particularly in Ireland, commercial farms usually employ AI technicians who conduct AI once per day at a similar time each day.

1.7 Sex determination in the cattle offspring

In most mammals, somatic cells are diploid, having two homologous copies of each chromosome, one of parental origin and the other of maternal origin. For instance, in domestic cattle, each somatic cell carries 30 pairs of chromosomes: 29 pairs of autosomes and one pair of sex chromosomes (Popescu, 1990). The pair of sex chromosomes determine the sex of the animal: somatic cells in females carry two X-chromosomes (XX), and somatic cells in males carry one X-chromosome and one Y-chromosome (XY). Since gametes (i.e., sperm and oocytes) are haploid cells, they carry only one sex chromosome; consequently, females produce oocytes that all carry an X-chromosome, whereas males produce sperm that carry an X- or a Y-chromosome. Therefore, the sex of the offspring is determined by the sex chromosome carried by the sperm that successfully fertilises the oocyte.

Approximately equal numbers of each type of sperm (X- and Y-sperm) are contained in a bull ejaculate (Garner et al., 1983; Di Berardino et al., 2004; Habermann et al., 2005; Parati et al., 2006) and, as consequence, in a straw of conventional sperm. Thus, approximately the same number of females and males are expected to be born (ratio 50:50) after natural mating or AI with conventional straws. In fact, the use of AI slightly increases the probability of producing a male calf compared to natural mating (Berry and Cromie, 2007), as does the use of frozen sperm (Xu et al., 2000). In contrast, a straw of sex-sorted sperm is enriched in one of the two sperm populations, containing around 90% of X- or Y-sperm. After insemination with X-sorted sperm, 90% of the offspring are females, while only 10% are males.

1.8 History of sex-sorting technology

The history of sex-sorting technology has been reviewed by, among others, Garner and Seidel (2008a), Seidel (2014), and Vishwanath and Moreno (2018).

In the early 1900s, independent experiments conducted in invertebrates by Nettie Stevens (1861–1912) and Edmund Wilson (1856–1939) lead to the discovery of the chromosomal XY sex-determination system. Sex chromosomes were observed in mammals as early as 1910 (Guyer, 1910). In mammals, the X-chromosome is larger than the Y-chromosome. Moruzzi (1979) used these differences in length to estimate

the difference in chromatin content between X- and Y-chromosome bearing sperm of 524 species of mammal. After a more exact measurement of axial chromatid length for 100 karyotypes, the author found 24 species in which the difference between X and Y chromosomes was greater than 6.2%, and suggested that such species would be the best for attempts to separate the X- and Y-bearing sperm (Moruzzi, 1979). Otto et al. (1979) measured the relative DNA content of human sperm and distinguished the two sperm populations (X- and Y-sperm) using a coaxial flow cytometer. Pinkel et al. (1982a) distinguished for the first time the two sperm populations in mice, bull and rabbit sperm, and overcame optical difficulties in fluorescence measurement due to the flat and highly refractive sperm head by using an orthogonal flow cytometer that hydrodynamically controlled the orientation of the sperm in the flow axis. Mean differences in DNA content of the X- and Y-sperm of around 4% were reported for bull sperm of different breeds (Holstein, Hereford, Jersey, Angus, and Brahman) by Garner et al. (1983). The X- and O-sperm of the creeping vole *Microtus oregoni* (Pinkel et al., 1982b), which have a 9% DNA content difference, were the first to be sex-sorted. However, in these first attempts of sperm sorting, sperm were killed in the process of making them permeable to the membrane impermeant fluorescent dye, 40-6-diamindino-2-phenylindole (DAPI). Johnson et al. (1987) replaced DAPI with Hoechst 33342, a membrane permeant bisbenzimidazole fluorescent dye, so the permeabilization was not necessary anymore and sperm remained viable after sorting. Subsequently, the first offspring were born after surgical insemination of rabbits with sex-sorted sperm (Johnson et al., 1989). Offspring from sex-sorted sperm were also obtained in swine after surgical insemination (Johnson, 1991).

The sperm sorting technology was then patented by the USDA Beltsville Research Center group led by Larry Johnson (US Patent #692958, 04/26/ 1991). Initially, sex-sorting was very slow (around 400,000 sperm/h), with IVF being used to overcome the limitation in sperm number. The first calves were obtained after transferring fresh (Cran et al., 1993) and frozen embryos (Cran et al., 1995) resulting from IVF of oocytes with sex-sorted sperm. Seidel et al. (1996) inseminated 29 oestrous-synchronized heifers with fresh sex-sorted sperm; 41% were pregnant 8 weeks after insemination. Based on the encouraging results of that trial, USDA granted a license to the Colorado State University Research Foundation (CSURF) to begin with the commercialization of the Beltsville Sperm Sexing Technology, and the company XY Inc. was formed. The original sperm sorter developed in Beltsville was improved

over the years with several modifications and sorting rates were increased to at least 6 million sperm per h for each population at 90% accuracy (Johnson and Welch, 1999). For example, a more efficient sperm orientation system was implemented (Rens et al., 1998), and Hoechst was “quenched” with propidium iodide (later substituted by red food dye FD&C40 to avoid potential mutagenic effects of propidium iodide) so sperm with damaged membranes could be discarded (Johnson et al., 1994). Sex-sorted sperm was successfully cryopreserved using conventional cryopreservation methods (Schenk et al., 1999a), and numerous field trials were conducted to determine the commercial potential of cryopreserved sex-sorted bovine sperm (Amann, 1999; Johnson and Seidel, 1999; Seidel, 1999; Seidel et al., 1999). The first commercial license for AI was granted by XY Inc. to Cogent in the United Kingdom in 2002 (Seidel, 2014). In 2007, XY Inc. was sold to Sexing Technologies (Seidel, 2014), who greatly broadened commercialization and have continued to improve sex-sorted sperm processing. The XY technology used previously (Johnson and Welch, 1999; Schenk et al., 1999b) was modified and evolved into the SexedULTRA™ technology; this new technology was globally introduced in 2013 (Vishwanath and Moreno, 2018).

Most of the intellectual property associated with sex-sorted sperm as currently used commercially is owned and controlled by Sexing Technologies or related companies. Currently, sort speeds of > 9000 cells/s can be achieved for each sperm population, and multiple head sorters, such as Genesis III, are simultaneously used to increase productivity (Vishwanath and Moreno, 2018). An analysis of the long-term usage of sex-sorted sperm in the USA showed an increase in usage in heifers from 9.4% in 2007 to 30.7% in 2015, and in cows from 0.2% in 2007 to 1% in 2015 (Hutchison and Bickhart, 2016). Sexing Technologies introduced to the market SexedULTRA-4M™ in 2017; this new product contains 4 million sex-sorted sperm per straw instead of the traditional 2 million dose. Also, in 2017, Genus ABS launched a competitor product on the market, Sexcel™, where sex-sorted sperm are obtained by a laser-based method of cell destruction of the unwanted X- or Y-sperm (Faust et al., 2016a; Faust et al., 2016b; Perry et al., 2018).

Major milestones in the development of AI and sex-sorting technology in cattle are summarised in Table 1.2.

Table 1.1. Major milestones in development of AI and sex-sorting technology in cattle. Modified from Lonergan (2018).

Year	Event
1776	Spallanzani reports that sperm frozen in snow retained viability.
1784	Spallanzani reports the first successful AI (in the dog).
1790	Hunter reports the first AI in humans.
1897	Heape describes AI in rabbits, dogs, horses.
1900	Ivanov is hired by the Russian throne to develop AI for horses. By 1933 he had developed methods for collecting semen and inseminating horses, cattle, sheep, and swine.
1910	Sex chromosomes are observed in mammals.
1931	The first large-scale AI breeding of cows is conducted in Russia.
1933	Walton publishes 'The Technique of Artificial Insemination'.
1936	Sorenson and Gylling-Holm establish the first AI cooperative, in Denmark.
1937	Danish veterinarians developed the rectovaginal/cervical fixation method of AI.
1938	Milanov develops the artificial vagina for semen collection.
1938	Perry establishes the first dairy cooperative in U.S.
1939	Phillips and Lardy discover that egg yolk protects sperm cells against cold shock.
1941	Salisbury improves extender media by buffering the egg yolk with sodium citrate.
1943	Trimberger and Davies describe the relationship between oestrus and ovulation and timing of insemination to optimize fertility.
1945	Perry publishes 'The Artificial Insemination of Farm Animals'.
1949	Polge, Smith and Parkes discover the cryoprotective properties of glycerol in frozen sperm technology.
1950	Foote and Bratton develop the 'Cornell extender', which contains penicillin, streptomycin and polymyxin B.
1951	Stewart reports, in cooperation with Polge and Smith, the birth of the first calf from AI with frozen sperm (in the U.K.).
1953	Birth of 'Frosty' – the first calf from frozen sperm in the U.S.
1960	Adler develops the first technique for freezing sperm in straws using liquid nitrogen vapour.
1963	Shannon introduces 'Caprogen' as a liquid sperm extender.
1979	Moruzzi estimates the difference in chromatin content between X- and Y-chromosome bearing sperm of 524 species of mammal.
1979	Otto <i>et al.</i> measure the DNA content of human sperm by flow cytometry.
1982	Pinkel <i>et al.</i> measure the DNA content of mice, bull and rabbit sperm using flow cytometry, and sort X- and O-sperm of <i>Microtus oregoni</i> . The sex-sorted sperm is not viable, however.
1983	Garner <i>et al.</i> report mean differences in DNA content between the X- and Y-sperm measured by flow cytometry of around 4% for bull sperm of different breeds.
1989	Johnson <i>et al.</i> successfully obtain viable sperm after sex-sorting and report the first live offspring from sex-sorted sperm (in the rabbit).
1993	Cran <i>et al.</i> report the first calves from IVF of oocytes with sex-sorted sperm.
1995	XY Inc. is funded and begins the commercialization of the Beltsville Sperm Sexing Technology.
2002	The first commercial license for AI is granted by XY Inc. to Cogent (U.K.).
2007	XY Inc. is sold to Sexing Technologies.
2013	Sexing Technologies introduces the SexedULTRA™ technology.
2017	SexedULTRA-4M™ and Sexcel™ are launched to the market.

1.9 Sex-sorting process

Ejaculates are collected and examined for volume, sperm concentration and motility, and aliquots of accepted ejaculates are diluted to approximately 200×10^6 sperm per mL in a modified TALP medium (staining TALP; Schenk et al., 1999b) and incubated with Hoechst 33342 for 45 min in a water bath at 34°C. Hoechst 33342 is a live-cell dye that diffuses through the sperm membrane and emits blue fluorescence (350/ 460 nm) when selectively bounds to A–T base pairs along the minor groove of the double stranded DNA. Since there is a difference of around 4% in the amount of DNA between X- and Y-sperm in cattle (Garner et al., 1983), the two sperm populations can be distinguished by measuring the fluorescence emitted by each sperm. After removal from the water bath, TALP medium containing egg yolk is added. Stained aliquots are filtered to remove clumped sperm, media aggregates and seminal debris. Filtered aliquots are further diluted to 100×10^6 sperm per mL and stained with a food dye (FD&C40), which quench the Hoechst 33342 fluorescence in sperm with damaged membranes so that they can be removed during the sorting process by gating. Sperm are then evaluated and sorted in a fluorescence-activated cell sorter (Fig. 1.2).

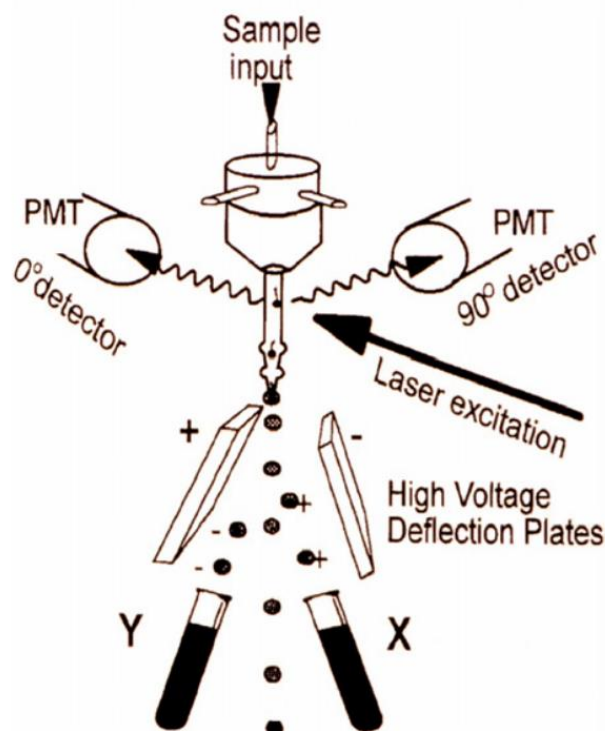


Figure 1.2. Schematic diagram of a fluorescence-activated cell sorter. From Seidel (2007).

First, sperm pass two fluorescence detectors at 90° angles to each other, and each detector measures the intensity of fluorescence emitted by the molecules of Hoechst 33342 bounded to the sperm DNA when excited by a laser. Sperm are orientated by hydrodynamic forces created by the pressurized sheath fluid (TRIS-based buffer; Garner et al., 2013) that surrounds the sample stream. Forward fluorescence measures sperm DNA content and side fluorescence measures cell orientation of sperm in the exiting stream. Then, the stream is broken off into droplets at controlled intervals by a piezo-electric crystal vibrator in such a way that each droplet ideally contains one sperm. Each droplet is positively or negatively charged according to the type of sperm that carries. The drops travel between two charged plates, one positive and one negative, and the two sperm populations are deflected into opposite streams for collection. Dead, moribund and non-oriented sperm are gated out, so the droplets contained them are uncharged and drop straight into the waste stream. Droplets without sperm or with more than one sperm are also discarded.

Sorted sperm are collected into tubes containing TALP medium with egg yolk (catching media). Tubes are slowly cooled to prevent cold shock by placing the catch tubes in beakers with water at room temperature prior to place the beaker in a 5°C cold room for 1.5 h. Then, 20% glycerol is added in two equal portions 15 min apart. Due to the mixing of the diverse media (staining, sheath, catching) used during the sorting medium, sorted sperm are highly diluted at this point (around 8×10^6 sperm per mL), and so must be concentrated by centrifugation at 850 g for 20 min. After discarding the supernatant, sperm concentration is determined, and additional extender containing 20% egg yolk and 6% glycerol is added to obtain the desired final concentration. Sperm are usually packaged into 0.25-mL straws (with a volume of 0.21 mL) at a concentration of 10×10^6 sperm per mL, which results in a dose of 2.1×10^6 sperm per straw. Straws are frozen in a programmable freezer. Sperm that accumulate every 3–4 h are frozen as a batch. Every batch undergoes quality control analysis before being released, which involves assessment of sperm motility and acrosome integrity after 3 h of incubation at 35°C and analysis of purity (i.e., proportion of the desired sperm population, typically of around 90%).

Of note, the previous description of the sorting process is based on the protocol followed before the introduction of SexedULTRA™ technology, as described by Seidel and Garner (2002) and Garner et al. (2013). Although the principles and the general

protocol followed with SexedULTRA™ technology are quite similar to those described here, Sexing Technologies has introduced some modifications, mostly in the media composition (Vishwanath and Moreno, 2018). However, since the technology and media are property of Sexing Technologies, the nature of those changes is not publicly known.

1.10 Advantages of the use of sex-sorted sperm in dairy herds

In the cattle industry, the future characteristics and fate of the offspring are dependent on the phenotypic sex. In dairy farms, heifer calves are highly desired since they are the future “milk producers”, and are used as replacements of dead or culled cows and for herd expansion purposes. In contrast, since each ejaculate can be divided into several insemination doses and used to breed a large number of cows, few bull calves are needed. For example, only 0.1% of bull calves are needed to produce sufficient sires for the US dairy industry (De Vries et al., 2008). In most countries, including Ireland, surplus male dairy calves are used in some form of calf-to-beef production system (Holden and Butler, 2018). The profit obtained when a dairy bull calf is sold is usually lower (mean of €75/head; Cottle et al., 2018) than with any other kind of calf (mean of €200/head for beef bull calves and €180/head for dairy heifer calves; Cottle et al., 2018). Therefore, it is not surprising that dairy farmers prefer heifer calves over bull calves and that sex-sorted sperm (X-sorted) is a highly attractive product with multiple potential advantages for the dairy industry.

With the use of sex-sorted sperm herd replacements and additional heifers for herd expansion can be produced at a faster rate and from a smaller proportion of the herd. Since all replacements can be generated on-farm, biosecurity is improved by eliminating the risks associated with the entrance of animals from different herds (Weigel, 2004). Bio-economic studies have demonstrated that the use of sex-sorted sperm, either fresh or frozen, can facilitate faster and more profitable herd expansion in pasture-based dairy herds (Hutchinson et al., 2013b; a; Butler et al., 2014a). Using sex-sorted sperm on heifers and genetically superior cows to generate replacements facilitates concurrent sire and dam selection, which could accelerate the rate of genetic gain (Hohenboken, 1999; Weigel, 2004; Abdel-Azim and Schnell, 2007; Khalajzadeh et al., 2012) but it is dependent on the availability of sex-sorted sperm from the highest

genetic merit sires (Butler et al., 2014a). Profit from calf sales can be increased by selling the surplus heifer calves where there is a good market for such calves (De Vries et al., 2008) or by breeding the remaining of the herd with sperm from beef sires (McCulloch et al., 2013). Alternatively, after generating the required number of replacements using sex-sorted sperm, short gestation sires could be used on repeat breeders and at first AI in late-calving cows to shorten the calving interval and condensed the calving period (Butler et al., 2014a).

The use of sex-sorted sperm reduces the proportion of unwanted male dairy calves, which may be subjected to poorer management practices than female calves (Renaud et al., 2017; Shivley et al., 2019) because their low economic value does not incentivise good husbandry, and have the potential to become a major animal welfare issue (von Keyserlingk and Weary, 2017; Holden and Butler, 2018; Ritter et al., 2019). In addition, as fewer male calves are born, the incidence of dystocia could be reduced, as well as its negative impact in cow health and subsequent return to maximum fertility potential (Holden and Butler, 2018).

1.11 Fertility of sex-sorted sperm

Early studies indicated that insemination of heifers with sex-sorted sperm resulted in lower conception rates than with conventional sperm (Seidel et al., 1999). The impaired fertility of sex-sorted sperm was later confirmed by several large field studies, where conception rates achieved after AI of dairy heifers and cows with frozen sex-sorted sperm at 2×10^6 sperm per dose were $\leq 81\%$ of those achieved with frozen conventional sperm at 15 to 20×10^6 sperm per dose (DeJarnette et al., 2009; Chebel et al., 2010; DeJarnette et al., 2010; Norman et al., 2010; DeJarnette et al., 2011; Sales et al., 2011; Healy et al., 2013; Djedović et al., 2016). Superovulated heifers and cows inseminated with sex-sorted sperm had lower fertilisation rates and embryo quality than those inseminated with conventional (Sartori et al., 2004; Schenk et al., 2006; Larson et al., 2010). As sex-sorted straws are an expensive product, recommendations were traditionally provided to limit their use to first or second service of virgin heifers in standing oestrus, and to avoid the use of sex-sorted straws on animals with lower fertility *per se*, such as lactating cows, in fixed time AI protocols or with problem breeders.

Fresh sperm is mostly used in countries with seasonal pasture-based dairy production systems (e.g., Ireland, New Zealand), where it is used to accommodate the high semen demand during the peak breeding season (Murphy et al., 2017). One study in New Zealand (Xu, 2014) reported non-return rates at 24 d after AI in dairy cows inseminated with fresh sex-sorted sperm (1×10^6 sperm per straw) over three seasons (2011, 2012 and 2013) that were 94.3% (SS = 69.4% vs. CONV = 73.6%), 94.2% (SS = 68.1% vs. CONV = 72.3%) and 95.1% (SS = 69.9% vs. CONV = 73.4%), respectively, of those achieved with fresh conventional sperm (1.25, 1.75, or 2×10^6 sperm per straw to be used on d 1, 2, or 3 after collection, respectively).

The reduced fertility of frozen sex-sorted sperm has been mainly attributed to a combination of two factors: the low sperm dose packaged in sex-sorted straws, and sperm damage due to the sex-sorting and freezing processes.

1.11.1 Low sperm dose

The inefficiency of the sex-sorting process is the main reason why a low sperm dose is typically used. During sex-sorting process, a high percent of sperm is lost or discarded at each step (e.g., sperm discarded due to coincidence in the same droplet, sperm in supernatant after centrifugation, sperm used for quality controls, dead sperm, non-oriented sperm, sperm with DNA content within the overlap or in the extremes of the X- and Y-populations,...). At the end of the process, only approximately 20% of the initial sperm population is successfully sorted and frozen, half of each sex (10% X-sperm and 10% Y-sperm) (Seidel and Garner, 2002). To deal with this inefficiency and make sex-sorted sperm economically viable, which essentially means producing a similar number of straws per volume of semen than with semen processed under conventional conditions, frozen sex-sorted sperm is routinely packaged in a lower sperm dose (2 to 4×10^6 sperm per straw) than frozen conventional sperm (15 to 20×10^6 sperm per straw) (Vishwanath and Moreno, 2018).

When frozen-thawed conventional sperm is used, the number of sperm needed to achieve 95% of the maximum conception rate value of most Holstein sires used in AI ranged from 1 to 11×10^6 sperm per dose (Den Daas et al., 1998). Therefore, AI companies typically use excessive sperm numbers in each straw (15 to 20×10^6 sperm) to protect against the risk of reduced fertility due to individual bulls with

compensable defects (Fair and Lonergan, 2018). However, sex-sorted straws contain only 2 to 4 x 10⁶ sperm per straw (Vishwanath and Moreno, 2018). As mentioned previously, during their journey through the female tract, sperm encounter a series of barriers that act as selection steps and reduce the number of sperm that finally reach the oviduct (Miller, 2018). Lower sperm cell number per dose can negatively impact fertility (Den Daas et al., 1998; DeJarnette et al., 2008). The low sperm dose in sex-sorted straws may not be enough to compensate this reduction on sperm numbers for certain bulls.

1.11.2 Sperm damage

Sex-sorting is a highly invasive procedure that includes many steps that challenge the sperm and can potentially cause sperm alterations and compromise their functionality (Vishwanath and Moreno, 2018). In addition, during the freezing-thawing processes, sperm suffer alterations to the membrane structure-function and cell metabolism (Hammerstedt et al., 1990) and, consequently, a large proportion of sperm do not survive; furthermore, the fertility and lifespan of the surviving sperm is reduced. In a study evaluating the effects of low sperm numbers per dose with and without sorting on bull fertility, it was estimated that, although two-thirds of the decrease in fertility when using sex-sorted sperm was due to the low sperm dose, the other third was consequence of the sorting process (Frijters et al., 2009). The role of both factors, low sperm dose and damage due to sorting, was further confirmed by DeJarnette et al. (2011) who reported that pregnancy rates of sex-sorted sperm were improved but still lower than those of conventional sperm at an equivalent sperm dose (10 × 10⁶ sperm).

Carvalho et al. (2013) reported that the process of sex-sorting can alter the sperm head shape, and some surface characteristics, such as the roughness of the plasma membrane (Carvalho et al., 2013). Moreover, it has been reported that sex-sorting induces altered motility patterns (Suh et al., 2005; Carvalho et al., 2010), plasma membrane damage and acrosome reaction (Mocé et al., 2006; Carvalho et al., 2010), membrane destabilization (Carvalho et al., 2018), and changes resembling *in vitro* capacitation (Bucci et al., 2012; Umezu et al., 2017). Moreover, sex-sorting compromised the capacity of ram sperm to remain bound to an oviductal epithelial cell

monolayer (Hollinshead et al., 2003) and of bull and boar sperm to remain bound to oviduct cells explants (Carvalho et al., 2018; Winters et al., 2018).

Embryos produced *in vitro* with sex-sorted sperm had delayed first cleavage (Bermejo-Alvarez et al., 2010) and lower blastocyst rates (Palma et al., 2008; Blondin et al., 2009; Bermejo-Alvarez et al., 2010) than those produced with conventional sperm. However, in other studies, embryos produced with sex-sorted sperm had similar cleavage and blastocyst rates than those produced with conventional sperm (Xu et al., 2009; Carvalho et al., 2010). Some studies reported abnormalities in terms of mRNA abundance or ultrastructure in embryos produced with sex-sorted sperm (Morton et al., 2007; Palma et al., 2008).

1.12 Introduction of SexedULTRA™ technology

Sexing Technologies updated the original sex-sorting technology (known as XY technology) by introducing a series of changes, mostly involving modifications to the protocol and optimization of media; this new technology is known as SexedULTRA™ (Vishwanath and Moreno, 2018). The results of an initial *in vitro* study of SexedULTRA™ fresh and frozen sperm showed that sex sorting resulted in a more functional sperm population in terms of lower levels of oxidative stress (Holden et al., 2016). However, high levels of agglutination were reported for fresh sperm, and it was suggested that the diluent used for the fresh sperm extension in that study was not the optimum for that type of sperm (Holden et al., 2016). Later studies demonstrated an improvement in both post-thaw sperm quality and *in vitro* fertility (González-Marín et al., 2016), as well as in conception rates after insemination of heifers (Lenz et al., 2016), when frozen sperm produced by the latest SexedULTRA™ technology was compared with sperm produced by XY technology. In a more recent study, González-Marín et al. (2018) reported that both fresh and frozen SexedULTRA™ sperm had lower DNA fragmentation, and equal or better *in vitro* quality (based on motility and percent sperm with intact plasma membrane and intact acrosome) than their counterpart conventional sperm at every post-incubation time point analysed.

While conception rates achieved with XY sperm were not compensable by increasing the number of sperm per straw (DeJarnette et al., 2011), greater 56-day non-return rates (Lenz et al., 2016) and conception rates (Utt et al., 2017) were

reported when heifers were inseminated with frozen SexedULTRA™ sperm at 4×10^6 sperm than when they were inseminated with the traditional 2×10^6 sperm dose. Also, for the first time, sex-sorted sperm achieved equivalent field fertility than conventional sperm (Lenz et al., 2016). Based on these promising results, Sexing Technologies launched a new product with 4×10^6 sperm per straw (SexedULTRA-4M™) in 2017 (Vishwanath and Moreno, 2018).

1.13 ICBF database and EBI

The Irish Cattle Breeding Federation (ICBF), established in 1997, is an independent non-profit organisation that provides cattle breeding information services to the Irish dairy and beef industries. The ICBF is owned by the cattle industry, with 46% of shares held by farm organizations, 18% by AI companies, 18% by milk recording companies and 18% by herdbook organizations. The ICBF board is formed by 15 people appointed by the shareholders and one person appointed by the Department of Agriculture, Food and the Marine (DAFM). Among other functions, the ICBF is charged with developing the cattle-breeding infrastructure in Ireland, providing genetic evaluation services, providing information useful for cattle-breeding decisions, and operating the national cattle breeding database (Wickham et al., 2012).

The ICBF database holds information on practically all animals registered nationally, and any available performance data relating to those animals and the herds in which they reside. Data are gathered from a variety of sources, such as farms (e.g., birth and calving dates, weights, docility, calving difficulty), DAFM (e.g., registrations, movements, deaths, exports), meat factories (e.g., slaughter date, carcass weight, conformation and fat scores), marts (e.g., sale date, price and weight), AI companies (e.g., insemination date and sire), milk recording services and milk processors (e.g., milk weight and composition), laboratories (e.g., disease test results, genotypes), genetic evaluations (e.g., breeding values, reliabilities, economic indexes), veterinarians and farm technicians (e.g., pregnancies, diseases) and herd books (e.g., Pedigree certificates). Since all information is gathered in one database, administrative duplication is avoided, and data can be analysed to provide useful management information to the farmers. For example, the ancestry, performance, and genomic

information stored in the ICBF database is used to predict the most profitable animals under Irish dairy farming conditions.

The Economic Breeding Index is a single figure profit-based index developed by ICBF and introduced in 2001 to replace the Relative Breeding Index, which was a milk production-based index (Veerkamp et al., 2002). The EBI is expressed in economic terms (€) and ranks animals according to their genetic merit (i.e., ability to produce superior offspring for a range of selected performance traits) in the context of profitable seasonal pasture-based dairy production. Irish farmers use the EBI to identify the most profitable bulls and cows for breeding dairy herd replacements. Originally composed of only 2 sub-indexes (milk production and fertility), now comprises 7 sub-indexes, each of them having different emphasis on the overall index (Table 1.2).

Table 1.2. Economic Breeding Index composition (adapted from www.icbf.com).

Sub-index (emphasis)	Trait	Economic weight (€)	Trait emphasis (%)
Milk production (32%)	Milk	-0.09	9.9
	Fat	1.04	3.5
	Protein	6.64	18.6
Fertility (35%)	Calving interval	-12.43	23.5
	Survival	12.01	11.6
Calving (10%)	Direct calving difficulty	-3.52	3.4
	Maternal calving difficulty	-1.73	1.5
	Gestation length	-7.49	4.5
	Calf mortality	-2.85	0.6
Beef (8%)	Cull cow weight	0.15	0.7
	Carcass weight	1.38	4.1
	Carcass conformation	10.32	1.7
	Carcass fat	-11.71	1.5
Maintenance (7%)	Cull cow weight	-1.65	7.0
Management (4%)	Milking time	-0.25	2.1
	Milking temperament	33.69	1.9
Health (4%)	Lameness	-54.26	0.7
	Somatic cells count	-43.49	2.4
	Mastitis	-77.10	0.9

The EBI describes the expected profitability per lactation of the progeny of an individual. For example, the progeny of an individual with an EBI of €200 will yield, on average, €50 more profit per lactation than the progeny of an individual with an EBI of

€150, providing that the progeny are in similar environments. Each prediction value is accompanied by a reliability, which is a measure of confidence surrounding the predicted genetic merit. A low reliability (e.g., 10%) indicates that the prediction of genetic merit is likely to change substantially as additional data for that trait are introduced in the database, whereas a high reliability (95%) indicates that the prediction is unlikely to change as more data are added. Evaluations are run four times per year and EBI updated as more data becomes available. The success of the EBI in the selection of favourable genetics for the Irish pasture-based system (high capability of intake of grazed pasture, positive energy balance and maintenance of adequate BCS, and high milk solids) has been confirmed by the results of recent studies (O'Sullivan et al., 2019a; O'Sullivan et al., 2019b).

1.14 The Irish pasture-based seasonal-calving system

The Republic of Ireland has a total agricultural area of 4,455,800 hectares. Of that, 92.1% (4,104,300 hectares) of the land area is dedicated to growing grass (CSO, 2018a). The mild climate in Ireland, characterised by abundant rainfall and lack of extreme temperatures, facilitates the growing of large quantities of grass and supports extended grass growing seasons. A pasture-based seasonal-calving system is used in practically every Irish dairy farm. This system differs from the year-round production system, the most common system used by dairy farmers in other countries, in two main aspects:

- 1) The diet offered to the cows, which is mainly grass-based (“pasture-based”). For instance, the daily diet at the start of the breeding season is mostly composed of grazed perennial ryegrass swards (12 to 18 kg/d), supplemented with concentrate feed (0 to 6 kg/day).
- 2) A compact calving period (“seasonal-calving”), which is achieved by limiting the inseminations to the breeding season. This aims to maximise the utilisation of grazed grass by matching the peak intake demands of the dairy herd to the flush of spring pasture growth (Dillon et al., 1995) (Fig. 1.3).

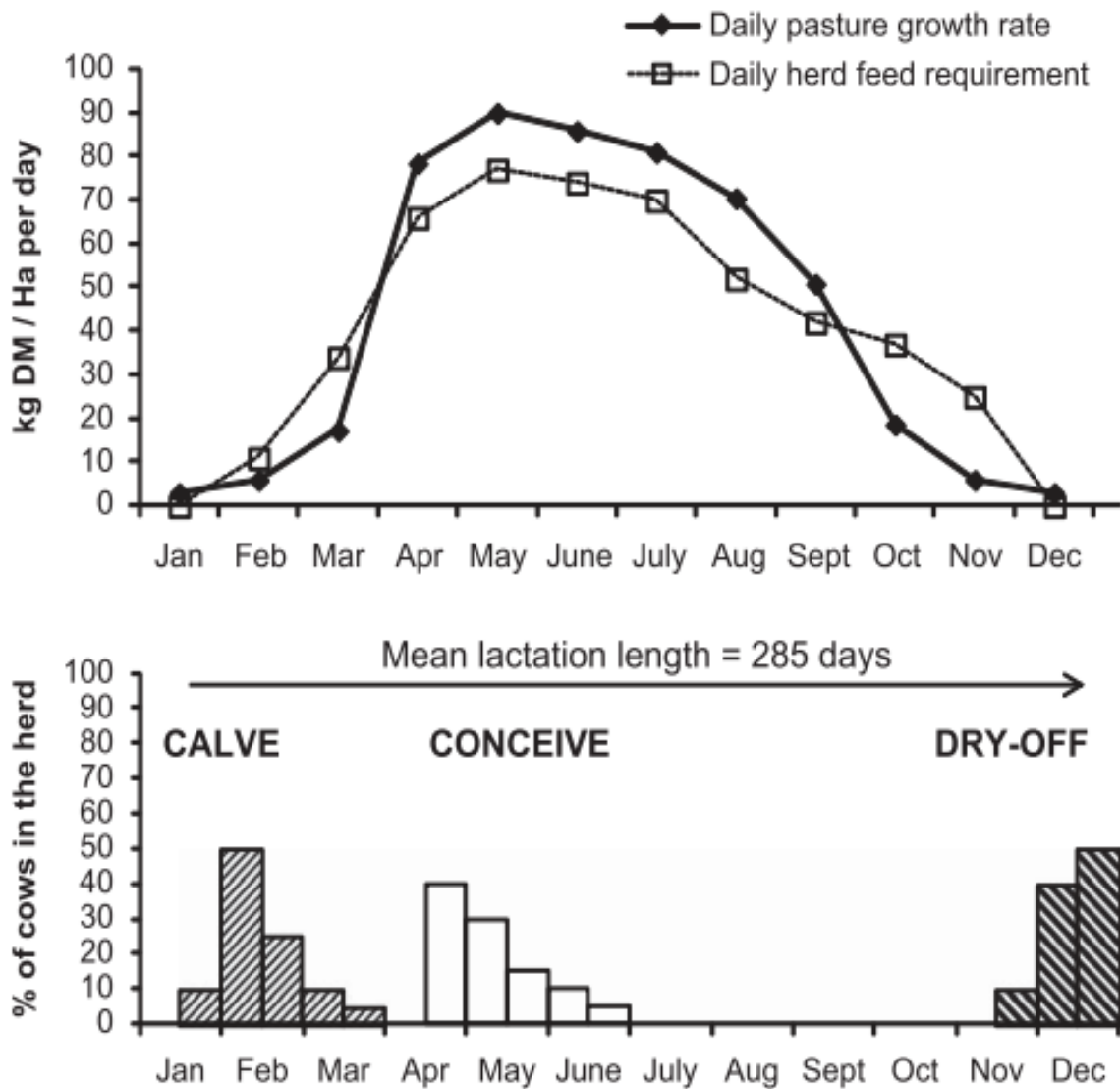


Figure 1.3. Pasture growth rate and herd feed demand (top panel), and corresponding calving, breeding and drying off periods (bottom panel) of the Irish pasture-based seasonal-calving system. From Butler (2014).

On most Irish dairy farms, the breeding season starts between mid-April and the first week of May; the mating start date (MSD) is selected so that calving the following spring coincides with planned turn-out to pasture. The breeding season ideally lasts 12 weeks (Fig. 1.4): AI is carried out during the first 6 weeks, generally after spontaneous oestrus (observed or detected with heat detection aids, such as tail paint), and then stock bulls are introduced during the following 6 weeks.

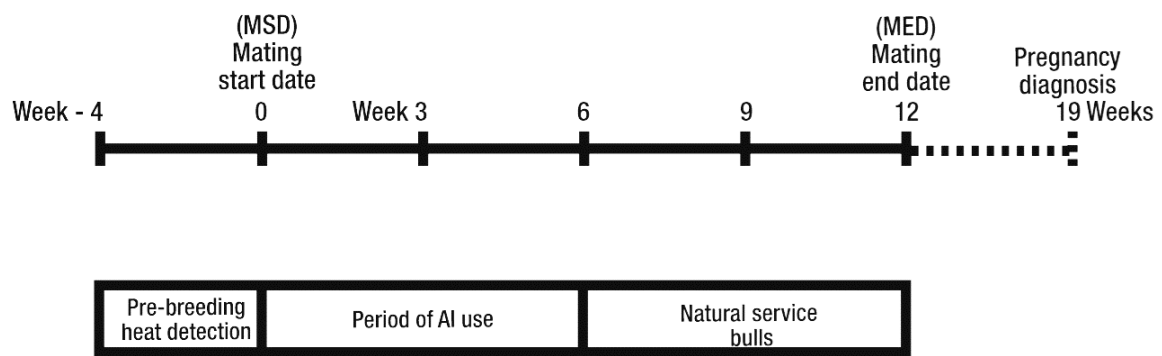


Figure 1.4. Breeding program for compact calving. From Butler (2011).

Since the length of the calving season is dictated by the length of the breeding season, in order to ensure a compact calving period, the aim is to rapidly establish pregnancy in as many cows as possible after MSD. The following breeding season targets have been recommended (Butler, 2014): a) every cow calved and $\geq 70\%$ of the cows cycling by MSD; b) $\geq 90\%$ of cows submitted for AI during the first 3 weeks of the breeding season; c) 6-week pregnancy rate of $\geq 70\%$ and 12-week pregnancy rate of $> 90\%$. Meeting these demanding targets requires excellent fertility, especially during the AI weeks. A delay in pregnancy establishment has consequences not only in the duration of the subsequent calving season and its synchrony with pasture growth, but also during the subsequent breeding seasons. Late calving cows have less time post-partum for recovery and resumption of oestrus cyclicity, being less likely to exhibit oestrus behaviour before MSD, and, less likely to establish and maintain pregnancy after AI. In addition, replacement heifers born from late calving cows may not have enough time to reach target weights at the beginning of their first breeding season and, as consequence, present poor fertility at MSD.

Table 1.3 provides national average values of selected dairy calving statistics in Ireland and their evolution from 2015 to 2019. There is a large variation between herds, however. For example, in 2019, calving interval, 6-wk calving rate, and the proportion of heifers calved between 22 to 26 months were, respectively, 363 days, 90%, and 100% in the top 5% herds, whereas they were 444 days, 25%, and 0% for the bottom 5% herds. Although ideally every cow should be calved by MSD, the calving pattern in Table 1.3 reflects that, in reality, there is still a small proportion of cows calving in May and June, thus, an overlap between calving and breeding season exists.

Table 1.3. National dairy calving statistics from ICBF database (www.icbf.com).

	2015	2016	2017	2018	2019
Calving interval (d)	391	388	390	387	390
6-wk calving rate (%)	57	59	64	64	65
Calves/cow/year	0.90	0.90	0.91	0.90	0.91
Calvings per cow	3.3	3.3	3.4	3.4	3.5
Heifers calved at 22-26 mo of age (%)	59	63	68	70	69
Calving pattern – cows calved (%) in					
January and February	44.6	46.6	45.8	47.1	45.0
March	24.2	23.4	24.9	24.1	26.1
April	13.2	12.5	12.5	12.4	12.8
May	6.3	6.2	6.0	5.8	5.9
June	3.0	2.7	2.5	2.5	2.3
Mortality – dead at birth (%)	1.79	1.83	1.63	1.62	1.30
Mortality – dead at 28 days (%)	3.89	4.04	3.61	3.88	3.20
Number of herds included	14,401	14,510	14,031	13,972	13,902

1.15 Justification and objectives of the thesis

The cattle industry plays a key role in the national economy of the Republic of Ireland. In 2016 there was 7.2 million of cattle in the Republic of Ireland (CSO, 2018a), more than the number of people living on the country (4,761,865) that same year (CSO, 2018b). Of them, 1.4 million were dairy cows. With the global population expected to increase to 9 billion by 2050, it has been predicted that the global demand for milk and meat protein will increase in the coming decades (Alexandratos and Bruinsma, 2012), thus greater numbers of dairy cows and more efficient milk and beef production from the dairy herd will be needed.

In a ten year strategy report for the Irish agri-food sector published in 2010 (Food Harvest 2020), the Irish Department of Agriculture, Food and the Marine set a target of increasing the value of primary agricultural output by 33% by 2020; the target for the dairy sector was to increase the volume of milk production (5,173 million litres in 2010; Shalloo and French, 2019) by 50% by 2020 (DAFM, 2010). A subsequent ten year strategy document, Food Wise 2025, published in 2015 predicted that Ireland would increase the value of exports by 85% to €19 billion, increase the value added to the agri-food sector by 70% to €13 billion and increase the value of primary production by 65% to €10 billion (DAFM, 2015). Prior to 2015, milk production and herd expansion in Ireland were curtailed by the EU milk quota regime. EU milk quotas were abolished on 1st April 2015. This fact, coupled with the ambitious targets set by Food Harvest 2020 and Food Wise 2025, present the opportunity of increase herd size and milk output to Irish dairy farmers.

Sex-sorted sperm reliably produces a 90% sex bias; thus, it can be used to rapidly produce high numbers of dairy heifers, and has the potential to accelerate herd expansion and increase profitability (Holden and Butler, 2018). Bio-economic modelling demonstrated that the use of sex-sorted sperm, either fresh or frozen, could facilitate faster and more profitable herd expansion in pasture-based dairy herds (Hutchinson et al., 2013b; a; Butler et al., 2014a). Therefore, it is considered as a useful technology that could help to achieve expansion of the Irish herd and milk production goals established.

Previous field studies demonstrated that frozen sex-sorted sperm generally achieved poorer field fertility when compared with conventional sperm (Butler et al.,

2014a), which limited its widespread adoption, particularly in pasture-based systems, where fertility is a key factor in determining efficiency and profitability (Verkerk, 2003; Beukes et al., 2010). However, aiming to close the gap in fertility between conventional and sex-sorted sperm, Sexing Technologies modified the sex-sorting technology used previously (XY technology, Johnson and Welch, 1999; Schenk et al., 1999b) into the newer SexedULTRA™ technology, which was introduced in 2013 (Vishwanath and Moreno, 2018). Moreover, because cryopreservation could have an additive damaging effect on the sperm already compromised by the sorting process, it has been suggested that fresh sex-sorted sperm has the potential to yield higher fertility than frozen sex-sorted sperm (Xu, 2014). Although straws of fresh sperm account for 5% of the total AI usage within the Irish dairy industry, their usage can rise to 25% during the peak breeding season from mid-April to May (Murphy et al., 2015).

Given the potentially severe impact of poor fertility in a seasonal system such as that operated in Ireland, there was a need to conduct field studies to evaluate the fertility of fresh and frozen sex-sorted sperm produced by SexedULTRA™ technology under Irish conditions. In addition, there is a real lack of data in the literature on the normalcy (viability, productivity) of the offspring produced. To this end, the objectives of these thesis were:

- 1) to investigate the phenotypic fertility performance of:
 - a) dairy heifers and lactating cows inseminated after spontaneous oestrus with fresh (1 or 2 million sperm dose) and frozen (2 million sperm dose) SexedULTRA™ sperm compared with fresh conventional sperm in seasonal-calving pasture-based dairy herds;
 - b) dairy lactating cows inseminated with frozen SexedULTRA-4M™ (4 million sperm dose) sperm compared with frozen conventional sperm in seasonal-calving pasture-based dairy herds;
- 2) to compare the survival and productivity characteristics of the offspring derived from AI with fresh and frozen sex-sorted sperm vs. conventional sperm.

CHAPTER 2: Fertility of fresh and frozen sex-sorted sperm in dairy cows and heifers in seasonal-calving pasture-based herds

C. Maicas¹, I. A. Hutchinson¹, J. Kenneally¹, J. Grant³, A. R. Cromie⁴, P. Lonergan², and S. T. Butler¹. 2019. Fertility of fresh and frozen sex-sorted semen in dairy cows and heifers in seasonal-calving pasture-based herds. *J. Dairy Sci.* 102(11):10530 - 10542.

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2.1 Abstract

The objective was to evaluate the reproductive performance of dairy heifers and cows inseminated with fresh or frozen sex-sorted sperm (SS) in seasonal-calving pasture-based dairy herds. Ejaculates of 10 Holstein-Friesian bulls were split and processed to provide: (i) fresh conventional sperm at 3×10^6 sperm per straw (CONV-3M); (ii) fresh SS at 1×10^6 sperm per straw (SS-1M); (iii) fresh SS at 2×10^6 sperm per straw (SS-2M); and (iv) frozen SS at 2×10^6 sperm per straw (SS-FROZEN). Generalized linear mixed models were used to evaluate the effect of sperm treatment and other explanatory variables on pregnancy per AI (P/AI) in heifers ($n = 3,214$) and lactating cows ($n = 5,457$). In heifers, P/AI was greater for inseminations with CONV-3M (60.9%) than with SS-FROZEN (52.8%) but did not differ from SS-1M (54.2%) or SS-2M (53.5%). Cows inseminated with CONV-3M had greater P/AI (48.0%) than cows inseminated with SS, irrespective of treatment (SS-1M, SS-2M, S-FROZEN; 37.6%, 38.9% and 40.6%, respectively). None of the SS treatments differed from each other in P/AI in either heifers or cows. The relative performance of SS compared with conventional sperm (CONV) was also examined (i.e., relative P/AI = $(\text{SS P/AI} \div \text{CONV P/AI}) \times 100$). SS-FROZEN achieved relative P/AI $> 84\%$ of those achieved with CONV-3M. Bull affected P/AI in both heifers and cows, but there was no bull by sperm treatment interaction. In heifers, P/AI increased with increasing Predicted Transmitting Ability for milk protein %. In cows, P/AI increased with increasing Economic Breeding Index (EBI) and with days in milk at AI (DIM) but decreased with increasing EBI milk sub-index, parity and with DIM². Cows in parity ≥ 5 had the lowest P/AI and differed from cows in parities 1, 2, or 3. Dispatch-to-AI interval of fresh sperm did not affect P/AI in lactating cows, but a dispatch-to-AI interval by bull interaction was detected whereby P/AI was constant for most bulls, but increased with greater dispatch-to-AI intervals for two bulls. In conclusion, frozen SS achieved P/AI relative to CONV that was greater than previously reported in lactating cows. Fresh SS did not achieve greater P/AI than frozen SS, regardless of whether the sperm dose per straw was 1 or 2×10^6 . A bull effect for all sperm treatments, as well as a dispatch-to-AI interval by bull interaction for fresh sperm, highlight the importance of using a large team of bulls for breeding management.

2.2 Introduction

Use of sex-sorted sperm (SS) increases the proportion of female dairy offspring from artificial insemination (AI), thereby enabling faster herd expansion, allows surplus breeding females to be sold for profit, facilitates greater usage of beef semen to increase the value of surplus calves sold for beef production and improves profitability (Murphy et al., 2016). In a seasonal calving system, use of SS at the start of the breeding period (e.g., first three weeks) ensures that all replacement heifers are born at the start of the following calving period. This provides advantages for block-rearing of replacement heifers and ensures that all heifers have reached target bodyweights at the time of both first mating and first calving. Additionally, fewer low-priced male dairy calves are born, with a consequent reduction in dystocia (Norman et al., 2010) and calf welfare issues (Hötzel et al., 2014).

Currently, the only commercially available methods to produce sperm straws that reliably result in a desired offspring sex bias rely on identification of differences in sperm DNA-content (Garner et al., 2013; Faust et al., 2016a). The most widely used method for producing flow cytometry-based SS results in approximately a 90% sex bias (Garner et al., 2013; Vishwanath and Moreno, 2018). The field fertility of SS is generally impaired, however; pregnancy per AI (P/AI) with frozen SS at 2×10^6 sperm per dose were $\leq 81\%$ of that achieved with conventional (i.e. non sex-sorted) sperm (CONV) (DeJarnette et al., 2009; Chebel et al., 2010; DeJarnette et al., 2010; Norman et al., 2010; DeJarnette et al., 2011; Sales et al., 2011; Healy et al., 2013; Djedović et al., 2016). The relative performance of SS compared with CONV is usually reported in studies that have evaluated sex-sorted sperm (i.e., $\text{relative P/AI} = (\text{SS P/AI} \div \text{CONV P/AI}) \times 100$), and the relative P/AI is generally similar in cows and heifers. A dose response study with increasing numbers of sperm per straw indicated that the reduced P/AI achieved with SS relative to CONV were non-compensable (DeJarnette et al., 2011).

Excellent fertility performance is an important driver of farm profitability, particularly in seasonal-calving pasture-based systems (Shalloo et al., 2014) in which the objective is to rapidly establish pregnancy in as many cows as possible to ensure a compact calving pattern is achieved during the subsequent spring calving period. Hence, one of the main barriers to adoption of SS in pasture-based systems is the unsatisfactory P/AI achieved.

Use of liquid (fresh, non-frozen) sperm has traditionally been restricted to countries with seasonal-calving pasture-based systems where inseminations are confined to a short breeding season, leading to a concentrated period of high sperm straws demand. For CONV sperm straws, fresh sperm has a distinct advantage over frozen sperm as the reduced sperm concentration per straw (approximately 3–5 vs. 15–20 $\times 10^6$ sperm, respectively; Murphy et al., 2015) allows for approximately 3 to 5 times more sperm straws to be produced per ejaculate. A limited number of studies have been carried out with fresh SS. One study in New Zealand (Xu, 2014) reported average non-return rates over three seasons (2011, 2012 and 2013) that were 94.6% of those achieved with conventional sperm (SS = 69.1% vs. CONV = 73.1%; SS/CONV = 94.6%) at 24 d after AI in dairy cows inseminated with fresh SS (1.25 to 2 $\times 10^6$ sperm per straw). Nevertheless, fresh SS is currently a niche product. With the current sperm sorting technology, sperm sorting speed is a limiting factor for the availability of fresh SS, and hence could not cater for widespread usage during a period of concentrated demand (~6 weeks) that occurs in countries with seasonal-calving systems. Hence, it is necessary to evaluate the fertility performance of both fresh and frozen sex-sorted sperm in a large controlled field trial.

Our objective was to investigate the phenotypic fertility performance of fresh and frozen SS compared with CONV in seasonal-calving pasture-based dairy herds. Ejaculates were split such that each treatment was represented in each ejaculate, and fertility performance of CONV and SS (fresh and frozen) was compared in both heifers and cows. We tested two hypotheses: (i) fresh SS would result in greater P/AI compared with frozen SS; and (ii) increasing the concentration of sperm in fresh SS straws would increase P/AI. Preliminary results based on pregnancy exams at 55 to 80 days post AI from a subsample ($n = 3,943$) of the animals used in the present study were previously published (Butler et al., 2014b; Butler et al., 2014a). Here, we present a more extensive and detailed study of the results based on data from a greater number of animals.

2.3 Material and methods

All animal work was approved by the Teagasc Animal Ethics Committee. The herds enrolled in the study were all seasonally calving pasture-based dairy herds. The cows were predominantly Holstein-Friesian breed, and some crossbred cows were also present (especially Jersey and Norwegian Red). The typical daily diet at the start of the breeding season in this system is primarily grazed perennial ryegrass swards (12 to 18 kg/d), supplemented with a variable quantity of concentrate feed depending on farm pasture availability (0 to 6 kg/day). Average cow milk production at this stage of lactation typically ranges between 1.8 to 2.2 kg milk solids (i.e., fat plus protein yield) or 25 to 28 kg milk volume per day.

2.3.1 Semen collection and processing

From April 15th to May 16th 2013, semen was collected from 18 Holstein-Friesian bulls using an artificial vagina at one of three EU approved semen collection centres: National Cattle Breeding Centre, Enfield, Co. Meath, Ireland (n = 6); Munster Cattle Breeding Group, Mallow, Co. Cork, Ireland (n = 6); Dovea Genetics, Thurles, Co. Tipperary, Ireland (n = 6). Individual bulls were collected every 3 days on a routine schedule for the duration of the 32-d collection period. Of the 18 bulls, nine bulls were identified as priority bulls, with the remaining nine bulls collected as reserve bulls for use in the event that a priority bull failed initial screening tests. Bulls were identified as priority or reserve at the time of study design, but if an individual bull was ultimately used in the study, no distinction was made between priority and back-up bulls in the data analysis. Raw ejaculates were transported in temperature-controlled boxes (18 °C) to a temporary semen laboratory operated by Sexing Technologies located on the Teagasc Moorepark Campus. Upon arrival at the laboratory, sperm concentration, subjective motility and sperm morphology of the raw (undiluted) semen were assessed. Sperm concentration was calculated using an automated cell counter (NucleoCounter; ChemoMetec A/S, Gydevang 43, 3450 Lillerød, Denmark); ejaculates containing $< 1 \times 10^9$ sperm/mL or $< 5 \times 10^9$ total sperm were rejected. Subjective motility was assessed using a microscope and a minimum of 65% motile sperm was required for processing. Sperm morphology was evaluated by differential interference contrast microscopy at 600x magnification in a 10 μ L sample of neat semen in 0.5 mL of Tyrode's albumin

lactate pyruvate buffer containing 0.2% formalin. Abnormalities related to sperm head defects were classified as primary abnormalities, and defects involving the sperm tail were classified as secondary abnormalities (Barth and Oko, 1989). Ejaculates with < 75% normal sperm morphology were rejected.

Each acceptable ejaculate was initially split into two aliquots; one of these aliquots was processed as fresh conventional sperm at 3×10^6 sperm per straw (CONV-3M), whereas the other aliquot was sex-sorted for X-chromosome bearing sperm with a ~90% purity using SexedULTRA™ sorting technology (Sexing Technologies, Navasota, TX) and following standard commercial procedures (Garner et al., 2013), and subsequently processed to provide fresh SS at 1×10^6 sperm per straw (SS-1M), fresh SS at 2×10^6 sperm per straw (SS-2M), or frozen SS at 2×10^6 sperm per straw (SS-FROZEN). Sperm aliquots were incubated with Hoechst 33342 (50.4 μ M) in a water bath at 34 °C for 45 min. Sex sorting was conducted on a high-speed flow cytometer at 40 psi with a 70- μ m nozzle, vanguard laser set at 200 mW and event rate of 20,000 to 21,000 events/s. Once sorted, SS-1M and SS-2M were processed (reconcentration, final extension and packaging in straws) at 18 °C, whereas SS-FROZEN was processed at 5 °C.

All diluents were proprietary media supplied by Sexing Technologies (Navasota, TX, USA). An optimized Tris-egg-yolk buffer containing 6% glycerol, was used as diluent for the SS-FROZEN. Fresh sperm (CONV-3M, SS-1M, SS-2M) was extended in a medium containing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and additives, which had previously been developed for use with conventional sperm.

SS-FROZEN straws were frozen in liquid nitrogen vapor at rates within documented tolerance limits (Robbins et al., 1976) before plunging them into liquid nitrogen. Fresh sperm straws (SS-1M, SS-2M and CONV-3M) were stored in temperature-controlled units (18 °C) until dispatch. All batches underwent quality control analysis before being released, including subjective assessment of sperm motility, acrosome integrity by differential interference contrast microscopy, and purity (i.e., X-chromosome bearing sperm population $\geq 87\%$; Garner et al., 2013). In addition, thermal stress tolerance test (reduction in motility of the sperm population after 3-h of incubation at 37 °C) was performed with the frozen straws and the motility of sperm in

the fresh straws was assessed 24 and 48 h after production. The semen collection schedule and processing protocol are illustrated in Fig. 2.1.

(a)

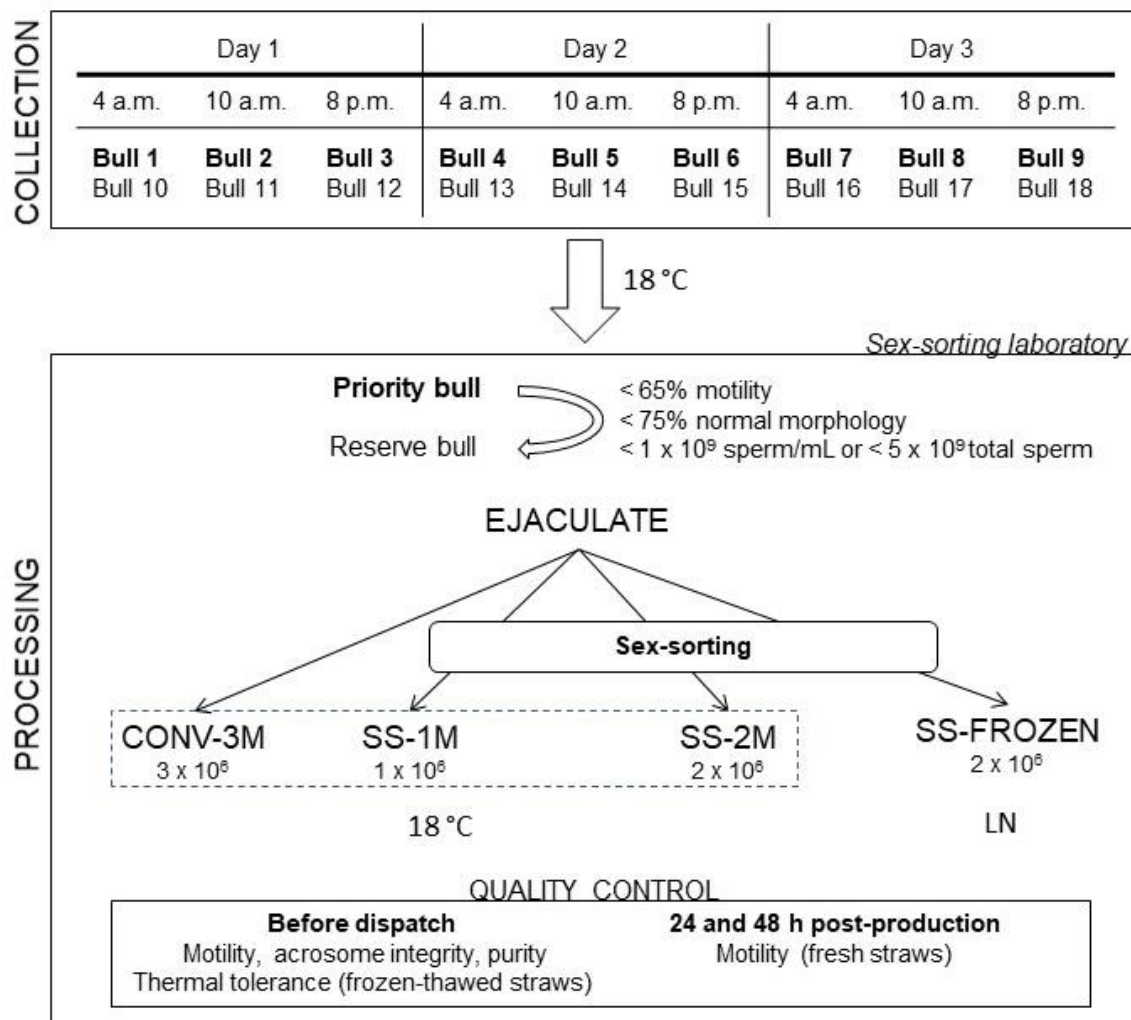


Figure 2.1. Experimental design: collection schedule (3-day cycle) and processing of the ejaculates. Quality control (QC); fresh conventional sperm (CONV-3M); fresh sex-sorted sperm at 1 x 10⁶ sperm/straw (SS-1M) or at 2 x 10⁶ sperm/straw (SS-2M); frozen sex-sorted sperm (SS-FROZEN); liquid nitrogen (LN).

2.3.2 Distribution, insemination and recording of straws

A courier network was established to distribute the sperm straws from the sorting laboratory to participating AI technicians for the duration of the trial (Fig. 2.2). Ejaculates were collected from bulls at the different studs at 20:00 (day -1), 04:00 (day 0) and 10:00 (day 0) and were delivered to the laboratory within 4 h of collection.

Ejaculates were processed and released for distribution to AI technicians at 18:00 (day 0) and inseminations were conducted starting at 07:00 (day +1) the following day and continuing until 19:00 the day after (day +2). The average time interval from semen collection to first use in the field was 35, 27 and 21 h for the first, second and third bulls on the collection schedule, respectively. The time interval from sperm dispatch to first use in the field was ~13 h. Each AI technician received an allocation of straws every second day in order to ensure balanced use of the fresh sperm on days +1 and +2. The sperm dispatch schedule ensured that every AI technician received straws from all bulls.

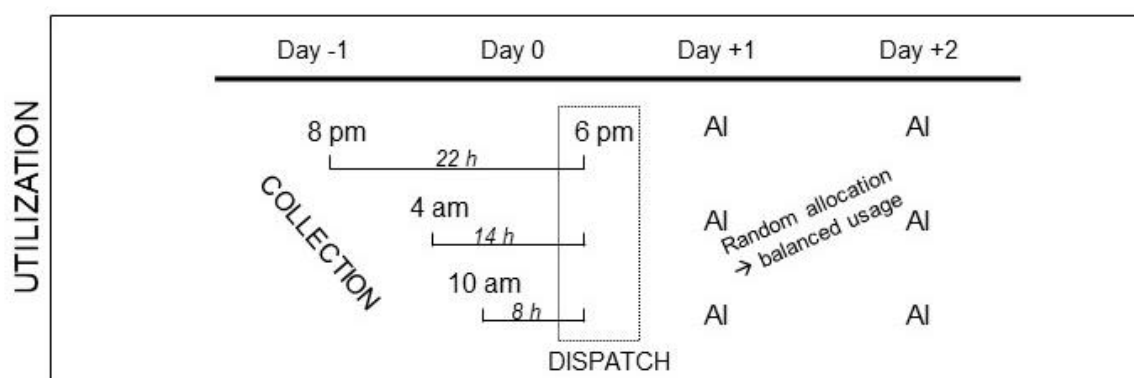


Figure 2.2. Experimental design: utilization. The day of dispatch is considered Day 0. Interval from collection to dispatch (hours) in *italics*.

A total of 110 AI technicians were involved in the study and were provided with guidelines on optimal handling techniques for both fresh and frozen SS. Inseminations were performed during a 34-day period from April 15th to May 18th, 2013. Every insemination was recorded on a handheld computer, and the information was subsequently exported to the Irish Cattle Breeding Federation (ICBF) database, which is a repository for animal records, events and performance data for all of the cattle registered in Ireland and the herds in which they reside. In total, 15,145 inseminations on 492 farms were recorded (9,153 for cows and 6,151 for heifers). At 55 to 80 days post AI, pregnancy status was determined by ultrasound scanning for 3,943 (26%) of those animals (2,160 cows and 1,783 heifers), as well as BCS (1 to 5 scale; Edmonson et al., 1989), which was recorded by one of two highly experienced personnel. Additional animal information was also retrieved from the ICBF database including calving date, parity, Economic Breeding Index (EBI) and Predicted Transmitting Ability

(PTA; i.e., average genetic value for a given trait that an animal transmits to its offspring) values for genetic traits of interest. The EBI is a single figure profit index used in Ireland to identify animals with superior genetic merit (Teagasc, 2014), and is composed of 7 sub-indexes with different weight (in parenthesis): fertility (35%), milk production (33%), calving performance (9%), beef performance (9%), cow maintenance (7%), cow management (4%), and health (3%) sub-indexes. The fertility sub-index is calculated based on calving survival (24%) and cow survival (i.e., longevity) (11%), whereas the milk production index is calculated based on milk protein kg (19%), milk kg (11%), and milk fat kg (3%).

2.3.3 Data handling

Observations with calving-to-AI intervals (i.e. DIM at AI) > 140 days were assumed to indicate cows that had calved before the current seasonal cohort (i.e., carryover cows from a previous autumn or spring calving period), and were removed from the dataset. After removing observations with missing values in the variables of interest, the data set consisted of 12,251 observations; of those, 3,222 observations included a BCS record. Only insemination events conducted on day +1 and day +2 after dispatch were retained for analysis (including frozen SS treatment). The data set was then checked for duplicates, and only cows that received a trial straw insemination at the first AI were retained. After this, the total number of observations was reduced to 11,400. The data set was subsequently constrained so that only bulls with ≥ 300 inseminations were used. There were 10 bulls remaining and the total observations were 11,274 at this point. After removing herds with < 16 inseminations (4 treatments x 4 replicates/herd), the total number of insemination records was reduced to 9,963. The data set was split into parity = 0 (heifers, n = 3,733) and parity ≥ 1 (lactating cows, n = 6,230). Pregnancy per AI (P/AI) at the first insemination using trial sperm straws was the primary outcome variable of interest. Pregnancy status was coded as 1 (i.e., successful) if either of two criteria were met: (a) cows were diagnosed pregnant following ultrasound examination; or (b) there was no record of a subsequent insemination and the interval from AI to subsequent calving was ≥ 270 and ≤ 292 d. Otherwise, pregnancy status was coded as 0 (i.e., unsuccessful).

2.3.4 Statistical analysis

Generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a binary distribution, with residual pseudo-likelihood as estimation technique and Kenward-Roger method for calculation of the degrees of freedom, were used to evaluate the effect of sperm treatment on P/AI in heifers and lactating cows. Data for heifers and lactating cows were analysed separately because some variables included in the analysis only affected cows (e.g., parity, DIM). The animal that received the insemination event (heifer or cow) was designated as the experimental unit. Sperm treatment, bull and treatment by bull interaction were always included in the model as fixed effects, and farm was included as a random effect. A number of covariates (such as EBI, EBI fertility and milk production sub-indexes; PTA for calving interval, for survival, for milk protein %, and for milk kg; parity and DIM), interactions and quadratic (i.e., squared) terms for continuous variables were tested for inclusion (backward stepwise regression) and retained as fixed effects for the final model when $P \leq 0.25$. ; The use of a relaxed P -value cut-off point is recommended (Mickey and Greenland, 1989; Sperandei, 2014) during pre-selection because it minimizes type II error and is less likely to overlook contributing covariates than lower significance levels for entry such as the traditional $P \leq 0.05$. Quadratic terms, such as DIM^2 , can be used when a curvilinear instead of linear relationship between variables (e.g., P/AI and DIM) is suspected. The inclusion of a quadratic term transforms a linear regression model into a curve without having to model a non-linear model, since the independent variable is squared but not the β -coefficient. Comparisons of least squares means (LSM) for P/AI between sperm treatments, parities, bulls and bull by sperm treatment interactions were performed using Tukey-Kramer adjustment for multiple comparisons. A significant difference or effect was considered to exist when $P < 0.05$. Results are reported as statistic $\pm$ standard error of the statistic (e.g. $\text{LSM} \pm \text{SEM}$).

2.3.4.1 Regression model for heifers

Fixed effects retained for the final heifer model were: sperm treatment, bull, sperm treatment by bull interaction, and PTA for milk protein %. Herd was included as a random effect.

2.3.4.2 Regression model for lactating cows

Fixed effects retained for the final cow model were: sperm treatment, bull, sperm treatment by bull interaction, parity (1, 2, 3, 4, > 4), DIM, DIM², EBI, EBI milk production sub-index. Herd was included as a random effect.

2.3.4.3 Dispatch-to-AI interval

An additional model was run to examine the effect that dispatch-to-AI interval had on P/AI of heifers and cows inseminated with fresh sperm (CONV-3M, SS-1M, SS-2M). The same fixed effects as in previous models for heifers (sperm treatment, bull, sperm treatment by bull interaction, and PTA for milk protein %) or cows (sperm treatment, bull, sperm treatment by bull interaction, parity, DIM, DIM², EBI, and EBI milk production sub-index) were included, with the addition of dispatch-to-AI interval and bull by dispatch-to-AI interval interaction. Herd was included as a random effect in both models.

2.3.4.4 Surface plots

Based on the data set of the lactating cows, surface plots for the probability of pregnancy (equivalent to P/AI, but expressed as a proportion) of young (parity ≤ 2) cows inseminated with fresh or frozen SS at 2×10^6 sperm per straw (SS-2M and SS-FROZEN) were generated (PROC G3D, SAS 9.4) pairing the variables BCS, DIM, and EBI fertility sub-index. Surface plots are three-dimensional graphs that show a functional relationship between a dependent variable (y-axis; probability of pregnancy), and two independent variables (x- and z-axis). For each surface plot, two independent variables were paired and plotted, and the remaining two were held at selected values that defined an elite group of cows: BCS ≥ 3, DIM > 60 d, and fertility sub-index > €100.

2.4 Results

2.4.1 Bulls

The final analysis included a total of 10 Holstein-Friesian bulls (≥ 3 yr old). Of these, 9 were initially identified as priority bulls and 1 as a reserve bull. Their average EBI in April 2013 was $\text{€}238 \pm 7$, with a reliability ranging from 51 to 89%.

2.4.2 Heifers

Of the 3,733 heifers with P/AI data, a complete dataset with all covariates of interest was available from 3,214 heifers on 199 herds for the final analysis. Sperm treatment affected ($P < 0.05$) P/AI in heifers. Heifers inseminated with CONV-3M had greater P/AI than those inseminated with SS-FROZEN, and tended ($P = 0.09$) to have greater P/AI than heifers inseminated with either of the fresh SS treatments (SS-1M or SS-2M). None of the SS treatments differed from each other in terms of P/AI (Table 2.1).

Table 2.1. Least square means (LSM) $\pm$ SEM for pregnancy per AI (P/AI) and performance of sex-sorted sperm relative to conventional (SS/CONV*100) after AI of dairy heifers with fresh conventional sperm (CONV-3M), fresh sex-sorted sperm at 1×10^6 sperm/straw (SS-1M) or at 2×10^6 sperm/straw (SS-2M), or frozen sex-sorted sperm (SS-FROZEN).

Sperm treatment	Heifers		
	N	P/AI (%)	SS/CONV (%)
CONV-3M	865	60.9 ± 2.3^a	-
SS-1M	811	54.2 ± 2.5^{ab}	88.9
SS-2M	726	53.5 ± 2.9^{ab}	87.8
SS-FROZEN	812	52.8 ± 2.5^b	86.7

^{a-b} LSM within a column with different superscripts differ ($P < 0.05$).

Bull affected P/AI in heifers ($P < 0.001$), which ranged from 31.6% to 64.3%. Bull 10 achieved the lowest P/AI and differed from all other bulls except Bull 9 (Fig. 2.3a). There was no interaction between bull and sperm treatment ($P = 0.157$). Nevertheless, LSM of P/AI for each sperm treatment within bull are plotted in Figure 2.3b; P/AI achieved did not differ ($P > 0.05$) between sperm treatments within any

individual bull. Pregnancy per AI increased ($P < 0.001$) with increasing PTA for milk protein %.

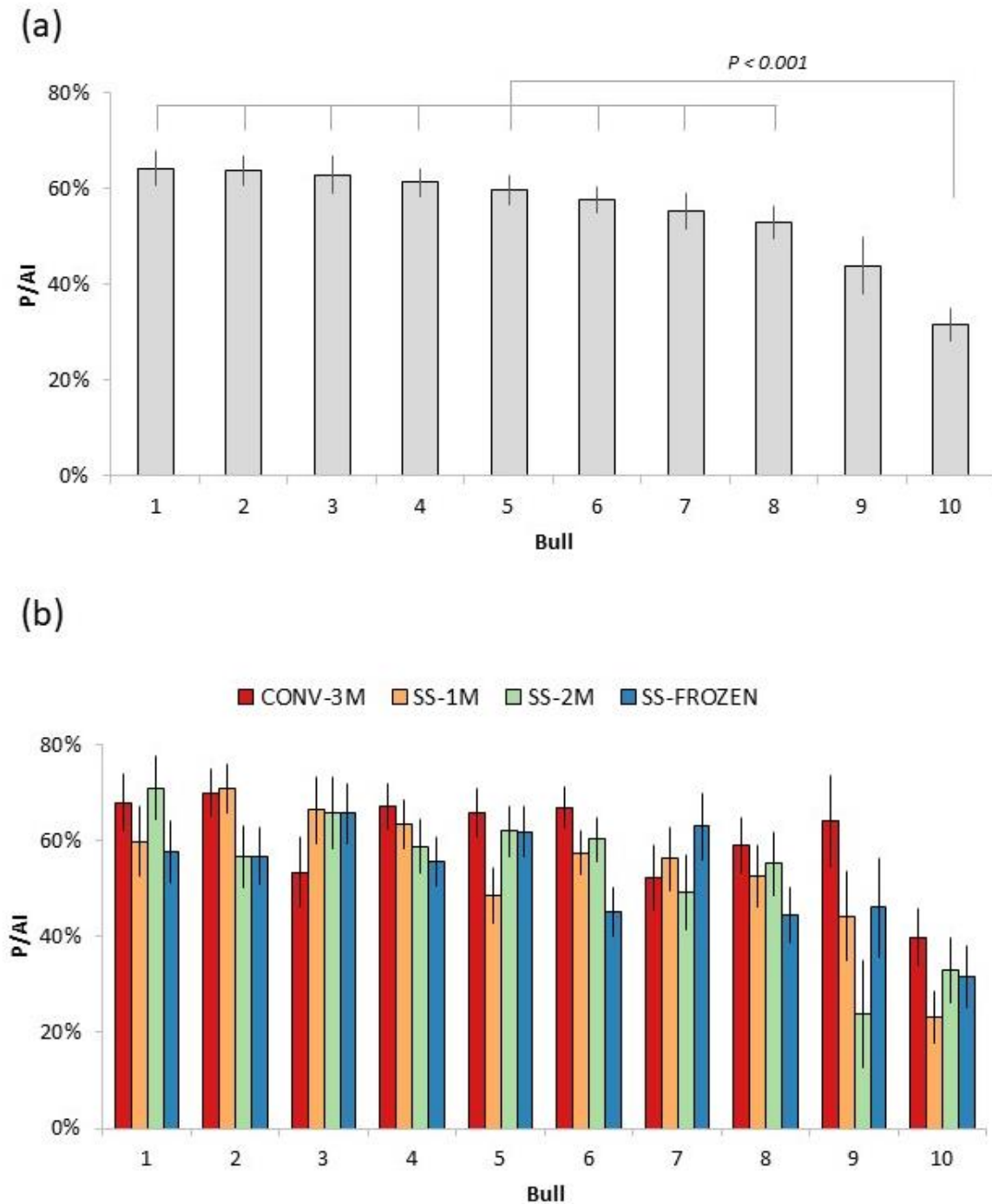


Figure 2.3. Effect of bull on pregnancy per AI (P/AI; LSM $\pm$ SEM) of heifers across the four sperm treatments (a) and by sperm treatment (b). Fresh conventional sperm (CONV-3M); fresh sex-sorted sperm at 1×10^6 sperm/straw (SS-1M); fresh sex-sorted sperm at 2×10^6 sperm/straw (SS-2M); frozen sex-sorted sperm (SS-FROZEN). In panel (b), only differences between sperm treatments within individual bulls are displayed; LSM did not differ ($P > 0.05$) between sperm treatments within any individual bull.

2.4.2.1 Effect of dispatch-to-AI interval on fresh sperm treatments

This analysis only used data from heifers inseminated with any of the fresh sperm treatments (CONV-3M, SS-1M and SS-2M; $n = 4,292$). Dispatch-to-AI interval did not affect P/AI in heifers inseminated with fresh sperm, and there was no dispatch-to-AI interval by bull interaction (both $P > 0.05$).

2.4.3 Lactating cows

Of the 6,230 cows with P/AI data, a complete dataset with all covariates of interest was available from 5,493 cows on 238 herds for the final analysis. Sperm treatment affected ($P < 0.001$) P/AI in lactating cows. Lactating cows inseminated with CONV-3M had greater P/AI than those inseminated with any of the SS treatments (SS-1M, SS-2M, S-FROZEN), but P/AI did not differ between the SS treatments (Table 2.2).

Table 2.2. Least square means (LSM) $\pm$ SEM for pregnancy per AI (P/AI) and performance of sex-sorted sperm relative to conventional (SS/CONV*100) after AI of dairy lactating cows with fresh conventional sperm (CONV-3M), fresh sex-sorted sperm at 1×10^6 sperm/straw (SS-1M) or at 2×10^6 sperm/straw (SS-2M), or frozen sex-sorted sperm (SS-FROZEN)

Sperm treatment	Cows		
	N	P/AI (%)	SS/CONV (%)
CONV-3M	1,593	48.0 ± 1.6^a	-
SS-1M	1,299	37.6 ± 1.7^b	78.4
SS-2M	1,428	38.9 ± 1.8^b	81.0
SS-FROZEN	1,173	40.6 ± 1.8^b	84.7

^{a-b} LSM within a column with different superscripts differ ($P < 0.05$).

Bull affected P/AI ($P < 0.001$) in lactating cows. For comparison purposes, bulls were numbered in order of the P/AI achieved with heifers previously illustrated in Figure 2.2 (Fig. 2.4a); Bull 10 achieved the lowest P/AI (14.4%) and was different from all the other bulls (range 39.9 to 47.9%). Of note, Bulls 4, 6 and 10 had the longest collection-to-dispatch interval (22 h). There was no bull by sperm treatment interaction ($P = 0.127$). Within bull, a significant effect of sperm treatment on P/AI was detected for one bull only (Bull 10, Fig. 2.4b), with SS-2M achieving a lower P/AI than CONV-3M (5.3 vs. 26.7%, respectively).

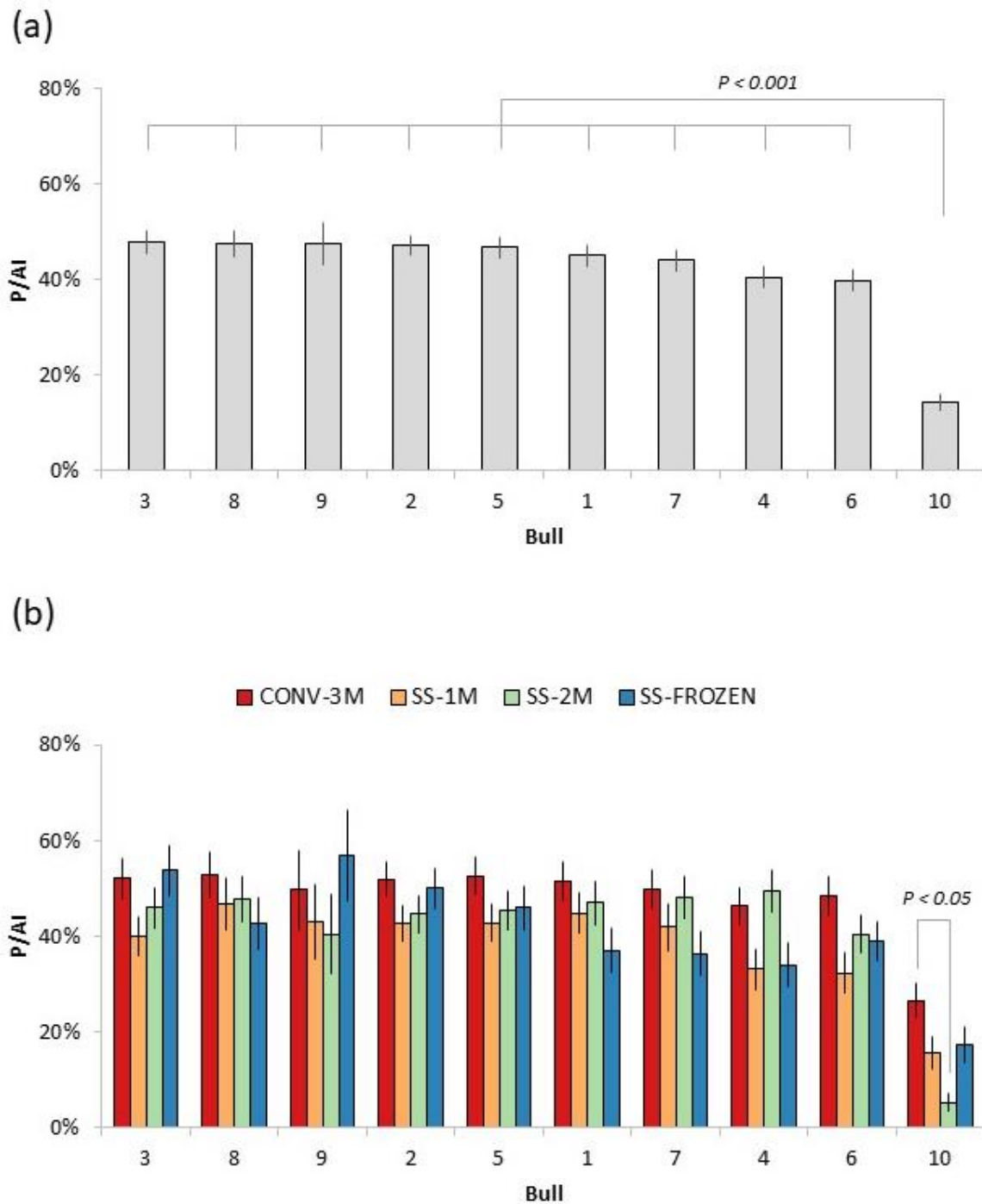


Figure 2.4. Effect of bull on pregnancy per AI (P/AI; LSM $\pm$ SEM) of lactating cows across the four sperm treatments (a) and by sperm treatment (b). Fresh conventional sperm (CONV-3M); fresh sex-sorted sperm at 1×10^6 sperm/straw (SS-1M); fresh sex-sorted sperm at 2×10^6 sperm/straw (SS-2M); frozen sex-sorted sperm (SS-FROZEN). In panel (b), only differences between sperm treatments within individual bulls are displayed; LSM only differ ($P < 0.05$) between sperm treatments (CONV vs. SS-2M) within Bull 10.

Parity influenced P/AI ($P < 0.001$); cows in parities 1, 2, and 3 had greater P/AI (45.2, 44.0, 42.8%, respectively) compared with cows in parity ≥ 5 (34.4%; all $P < 0.01$), and cows in parity 4 were intermediate (40.2%; not different from other parities). Pregnancy per AI of lactating cows increased with increasing EBI but decreased with increasing milk sub-index of the EBI (both $P < 0.001$). Pregnancy per AI of lactating cows increased with increasing DIM; however, it decreased when DIM was considered quadratic (DIM^2 ; both $P < 0.001$).

2.4.3.1 Effect of dispatch-to-AI interval on fresh sperm treatments

This analysis only used data from lactating cows inseminated with any of the fresh sperm treatments (CONV-3M, SS-1M and SS-2M; $n = 4,292$). Dispatch-to-AI interval did not affect ($P = 0.403$) P/AI of lactating cows inseminated with fresh sperm. However, a dispatch-to-AI interval by bull interaction existed ($P < 0.01$). Bulls 4 and 6 were combined (Group 1) and plotted (Fig. 2.5) against the rest of bulls also combined (Group 2): P/AI of Group 1 increased (slope = 0.021; $P < 0.001$) as dispatch-to-AI interval increased whereas P/AI of Group 2 slightly decreased although without statistical significance (slope = -0.003; $P > 0.05$).

2.4.3.2 Surface plots

This analysis used data from elite lactating cows (parity ≤ 2 and BCS, DIM, or EBI fertility sub-index held at selected values) inseminated with SS-2M or SS-FROZEN. For elite cows of BCS ≥ 3 ($n = 2,313$; Fig. 2.6a), those with a fertility sub-index $< \text{€}100$ did not reach equivalent probability of pregnancy to those whose fertility sub-index $\geq \text{€}100$. In elite cows with a fertility sub-index $> \text{€}100$ ($n = 848$; Fig. 2.6b), probability of pregnancy increased with greater BCS, especially when comparing cows of BCS < 3 to cows of BCS ≥ 3.0 , and independently of DIM. In elite cows with > 60 DIM at AI ($n = 1763$; Fig. 2.6c), probability of pregnancy was greater for those with a BCS ≥ 3.0 than for those with a BCS < 3.0 , independent of fertility sub-index. Additionally, probability of pregnancy increased with fertility sub-index for these elite when their BCS was ≤ 3 .

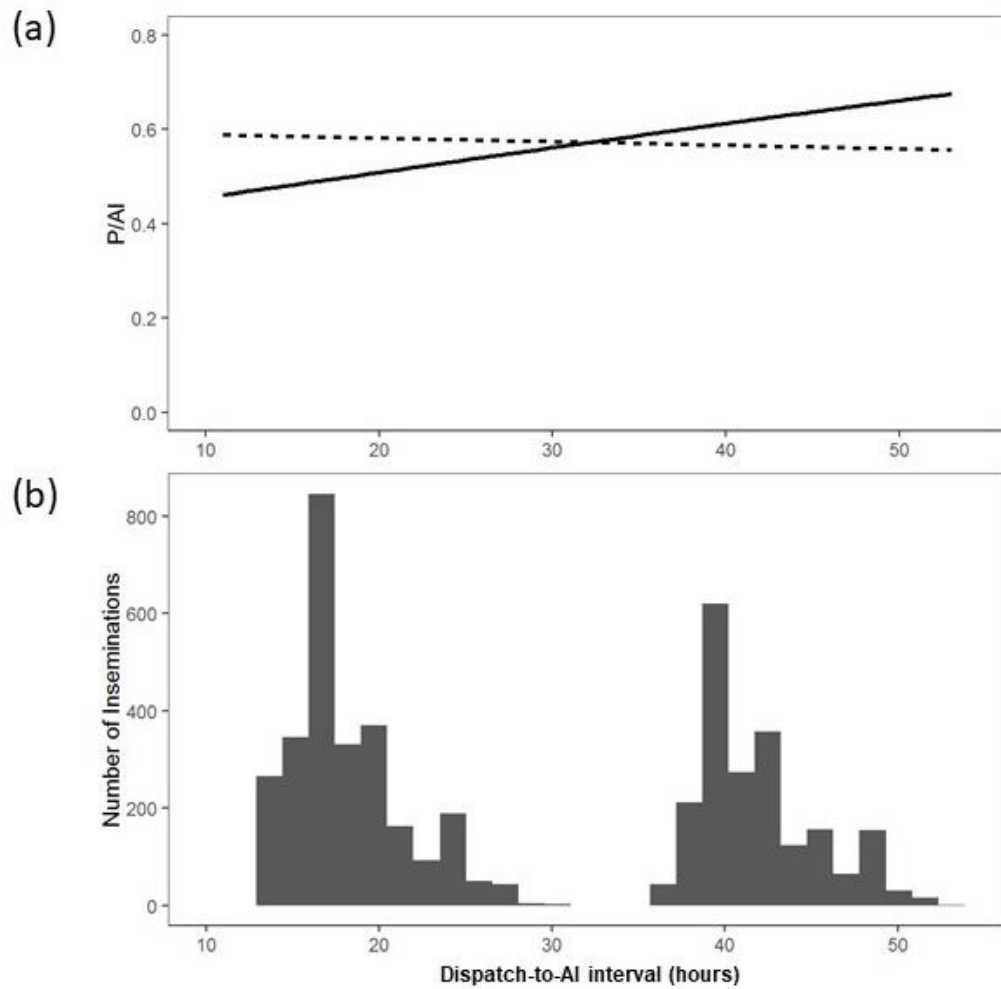


Figure 2.5. Effect of dispatch-to-AI interval on pregnancy per AI (P/AI) of lactating cows inseminated with fresh sperm treatments (CONV-3M, SS-1M, and SS-2M). (a) Bulls 4 and 6 are combined in Group 1 (solid line) whereas the rest of bulls are combined in Group 2 (dashed line); (b) histogram illustrating the time relative to dispatch when the fresh sperm was used for AI.

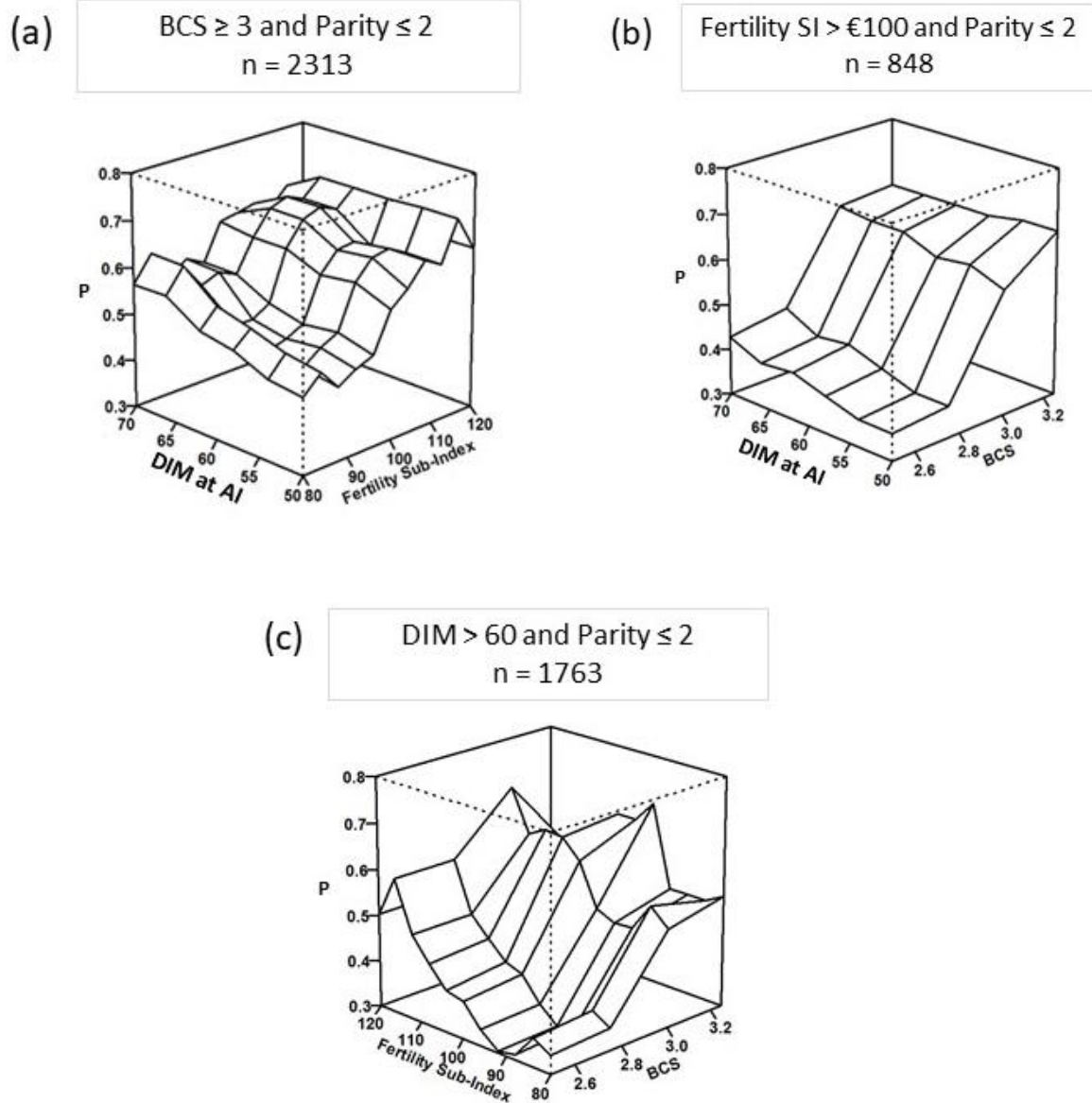


Figure 2.6. Surface plots for probability of pregnancy (P) of young lactating cows (parity ≤ 2) inseminated with SS-2M or SS-FROZEN and defined by BCS, DIM, and fertility sub-index of the Economic Breeding Index. The value for P is equivalent to pregnancy per AI (P/AI), but expressed as a proportion. The plots show possible pairings of the variables, holding the remaining two (box on the top) at selected values that define an elite group of cows: $BCS \geq 3$, $DIM > 60$ d, and Fertility Sub-Index > €100.

2.5 Discussion

This study used split ejaculates to compare P/AI achieved with different SS treatments compared with CONV in dairy heifers and lactating dairy cows. The main findings can be summarized as follows: (1) heifers inseminated with SS-FROZEN had lower P/AI than those inseminated with CONV-3M, whereas lactating cows inseminated with any of the SS treatments had lower P/AI than those inseminated with CONV-3M; 2) P/AI achieved with frozen SS did not differ from that achieved with fresh SS; 3) doubling the concentration of sperm in fresh SS straws did not improve P/AI; 4) SS achieved relative P/AI ranging from 78.4 to 88.9% of those achieved with CONV; and 5) there was a strong bull effect on P/AI achieved with SS.

There is ejaculate-to-ejaculate variation in sperm characteristics during quality assessment, as well as evidence of straw-to-straw variation within the same ejaculate (Utt, 2016; Harstine et al., 2018). This variation makes assessment of bull fertility, both *in vivo* and *in vitro*, challenging. One significant weakness in most previously published studies on SS is the fact that treatments were applied to different ejaculates. Indeed, in some studies, different bulls were used for the SS and CONV treatments (Chebel et al., 2010; Xu, 2014); in others, different ejaculates from the same bull were used (DeJarnette et al., 2009; Norman et al., 2010; DeJarnette et al., 2011). A recent trial in Germany (Lenz et al., 2016) aliquoted the ejaculate for the different SS treatments ($n = 4$), but used different contemporaneous ejaculates for the CONV treatment. To our knowledge, only a Danish field trial (Borchersen and Peacock, 2009) applied SS and CONV treatments to aliquots from the same ejaculate. In that study, however, only heifers were inseminated, and the number of inseminations was relatively low (2,087 doses in total, of which 735 were from Holstein bulls) and unbalanced between treatments (554 heifers for SS vs. 181 for conventional). Here, we used a rigorous split-ejaculate technique, in which all four treatments were applied to each ejaculate from each bull in order to mitigate against ejaculate-to-ejaculate variability. Moreover, all fresh sperm straws (SS-1M and SS-2M and CONV-3M) were processed at the same temperature (18 °C) and extended with the same diluent.

In lactating cows, both fresh SS treatments achieved lower P/AI than CONV-3M, and the P/AI achieved with SS relative to CONV-3M with either 1×10^6 or 2×10^6 sperm per straw was less than previously reported in a pasture-based system with 1×10^6 sperm per straw (24-d non-return rates with fresh SS = 69.1% vs. fresh CONV =

73.1%; SS/CONV = 94.6%; Xu, 2014). In heifers, although P/AI achieved by both fresh SS treatments were not statistically different from CONV-3M, there was a tendency to be lower ($P < 0.10$). The reason for different findings for fresh SS in the current study compared with Xu (2014) is unclear, but is likely related to differences in the media used (HEPES-based medium vs. Caprogen medium, respectively). Using representative straws generated for the current study, Holden et al. (2016) reported abnormally high levels of sperm agglutination ($> 20\%$) in both fresh SS treatments (SS-1M and SS-2M), which was associated with lower progressive motility. These high levels of agglutination could impact sperm transport to the site of fertilisation, and could have contributed to the somewhat lower reproductive performance achieved by the fresh SS treatments in the current study compared with Xu (2014).

Despite having a long lifespan, frozen sperm straws are costly to store because of the use of liquid nitrogen. The storage of fresh sperm straws is inexpensive and its fertility, when stored at ambient temperature, is maintained for 3-5 days, after which it gradually decreases (Vishwanath and Shannon, 1997); as a consequence, fresh sperm is generally used within 3 days after collection (Murphy et al., 2015). Cryopreservation alters sperm structure and function (Pini et al., 2018), and a significant proportion of sperm are non-viable after thawing. Hence, significantly more fresh straws can be produced per ejaculate as fewer sperm per straw are required to achieve similar P/AI to frozen sperm (Shannon and Vishwanath, 1995). Xu (2014) suggested that the improved reproductive performance observed with fresh SS (94% 24-d non-return rates relative to non-sorted) compared with frozen SS in previous studies may have been a consequence of a reduction in cryopreservation damage. Moreover, cryopreservation could have an additive damaging effect on the sperm already somewhat compromised by the sorting process. Xu (2014) did not include a frozen SS treatment against which to compare fresh SS. We hypothesized that fresh SS (1×10^6 and 2×10^6 sperm per straw) would result in greater P/AI than SS-FROZEN (2×10^6 sperm per straw). However, there was no difference in P/AI between fresh and frozen SS treatments. Moreover, frozen SS had a better P/AI relative to CONV ($> 84\%$) in heifers and lactating cows than previous studies (DeJarnette et al., 2009; Chebel et al., 2010; DeJarnette et al., 2010; Norman et al., 2010; DeJarnette et al., 2011; Sales et al., 2011; Healy et al., 2013; Djedović et al., 2016). To our knowledge, only two studies have reported greater P/AI relative to CONV ($\geq 90\%$) than the current study with frozen SS at 2×10^6 sperm per straw (Borchersen and Peacock, 2009; Lenz et

al., 2016), and both studies were conducted using heifers only. It is important to note, however, that since the current study was conducted, Sexing Technologies Inc, has increased the number of sperm in its commercially available SS from 2×10^6 to 4×10^6 sperm per straw. Non-return rates equivalent to CONV have been reported in dairy heifers when the sex-sorted sperm dose was increased to 4×10^6 sperm per straw (Lenz et al., 2016). Additional split-ejaculate studies are needed to examine the effects of the higher sperm dose in seasonal calving pasture-based dairy cows and heifers.

There was a significant bull effect on P/AI in both lactating cows and heifers. It is noteworthy that one bull (Bull 10) had poorer P/AI compared with all other bulls when used on cows and all other bulls except one when used on heifers, and was ranked poorest for both cows and heifers. Additionally, the P/AI of lactating cows inseminated with fresh sperm (CONV-3M, SS-1M, SS-2M) varied between bulls as dispatch-to-AI interval increased. Although a statistical difference was only found between two treatments within one bull (CONV-3M vs. SS-2M, Bull 10), P/AI varied between treatments within bull (Fig. 2.3b and 2.4b), in agreement with previous reports (Frijters et al., 2009; DeJarnette et al., 2010). Bulls are sometimes classified as “good freezers” or “poor freezers” regarding their sperm susceptibility to cryopreservation (i.e. freezability). We observed that the inter- and intra-bull variability may not be exclusive to the freezing process, appearing also after sorting as previously reported in boars (Parrilla et al., 2012). Even though all ejaculates passed the minimum quality control criteria to be released from the semen processing laboratory, we observed substantial variation between bulls in field fertility performance. Due to this inter- and intra-male variability, predicting whether an individual bull would achieve high or low P/AI with SS based on previous P/AI data with CONV is challenging. Therefore, in order to reduce the risk involved in using bulls of unknown fertility with SS, or until *in vitro* tests could predict bull *in vivo* competence with SS, it is recommended to use a large team of bulls for breeding management.

In the lactating cows in our study, P/AI increased with increasing EBI, which was expected due to the significant weighting on fertility in the index, but decreased with increasing milk sub-index, that is, without considering the fertility sub-index and only due to genetic merit for milk production. Because young cows usually have greater EBI than older cows, one could argue that the positive effect of EBI on P/AI was, in fact, a parity effect; however, the interaction between EBI and parity was among the effects

tested for inclusion during the backward stepwise regression, and it was excluded from the final model because it was not statistically significant. Regarding the positive relationship between P/AI and PTA for milk protein % in heifers, milk protein % has been positively associated with reproductive performance of pasture-based dairy cows (Patton et al., 2007; Yang et al., 2010; Morton et al., 2016). It has been hypothesized that this positive relationship could be explained by the energy balance status during early lactation (Yang et al., 2010) and other factors during the rest of the lactation (Morton et al., 2016). Glucose is critical for the proper functioning of the hypothalamic-pituitary-ovarian axis (Butler, 2014). Moreover, reduced blood concentrations of glucose, insulin, IGF-1 and leptin are all associated with impaired reproductive performance (Butler, 2014). Douglas et al. (2016) found that cows with average milk protein % between 3.22 and 3.40% had greater post-partum blood concentrations of glucose, insulin, IGF-1 and leptin compared with those whose average milk protein % was between 2.87 and 3.00%. Therefore, heifers with a higher PTA for milk protein % may be predisposed to having a better adaptation to lactation, and subsequently superior reproductive performance.

The high cost of SS underlines the importance of achieving high P/AI. Heifers and young lactating cows are typically genetically superior and achieve greater P/AI compared with older cows. In this study, heifers had greater P/AI than lactating cows, in agreement with previous studies (DeJarnette et al., 2010; Norman et al., 2010). Moreover, P/AI in lactating cows decreased with increasing parity. Therefore, heifers and young lactating cows should be prioritized for insemination with SS. In an attempt to provide additional criteria to identify young cows (parity ≤ 2) that are suitable for insemination with SS sperm, we identified cow characteristics associated with greater likelihood of pregnancy establishment: BCS ≥ 3 , DIM > 60 d, and fertility sub-index of the EBI $> \text{€}100$. The effect of these parameters and their interactions on probability of pregnancy are illustrated using surface plots (Fig. 2.6). Of the selected criteria, BCS was the most influential, followed by the fertility sub-index; elite cows with BCS ≥ 3 between 55 to 80 days post AI always had an enhanced probability of pregnancy compared to cows whose BCS < 3 , independent of their DIM (Fig. 2.6b) or fertility sub-index (Fig. 2.6c). Cows with poor BCS are more likely to have experienced negative energy balance during the early postpartum period, with adverse consequences for subsequent reproductive performance. This highlights the importance of achieving the correct BCS at calving (3.00 or 3.25) and minimizing postpartum BCS loss (≤ 0.5 units)

in pasture-based cows (Butler, 2014; Bedere et al., 2018). Defining the elite cows allows targeted use of SS for a more profitable breeding strategy when the number of SS straws is limited (e.g., due to the high price of SS or inadequate availability of bulls in high demand).

Reduced *in vivo* fertility for SS is one of the main factors limiting widespread adoption, particularly in seasonal-calving pasture-based systems. Starting with the original XY technology, modifications to the protocol and optimization of media led to a new product called SexedULTRA™ (Sexing Technologies, Navasota, TX), which was used for the present study, and previously during the last year of the 3-year study by Xu (2014). Additional changes to the SexedULTRA™ sperm processing methods have been implemented in recent years, resulting in better *in vitro* sperm quality parameters compared with the XY method (González-Marín et al., 2016) and CONV (González-Marín et al., 2018). In contrast to a previous study that indicated P/AI achieved with SS using the XY technology was not compensable by increasing the number of sperm per straw (DeJarnette et al., 2011), 56-day non-return rates for heifers inseminated with the latest version of frozen SexedULTRA™ sperm was dose-dependent, and when the number of sperm processed using the SexedULTRA™ technology was increased to 4×10^6 per straw, non-return rates were equal to CONV straws containing 15×10^6 sperm per straw (Lenz et al., 2016; Vishwanath and Moreno, 2018). Collectively, these studies have demonstrated that SexedULTRA™ improved P/AI relative to CONV versus historical reports using the XY technology, but SexedULTRA™ at 2×10^6 sperm per straw has not fully closed the fertility gap between CONV and SS. Further research is necessary to investigate the fertility performance of the latest generation of SexedULTRA™ containing 4×10^6 sperm per straw in heifers and cows managed under pasture-based systems.

In conclusion, frozen SS achieved P/AI relative to CONV that were greater than previously reported in lactating cows. However, fresh SS did not achieve greater P/AI than frozen SS, regardless of whether the sperm dose per straw was 1×10^6 or 2×10^6 . A bull effect for all sperm treatments as well as a dispatch-to-AI interval by bull interaction for fresh sperm underline the variable fertility performance observed, and hence highlight the importance of using a large team of bulls for breeding management. The use of SS does not have to be limited to heifers; when using SS in lactating cows, P/AI can be increased by targeting SS to animals with the greatest likelihood of

successful pregnancy establishment based on parity, BCS, EBI fertility sub-index, and DIM.

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CHAPTER 3: Fertility of frozen sex-sorted sperm at 4×10^6 sperm per dose in lactating dairy cows in seasonal-calving pasture-based herds

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3.1 Abstract

The objective was to evaluate the reproductive performance of frozen sex-sorted sperm at 4×10^6 sperm per dose (SexedUltra-4M™) relative to frozen conventional sperm in seasonal-calving pasture-based dairy cows. Semen from Holstein-Friesian ($n = 8$) and Jersey ($n = 2$) bulls was used. Four of the Holstein bulls used were resident at or near a sex-sorting laboratory (Cogent, UK or ST Benelux, The Netherlands). The remaining six bulls were located at studs in Ireland. For these six bulls, ejaculates were collected, diluted with transport medium and couriered to Cogent in parcel shippers. Transit time from ejaculation to arrival at the sorting lab was 6 to 7 h. For all bulls, ejaculates were split and processed to provide frozen conventional sperm at 15×10^6 sperm per straw (CONV) and frozen sex-sorted sperm at 4×10^6 sperm per straw (SS) and used to inseminate lactating dairy cows after spontaneous oestrus. Pregnancy diagnosis was performed by ultrasound scanning ($n = 7,246$ records available for analysis). Generalized linear mixed models were used to examine effects on pregnancy per AI (P/AI) at first artificial insemination, with sperm treatment (CONV vs. SS), bull ($n = 10$) and treatment by bull interaction as the fixed effects and herd ($n = 142$) as a random effect. Overall, P/AI was greater for cows inseminated with CONV than for those inseminated with SS (59.9% vs. 45.5%; 76.0% relative to CONV). This study was not designed to compare resident bulls vs. shipped ejaculates, but the magnitude of the difference between P/AI achieved by CONV and SS was apparently less for resident bulls (60.3% vs. 50.2%) than for shipped ejaculates (58.6% vs. 40.7%). There was a treatment by bull interaction for shipped ejaculates (P/AI ranged from 45 to 86% relative to CONV) but not for the resident bulls (P/AI ranged from 81 to 87% relative to CONV). Relative P/AI of SS compared with CONV was greater in cows with high or average fertility potential (76.1% and 78.3%, respectively) than cows with low fertility potential (58.1%). In 33.1% of the enrolled herds, the P/AI achieved with SS was $\geq 90\%$ of the P/AI achieved with CONV; this was mainly explained by herds where SS performed exceptionally well, but CONV performed poorly. In conclusion, SS had lower overall P/AI compared with CONV; however, P/AI achieved with SS was dependent on the bull, fertility potential of the cow, and herd. Strategies to improve the P/AI with SS in seasonal-calving pasture-based lactating dairy cows require further research.

3.2 Introduction

In cattle, as in other placental mammals, the female produces oocytes that bear an X-chromosome whereas the male produces sperm that bear an X- or a Y-chromosome. The sex of the offspring is determined by the sperm that successfully fertilises the oocyte since diploid cells carry two X-chromosomes (XX) in females and one X- and one Y-chromosome (XY) in males. Bull ejaculates contain approximately equal numbers of each type of sperm, as has been demonstrated by flow cytometry (Garner et al., 1983), fluorescence *in situ* hybridization (Di Berardino et al., 2004; Habermann et al., 2005) and PCR (Parati et al., 2006). Therefore, in the absence of differences between X- and Y-sperm in the ability to reach and fertilise the oocyte (i.e., primary sex ratio) or factors preferentially affecting the survival of embryo and fetuses of one sex over the other, one would expect approximately equal numbers of male and female offspring (i.e., secondary sex ratio) after natural mating or artificial insemination (AI) with conventional (i.e., non-sexed) sperm doses. Indeed, only slight deviations (< 2%) from the 50:50 ratio have been reported, with a higher percentage of male calves born from AI vs. natural mating (Berry and Cromie, 2007) or from AI with frozen vs. fresh sperm (Xu et al., 2000).

The Y-chromosome is considerably smaller than the X-chromosome (Moruzzi, 1979), which translates into a difference of ~4% in terms of DNA content between the X- and Y-sperm in cattle (Garner et al., 1983). The discovery of this difference in DNA content facilitated the development of sex-sorting by flow cytometry (Garner et al., 2013), which can produce populations of sperm enriched to contain $\geq 90\%$ X-sperm and, consequently, bias the female:male ratio in the offspring to 9:1. Sex-sorted sperm is of particular relevance to the dairy industry to generate female calves for replacements and for herd expansion (Holden and Butler, 2018). In addition, sex-sorted sperm can resolve the issue of unwanted male dairy calves, which have low economic value and may be subjected to poorer management practices than female calves (Renaud et al., 2017; Shivley et al., 2019), leading to increasing concerns regarding their welfare.

Sex-sorting of sperm involves multiple steps (Seidel and Garner, 2002; Garner et al., 2013), several of which can potentially cause cell damage, including dilution (Garner et al., 1997), centrifugation (Shekarriz et al., 1995), DNA staining (Garner and Seidel, 2008b), laser exposure (Garner, 2006) and mechanical pressures (Suh et al.,

2005). In addition, > 75% of sperm are typically discarded during the sex-sorting process (Seidel, 2014). As a consequence, commercially available sex-sorted doses contain fewer sperm per straw compared with conventional straws. Historically, frozen sex-sorted straws typically contained 2×10^6 sperm, whereas frozen conventional straws typically contain 12 to 20×10^6 sperm. The pregnancy per AI (P/AI) achieved with frozen sex-sorted sperm has historically been ~75% to 80% of that achieved with frozen conventional sperm (reviewed by Butler et al., 2014a). It has been hypothesized that the low sperm concentration and cell damage during the sorting and subsequent freezing processes could contribute to this lower fertility (Frijters et al., 2009), although others have reported that increasing the concentration of sex-sorted sperm per straw did not result in a corresponding increase in P/AI (DeJarnette et al., 2011). The technology has continued to evolve from the original XY technology, to a newer product, SexedULTRA™ (reviewed by Vishwanath and Moreno, 2018). Xu (2014) reported that New Zealand dairy cows inseminated with fresh SexedULTRA™ at 1×10^6 sperm per straw had 24-d non-return rates > 95% of those achieved with fresh conventional sperm. In a large-scale controlled field study in Ireland, frozen SexedULTRA™ at 2×10^6 sperm per straw achieved P/AI that were 84.7% and 86.7% relative to conventional sperm in lactating cows and heifers, respectively (Maicas et al., 2019).

Recent preliminary reports have indicated that P/AI was improved when dairy heifers were inseminated with increasing sperm concentration per straw (Lenz et al., 2016; Utt et al., 2017), leading to the launch of a new product with 4×10^6 sperm per straw (SexedULTRA-4M™) in 2017 (Vishwanath and Moreno, 2018). Since the introduction of this product, initial field data have suggested that the gap in fertility between sex-sorted sperm and conventional sperm has been reduced in lactating dairy cows (Vishwanath and Moreno, 2018). Given the importance of compact calving in a seasonal system, and the significant economic consequences of low fertility, the objective of this study was to evaluate the reproductive performance of frozen-thawed sex-sorted sperm at 4×10^6 sperm per straw (SexedULTRA-4M™) compared with conventional frozen sperm at 15×10^6 sperm per straw in seasonal-calving, pasture-based, lactating dairy cows.

3.3 Materials and methods

3.3.1 Semen collection and transportation

Semen from Holstein-Friesian (n = 8) and Jersey (n = 2) bulls was used in this trial. Jersey bulls were included in the study because there is a growing demand for Jersey x Holstein-Friesian crossbred cows in Ireland due to their favourable production, health, fertility and longevity characteristics. Ejaculates were collected using an artificial vagina at one of four EU-approved (Directive 88/407/EEC) semen collection centres (National Cattle Breeding Centre, Enfield, Ireland; Dovea Genetics, Thurles, Ireland; Cogent Breeding Ltd, Chester, United Kingdom; and ST Benelux, Deventer, Netherlands). Each ejaculate that was processed was from a single collection (or jump). Mean ($\pm$ SD) number of ejaculates processed per bull was 3.2 (range 2 to 6).

3.3.1.1 Resident bulls

Four of the Holstein bulls were resident at or near a sex-sorting laboratory (Cogent, UK or ST Benelux, Netherlands). Ejaculates from these bulls were collected and immediately delivered to the nearby sex-sorting laboratory for processing.

3.3.1.2 Shipped ejaculates

The ejaculates of six bulls residing at the two Irish AI centres were collected and shipped to the sex-sorting laboratory operated by Cogent Breeding Ltd. (UK) for processing. From the 12th of February to the 8th of March of 2018, the ejaculates of three bulls were collected in one of the Irish AI centres each morning. After collection, ejaculates were kept at room temperature (18 to 20 °C), evaluated under a microscope and the following standards were required for approval: sperm motility $\geq$ 65%, sperm primary defects $\leq$ 15%, sperm secondary defects $\leq$ 25%, sperm total defects $\leq$ 25%, and sperm concentration of $\geq 500 \times 10^6$ cells/mL. Approved ejaculates were gently diluted (1:1) in polypropylene tubes with transport medium (proprietary medium supplied by Sexing Technologies) at room temperature (18 to 20°C), sealed by a veterinarian from the Irish Department of Agriculture, Food and the Marine, inserted in foil pouches, and placed in parcel shippers (Crêdo Cube™, Pelican BioThermal). Ejaculates were couriered from the Irish AI centres to the Cogent sex-sorting laboratory, with an average transport time of approximately 6 to 7 h. Although no

differences were found in sperm chromatin integrity or *in vitro* embryo development after X-ray screening of bovine frozen straws at carry-on-luggage exposure doses (Hendricks et al., 2010), long term genetic and epigenetic effects have not been studied yet. Therefore, an exemption from X-ray screening of the ejaculates during the airport security control was provided by the Irish Aviation Authority. The parcel shippers containing the ejaculates were carried in the cabin of the plane to ensure stable temperature and motion conditions. On arrival at the Cogent sex-sorting laboratory, the mean ($\pm$ SD) temperature of the ejaculates was 11.1 ± 0.8 °C (range: 9.8 to 14.4 °C).

3.3.2 Semen processing

Upon arrival at the laboratory, ejaculates were subjected to quality control tests, where sperm concentration, subjective motility and sperm morphology were assessed. Ejaculates containing $< 1 \times 10^9$ sperm/mL or $< 5 \times 10^9$ total sperm were rejected. Minimum values of 65% motile sperm and 75% morphologically normal sperm were required for processing. Each ejaculate was split in two aliquots: one aliquot was used to generate frozen sex-sorted sperm (~90% X-chromosome-bearing sperm) at 4×10^6 sperm per straw (SS) using the SexedULTRA™ Genesis III sorting technology (Sexing Technologies, Navasota, TX) described by Vishwanath and Moreno (2018), while the other aliquot was processed as frozen conventional (un-sorted) sperm (CONV) at 15×10^6 sperm per straw. Both SS and CONV were packaged in 0.25 mL straws. Media used during the processing of CONV and SS were proprietary media from Sexing Technologies. All sperm batches underwent post-thaw quality control tests, including subjective assessment of sperm motility and acrosome integrity after 3 h of incubation at 35°C; analysis of purity was also assessed for the sex-sorted straws (i.e., X-chromosome bearing sperm population $\geq 87\%$) (Garner et al., 2013; Vishwanath and Moreno, 2018).

3.3.3 Distribution, insemination and recording of straws

After 30-days quarantine (required by EU directive), AI straws were shipped to Ireland in liquid nitrogen and used to inseminate lactating cows after spontaneous oestrus during the first 3 weeks of the breeding season in 142 participating herds. Each herd was allocated up to 60 straws (30 SS and 30 CONV) comprising 12 straws from

each of five bulls, and these straws were randomly used on cows that were enrolled in the study. The AI technicians and the farmers were blind to the sperm treatment. The participating farmers were instructed to use study straws at the first insemination of the breeding season, in younger cows (parity ≤ 3) that were ≥ 60 days calved (DIM) and in good body condition (BCS ≥ 3.0).

Each insemination was recorded on a handheld computer, and subsequently exported to the Irish Cattle Breeding Federation (ICBF) database, which is the national repository for cattle data in Ireland. Insemination data and other animal information including date of previous calving, parity, Economic Breeding Index (EBI) and PTA values for genetic traits of interest were retrieved from the ICBF database. The EBI is a single figure profit index used in Ireland to identify animals with superior genetic merit (Teagasc, 2014), and is composed of 7 sub-indexes with different weight (in parenthesis): fertility (35%), milk production (33%), calving performance (9%), beef performance (9%), cow maintenance (7%), cow management (4%), and health (3%) sub-indexes.

3.3.4 Ultrasound scanning

Ultrasound scanning for pregnancy diagnosis was performed by 6 experienced veterinarians on the cows enrolled in the study during the breeding season. Pregnancies resulting from the first AI were distinguished from those resulting from later services based on insemination dates and foetal crown-rump length. Body condition score data were recorded at pregnancy diagnosis on 7,238 cows; however, because BCS was recorded by several different veterinarians and the measurement was conducted several weeks after the insemination event, it was not further considered in the analysis.

3.3.5 Statistical analysis

Only scanned cows with a study straw used at the first AI of the breeding season ($n = 7,246$) were included in the final analysis. Cow was considered the experimental unit. Generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a binary distribution, with residual pseudo-likelihood as estimation technique and

containment method for calculation of the degrees of freedom, were used to evaluate the effect of sperm treatment and other variables on P/AI. A number of covariates and interactions were tested for inclusion and retained as fixed effects for the final model when $P \leq 0.25$; the use of a relaxed P -value cut-off point is recommended (Mickey and Greenland, 1989; Sperandei, 2014) during pre-selection because it minimizes type II error and is less likely to overlook contributing covariates than lower significance levels for entry such as the traditional $P \leq 0.05$. In every model, comparisons of least squares means (LSM) for P/AI between levels of the categorical variables of interest (e.g., sperm treatment, bulls, quintiles for fertility sub-index of EBI, categories of fertility potential) and interactions between them were performed using Tukey-Kramer adjustment for multiple comparisons. A P -value < 0.05 was considered significant. Results are reported as statistic $\pm$ standard error of the statistic (e.g. LSM $\pm$ SEM).

3.3.5.1 Model for the effect of sperm treatment and other variables on P/AI

The final model included the following variables as fixed effects: sperm treatment (CONV vs. SS), bull ($n=10$), parity, fertility sub-index of EBI, DIM at AI, and two-way interactions between sperm treatment and bull, fertility sub-index, and DIM. The model was first run with the fertility sub-index of the EBI as a continuous variable and, secondly as a categorical variable after being stratified into quintiles (≤ 28 , 29 to 44, 45 to 55, 56 to 69, and > 69). Herd ($n = 142$) was included as a random effect.

3.3.5.2 Models for resident bulls and shipped ejaculates

Bull was confounded with location (i.e., straws were from resident bulls or from shipped ejaculates, and no bull had straws from both locations). As a result, it was not possible to include bull and location in the same model, and two separate models were run; one for resident bulls and another for bulls with shipped ejaculates.

3.3.5.3 Model for the effect of fertility potential on P/AI

In order to incorporate additional factors known to affect fertility performance, cows were classified into three categories: (1) high fertility potential ($n = 813$): cows that were ≥ 70 DIM at AI, in parity ≤ 2 and with an EBI fertility sub-index > 60 ; (2) low fertility potential ($n = 718$): cows that were < 70 DIM at AI, in parity > 2 and with a EBI fertility sub-index ≤ 60 ; and (3) average fertility potential ($n = 5,715$): cows meeting any of the other possible combinations of DIM at AI, parity and EBI fertility sub-index.

The final model included the following variables as fixed effects: sperm treatment, bull, fertility potential (high, average or low), and the two-way interaction between sperm treatment and bull, and fertility potential. Herd was included as a random effect.

3.3.5.4 Model for the effect of selected sperm pack on P/AI

Farmers chose to use sperm packs only composed of straws from Holstein-Friesian bulls (black-and-white, BW pack), or sperm packs composed of straws from both Holstein-Friesian and Jersey bulls (crossbred, XB pack). More than 75% of the herds ($n = 109$) used a BW pack, whereas 33 herds used a XB pack. Therefore, the sperm pack selected affected the bull allocation to herds (i.e., straws from Jersey bulls were used only on the 33 herds that signed up for the XB pack, while straws from Holstein-Friesian bulls were used on all herds). A model was run to evaluate the effect of sperm pack on P/AI. The final model included the following variables as fixed effects: sperm treatment, bull, sperm pack (BW vs. XB), and the sperm treatment by sperm pack interaction. Herd was included as a random effect. The bull with the greatest reduction in fertility after sorting was a Jersey bull (Bull 10). As this bull contributed to the greater relative difference between the CONV and SS inseminations for the XB pack, the model was re-run without Jersey bulls (Bulls 9 and 10), in order to test the P/AI of SS relative to CONV on herds designated as BW or XB, but using only the bulls that were used in both BW and XB herds.

3.3.5.5 Model for the effect of herd on P/AI

A generalized linear mixed model (PROC GLIMMIX, SAS 9.4) for data with a binary distribution was used to evaluate the effect of herd on P/AI of the scanned cows at first AI. In this case, herd was considered as fixed factor. Herds were ranked in uppermost, middle and lowest tertiles (i.e. groups that contain a third of the samples) defined by P/AI achieved by CONV, by SS, or by relative P/AI (i.e., $[P/AI \text{ for SS} \div P/AI \text{ for CONV}] \times 100$). Then, herds with relative P/AI > 90% were examined.

3.4 Results

3.4.1 Characteristics of the animals used in the study

The average number of inseminations per bull was 725 (range 204 to 1521). The characteristics of the 10 bulls included in the final analysis are summarized in Table 3.1. An average of 51 cows (range 14 to 61) were inseminated and scanned on each farm. Of the scanned cows ($n = 7,238$), 52.3% had BCS ≥ 3.00 . Of the cows with known parity ($n = 6,754$), 77.1% were in parity 1, 2 or 3. Of the cows with a known calving date ($n = 6,726$), 81.8% were ≥ 60 DIM at the time of AI.

Table 3.1. Characteristics of the bulls used in the study. The bulls are sorted according to the P/AI at first AI in lactating cows obtained in the study for both treatments, CONV and SS, combined. Frozen conventional sperm at 15×10^6 sperm/dose (CONV) and frozen sex-sorted sperm at 4×10^6 sperm/dose (SS). R: resident bull; S: shipped ejaculate. DOB: date of birth. HO: Holstein; JE: Jersey.

Bull	Location	DOB	Main Breed	Number of AI (CONV + SS)
1	R	Jan-16	HO	861 (430 + 431)
2	R	Jan-15	HO	870 (445 + 425)
3	R	Jan-16	HO	974 (477 + 497)
4	R	Jan-15	HO	1,521 (773 + 748)
5	S	Jan-13	HO	723 (379 + 344)
6	S	Feb-15	HO	1,076 (540 + 536)
7	S	Feb-15	HO	254 (131 + 123)
8	S	Feb-15	HO	204 (105 + 99)
9	S	Sep-14	JE	383 (197 + 186)
10	S	Feb-14	JE	380 (189 + 191)
Total				7,246 (3,666 + 3,580)

3.4.2 Conventional vs. sex-sorted sperm

The results are presented as P/AI recorded for both the CONV and SS treatments and also as the relative performance of SS compared with CONV (i.e., relative P/AI = $[P/AI \text{ for SS} \div P/AI \text{ for CONV}] \times 100$). Overall, sperm treatment had an effect on P/AI ($P < 0.001$). Cows inseminated with CONV had a greater ($P < 0.001$) P/AI than those inseminated with SS ($59.9 \pm 1.2\%$ vs. $45.5 \pm 1.3\%$). Therefore, the overall relative P/AI was 76.0%. Bull affected P/AI ($P < 0.001$; Fig.3.1a), with greater variation observed for bulls with shipped ejaculates (range 43.9% to 55.4%) than for

resident bulls (range 52.6% to 58.4%). There was an interaction between bull and sperm treatment ($P < 0.01$; Fig 3.1b), with greater differences between CONV and SS treatments noted for bulls with shipped ejaculates. All four resident bulls and one of the bulls with shipped ejaculates (Bull 5) achieved relative P/AI that exceeded 80%; the other five bulls with shipped ejaculates achieved relative P/AI that were $\leq 75\%$. Whilst P/AI achieved by CONV did not differ ($P > 0.05$) between bulls (range 53.2 to 64.2%), SS from Bull 10 achieved lower P/AI ($27.5 \pm 3.4\%$; $P < 0.001$) than SS from Bulls 1 to 6. Bull 10 also exhibited the greatest difference ($P < 0.001$) between CONV and SS treatments, a decrease of 34.1 percentage points in P/AI.

P/AI decreased with increasing parity ($P < 0.05$), but increased with increasing DIM ($P < 0.001$) and with increasing EBI fertility sub-index ($P < 0.001$). There were no interaction effects ($P > 0.05$). The relative P/AI for EBI Fertility sub-index quintiles were 73.1, 71.0, 80.1, 77.2 and 75.3% (from the lowest to the highest fertility sub-index quintiles, respectively).

3.4.3 Resident bulls vs. bulls with shipped ejaculates

Ejaculates from resident bulls and shipped ejaculates accounted for 58.3% and 41.7% of the total number of inseminations, respectively. Both resident bulls and shipped ejaculates had greater P/AI for CONV than for SS ($60.3 \pm 1.3\%$ vs. $50.2 \pm 1.4\%$ and $58.6 \pm 1.8\%$ vs. $40.7 \pm 1.8\%$, respectively; both $P < 0.001$). The relative P/AI was apparently better for resident bulls than for shipped ejaculates (83.3% and 69.5%, respectively). An interaction between sperm treatment and bull was detected for shipped ejaculates ($P < 0.01$) but not for the resident bulls ($P = 0.70$). The interaction between sperm treatment and bull for shipped ejaculates was primarily due to the large divergence in the P/AI achieved for CONV and SS for Bull 10 (Fig 3.1b).

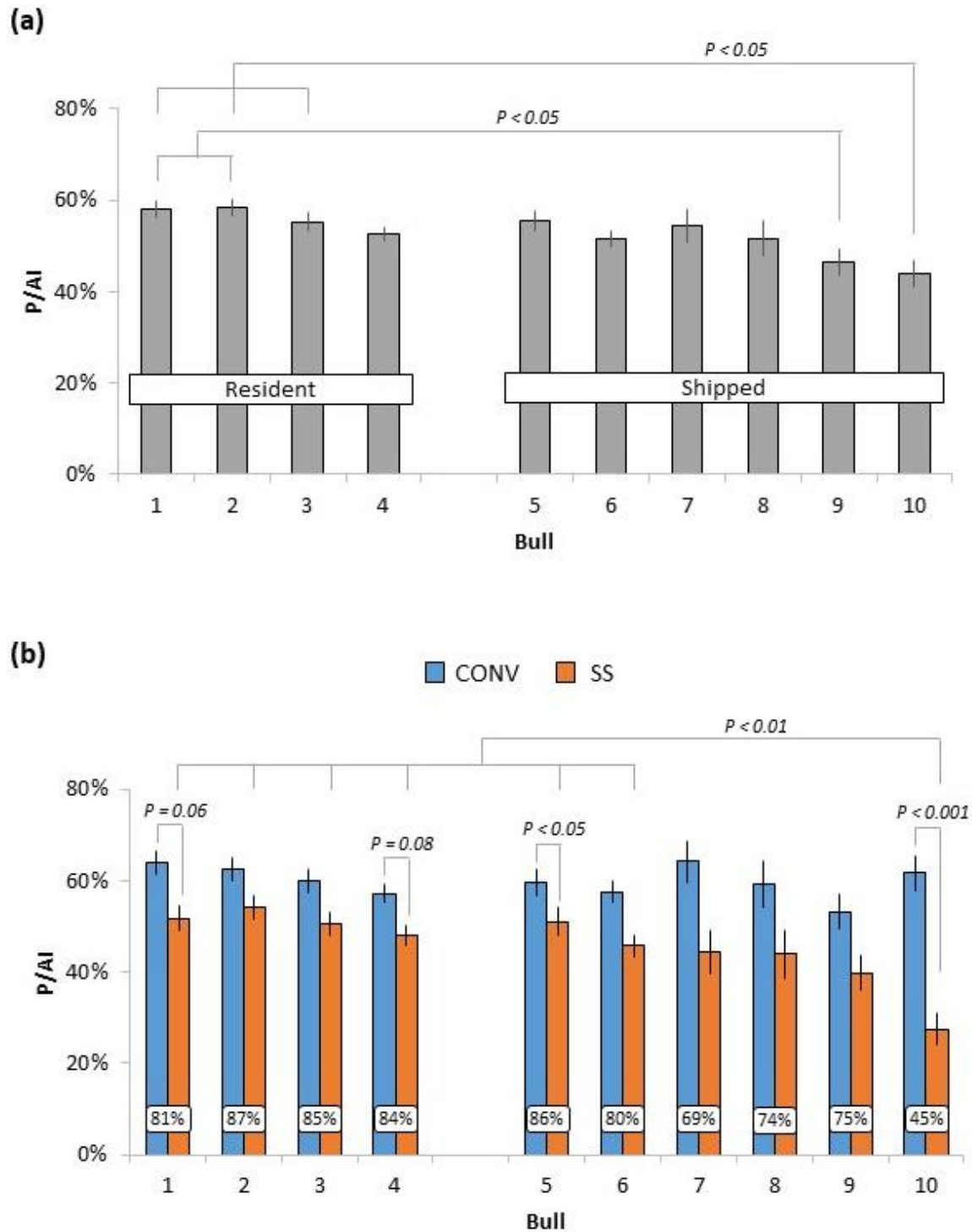


Figure 3.1. Effect of bull and treatment x bull interaction on P/AI at first insemination. (a) Mean P/AI per bull; and (b) and mean P/AI for each treatment within bull. Bulls were sorted by decreasing P/AI separately for the resident bulls and the shipped ejaculates. Sperm treatments were frozen conventional sperm at 15×10^6 sperm/dose (CONV) and frozen sex-sorted sperm at 4×10^6 sperm/dose (SS). Relative P/AI (i.e., $[P/AI \text{ for SS} \div P/AI \text{ for CONV}] \times 100$) values for each bull are displayed in white boxes at the base of the bars. P/AI achieved by CONV did not differ ($P > 0.05$) between bulls.

3.4.4 Fertility potential (high vs. average vs. low) of the cows

Fertility potential had an effect on P/AI ($P < 0.001$). There was a tendency for an interaction between fertility potential and sperm treatment ($P = 0.059$). Cows with high and average fertility potential had greater P/AI than cows with low fertility potential for both CONV ($65.3 \pm 2.5\%$ and $60.2 \pm 1.3\%$ vs. $52.0 \pm 2.8\%$, respectively; $P < 0.05$) and SS ($49.7 \pm 2.7\%$ and $47.1 \pm 1.4\%$ vs. $30.2 \pm 2.6\%$, respectively; $P < 0.001$). Therefore, the relative P/AI results were similar for cows with a high or average fertility potential (76.1% and 78.3%, respectively), but markedly poorer for cows with low fertility potential (58.1%).

3.4.5 Selected sperm pack (BW vs. XB pack)

When every bull was included in the analysis, the sperm pack chosen tended to affect P/AI ($P = 0.089$), but there was an interaction between sperm treatment and sperm pack ($P < 0.05$); when CONV was used, P/AI was greater ($P < 0.05$) with the XB pack than with the BW pack ($63.8 \pm 2.3\%$ vs. $56.6 \pm 1.4\%$, respectively), whereas there was no difference between packs when SS was used ($47.5 \pm 2.5\%$ vs. $46.4 \pm 1.4\%$, respectively). Therefore, SS had a lower relative P/AI for XB herds compared with the BW herds (74.3% vs. 81.8%). The two Jersey bulls used on the study had the lowest P/AI for sex-sorted sperm (Bulls 9 and 10, Figure 3.1b). When the two Jersey bulls were omitted from the analysis, the sperm pack chosen did not affect P/AI (CONV: $63.1 \pm 2.7\%$ vs. $58.8 \pm 1.3\%$, $P > 0.05$; SS: $51.4 \pm 2.9\%$ vs. $48.9 \pm 1.3\%$, $P > 0.05$; XB vs. BW, respectively), and there was no interaction between sperm treatment and sperm pack ($P > 0.05$). The relative P/AI was similar for XB herds and BW herds (81.5% vs. 83.2%).

3.4.6 Herd to herd variation in relative P/AI

Herd had an effect on P/AI ($P < 0.001$); mean herd P/AI ranged from 24% to 79%. Of the 142 herds, 101 (71.1%) had mean P/AI $\geq 50\%$ (for both sperm treatments combined). Mean herd P/AI with the CONV inseminations only explained 7.7% of variation in mean herd P/AI with the SS (Fig. 3.2a).

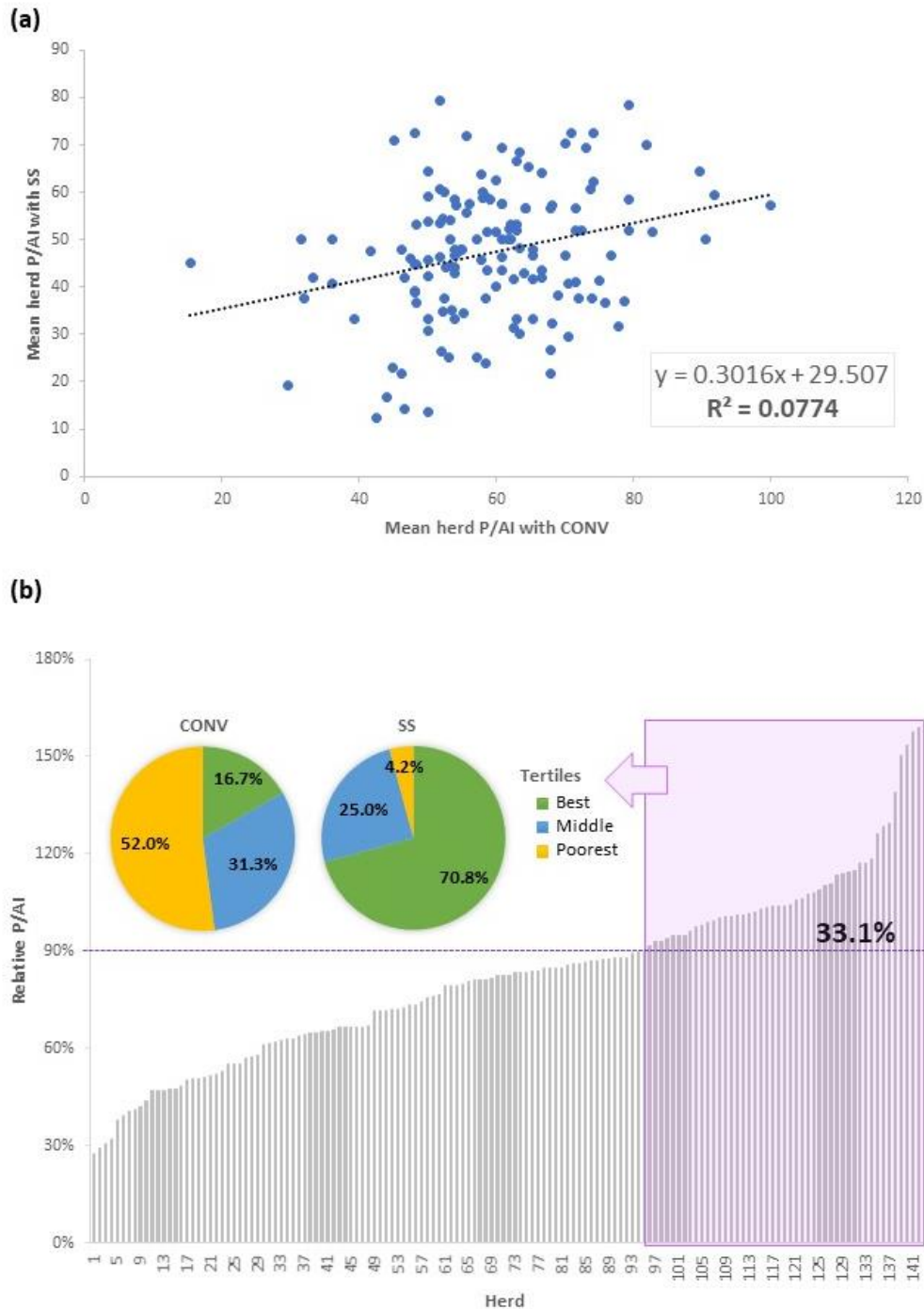


Figure 3.2. Effect of herd on P/AI: (a) Scatter plot of mean herd P/AI achieved with SS (y-axis) versus mean herd P/AI achieved with CONV (x-axis), regression line and coefficient of determination (R^2); (b) The bar chart shows the relative P/AI for each of the 142 participant herds, sorted from lowest to highest. Herds with a relative P/AI > 90% are highlighted by the rectangle. Of the herds with relative P/AI > 90%, the proportion of cows in the best, middle and poorest tertiles for P/AI following AI with SS or CONV are depicted in pie charts (top left): most of these herds had excellent performance with SS (i.e., 70.8% of them were in the best tertile; in green) but relatively poor with CONV (i.e., 83.3% [52.0 + 31.3%] of them were in the poorest and middle tertiles; in yellow and in blue, respectively).

In 47 of the 142 herds (33.1%), the relative P/AI was > 90% (Fig. 3.2b). The majority of these herds (70.8%) were in the tertile with the greatest P/AI (i.e., best tertile) for SS, but only 16.7% were also in the tertile with the greatest P/AI for CONV (Fig. 3.2b, pie charts). Hence, herds with relative P/AI > 90% were primarily a reflection of herds that had excellent performance with SS, but performed relatively poorly with CONV. Cow characteristics and sperm source for the herds with relative P/AI > 90% were similar to the cow characteristics and sperm source for all herds (Table 3.2).

Table 3.2. Characteristics of the lactating cows used in the study from every herd vs. from herds with relative P/AI¹ > 90%.

Every herd (n = 142)				
% AI from resident bulls = 58.3%				
	N	Mean ± SEM	Median ± IQR	Range
DIM at AI	6,726	78.1 ± 0.2	82 ± 25	(2, 137)
BCS	7,238	2.9 ± 0.0	3 ± 0.3	(0.8, 4.5)
Parity	6,754	2.6 ± 0.0	2 ± 2	(1, 12)
EBI ² (€)	6,659	125 ± 0.6	132 ± 58	(-136, 292)
Fert SI ³ (€)	6,692	48 ± 0.3	50 ± 32	(-133, 175)
Prod SI ⁴ (€)	6,662	42.7 ± 0.3	45 ± 37	(-98, 137)

Herds with relative P/AI > 90% (n = 47)				
% AI from resident bulls = 59.8%				
	N	Mean ± SEM	Median ± IQR	Range
DIM at AI	2,128	78.2 ± 0.4	82 ± 24	(2, 132)
BCS	2,404	2.9 ± 0.0	2.8 ± 0.3	(0.8, 4.5)
Parity	2,136	2.5 ± 0.0	2 ± 2	(1, 10)
EBI (€)	2,092	126.5 ± 1.0	135 ± 58	(-136, 272)
Fert SI (€)	2,099	48.8 ± 0.6	50 ± 31	(-133, 170)
Prod SI (€)	2,092	43.8 ± 0.6	46 ± 39	(-67, 135)

¹ Relative P/AI = (SS P/AI ÷ CONV P/AI) × 100

² EBI: Economic Breeding Index

³ Fert SI: fertility sub-index of the EBI

⁴ Prod SI: milk production sub-index of the EBI

3.5 Discussion

This study used split ejaculates to compare P/AI at spontaneous oestrus in pasture-based dairy cows following insemination with either frozen sex-sorted sperm at 4×10^6 sperm per straw (SexedUltra-4M™) or frozen conventional sperm at 15×10^6 sperm per straw. The main findings were: (1) overall, SS achieved 76.0% relative P/AI; (2) the effect of sex-sorting on P/AI was highly variable between bulls, with greater variation amongst bulls whose ejaculates were shipped to the sorting centre compared with resident bulls; (3) SS resulted in greater relative P/AI in cows with high and average fertility potential than in cows with low fertility potential; and (4) the relative P/AI was > 90% in a third of the enrolled herds.

Lactating cows inseminated with frozen SexedULTRA™ product at 2×10^6 sperm per straw had a relative P/AI of 84.7% when compared to fresh conventional sperm in a previous study from our group (Maicas et al., 2019). SexedULTRA-4M™ (SS in this study) has double the sperm dosage of its predecessor SexedULTRA™ 2M, as well as optimized media and process conditions that have been reported to result in improved *in vitro* sperm quality (González-Marín et al., 2018) and conception rates for both heifers and cows in field data from commercial farms in the USA (Vishwanath and Moreno, 2018). In the current study, P/AI results for both CONV (59.9%) and SS (45.5%) were better than those achieved in our previous study (48.0% vs. 40.6%, respectively; Maicas et al., 2019), but the relative P/AI was not improved either across all bulls, or when the analysis was restricted to resident bulls alone. A preliminary report from Xu et al. (2018) indicated comparable results in a similar field trial with lactating cows in New Zealand (54.2% vs. 40.9% P/AI for CONV vs. SS, respectively; relative P/AI = 75.5%). The impact of increasing the sperm dose from 2 to 4 million cannot be quantified, since none of these recent studies were designed to answer a dose-response question. Nevertheless, Utt et al. (2017) reported that SS at 4-million sperm per dose was superior to SS at 2-million sperm per dose, but failed to achieve P/AI equivalent to CONV. The split-ejaculate technique (i.e., SS and CONV are derived from the same ejaculate) was used in the current study and in three other recent studies (Utt et al., 2017; Xu et al., 2018; Maicas et al., 2019). Collectively, these results demonstrate non-equivalence in the P/AI achieved with SS versus CONV, and indicate that this is due to the sex-sorting process and (or) to the lower final sperm dose, and is not due to ejaculate-to-ejaculate variation.

The detrimental effect of the sex-sorting process varied between bulls, but this was evident only for the bulls with shipped ejaculates. The shipped ejaculates were exempted from X-ray airport controls, transported in insulated parcel shippers using a rapid courier (average transport time ~6 to 7 h) to reduce the risk of sperm damage during shipping. Every ejaculate passed minimum pre-sorting and post-thaw quality control criteria. Despite this, the decrease in P/AI due to the sex-sorting varied depending on the bull, in agreement with previous studies (Frijters et al., 2009; DeJarnette et al., 2010; Maicas et al., 2019; Thomas et al., 2019). It is unclear from the current study why this was the case, but there are some plausible potential explanations: (1) transport conditions used for the shipped ejaculates (media, temperature, door to door transit time) were not optimal to achieve high fertility performance, especially for sperm that go through the sorting process; (2) it was simply a bull effect, whereby the bulls that had ejaculates shipped had inherently poorer fertility and/or were inherently less suited to sex-sorting compared with the resident bulls; or (3) some bulls had a low number of inseminations, and the estimate for P/AI in these bulls is potentially inaccurate.

Bulls can differ in susceptibility to stressing procedures (e.g., incubation time in transport media, sex-sorting and freezing), and therefore, no conclusions can be drawn from this study about the effect of shipping on P/AI of SS. Nonetheless, our results indicate that sex-sorting and subsequent freezing had a more adverse impact on P/AI for most of the shipped ejaculates than for ejaculates from resident bulls. This may indicate that sperm from certain bulls were more affected by the prolonged incubation time in transport medium, becoming even more susceptible to the detrimental effects of sex-sorting and subsequent freezing. It is noteworthy that neither the detrimental effect caused by the incubation period during shipping, nor that caused by incubation periods associated with sex-sorting itself, were detected by quality controls routinely conducted with sex-sorted sperm. However, these controls rely on subjective assessment of sperm motility and acrosome integrity. Premature sperm capacitation-like modifications could be responsible for these detrimental effects (Lu and Seidel, 2004; Bucci et al., 2012; Umezu et al., 2017). The inclusion during the post-thaw quality control of assays monitoring sperm capacitation-like modifications, such as flow cytometric analysis of plasma membrane stability (Merocyanine 540 dye, Hallap et al., 2006) or image-based flow cytometric analysis of zinc signature (Kerns et al., 2018), may increase the ability to detect and discard affected ejaculates and bulls. In addition,

further research on sperm function changes due to sex-sorting could also discover currently unknown differences between conventional and sex-sorted sperm, which ultimately would help to improve routine quality controls.

In addition to the bull effect, cow characteristics and herd management are two main factors that could influence the likelihood of pregnancy success when using sex-sorted sperm. As sex-sorted sperm is expensive and usually has lower fertility than conventional sperm, more profitable breeding strategies can be achieved by targeting its use to genetically superior and more fertile female cattle (i.e., elite heifers and cows). Although use of sex-sorted sperm was traditionally limited to heifers, more recent studies demonstrated that it can also be successfully used in lactating cows (Xu, 2014; Vishwanath and Moreno, 2018; Xu et al., 2018; Maicas et al., 2019). In a previous study Maicas et al., 2019, we identified cow characteristics that defined elite cows among those inseminated with fresh or frozen sex-sorted sperm. Those elite cow characteristics were parity ≤ 2 , BCS ≥ 3 , DIM at AI > 60 d, and EBI fertility sub-index $> \text{€}100$. Using a similar approach in the current study, we identified cows of high, average and low fertility potential. Of note, the values selected for elite cow characteristics were slightly different (parity ≤ 2 , ≥ 70 DIM at AI, and EBI fertility sub-index $> \text{€}60$) and BCS was not included, reflecting differences in the cow population enrolled in the different studies, and a base change in the EBI during the interval between the two studies. In agreement with our previous study, SS performed better in cows with high and average fertility potential than in cows with low fertility potential. Moreover, relative P/AI was greater in cows with high or average fertility potential (76.1% and 78.3%, respectively) than cows with low fertility potential (58.1%). Thus, targeting the use of SS to elite cows based on parity, DIM at AI and fertility breeding indexes are, once more, highlighted as suitable criteria for identifying lactating cows suitable for insemination with sex-sorted sperm.

Approximately one third of the enrolled herds had a relative P/AI $> 90\%$, and within this subset, most exceeded 100% (i.e., SS performed better than CONV in those herds). One possible explanation is that the cows in those herds had better reproductive characteristics which would have benefited P/AI of SS, but a cursory examination of the cow characteristics in these herds does not support that possibility (Table 3.2). Interestingly, the herds that achieved high relative P/AI primarily reflect herds that had excellent performance with SS, but moderate or poor performance with

CONV. All of the cows enrolled in the study were identified as being in oestrus and nominated for insemination by the farm owner (or farm staff), and all of the inseminations were conducted by professional AI technicians that were blind to the sperm treatment. It is possible that the decisions regarding identification of cows in oestrus and nomination of cows for AI were particularly suited to sex-sorted sperm on some farms, and particularly suited to conventional sperm on other farms (at least for the cows enrolled in the study). Capacitation-like modifications due to sex-sorting may result in a reduced lifespan of the sex-sorted sperm, and hence AI closer to the time of ovulation has been proposed as potentially beneficial when using sex-sorted sperm. Indeed, improved P/AI has been reported for delayed AI when sex-sorted sperm was used in conjunction with FTAI (Sales et al., 2011) and activity collars used for automated oestrus detection (Bombardelli et al., 2016). This warrants further investigation in seasonally calving pasture based dairy cows.

In conclusion, SS achieved a mean relative P/AI of 76.0% compared to CONV, but the relative P/AI was apparently greater for resident bulls (83.3%) compared with shipped ejaculates (69.5%). The P/AI achieved with SS was highly dependent on the bull, herd and fertility potential of the cow. The likelihood of successful pregnancy establishment when using SS can be increased by targeting use on early calving, young cows with the greatest fertility potential. In addition, the inclusion of an analysis of capacitation-like modifications in the post-sorting quality control may help to detect and discard affected ejaculates and bulls before distribution of the straws. Research is needed on the importance of timing of AI relative to oestrus onset or expected time of ovulation with SS in lactating cows managed under seasonal-calving, pasture-based systems.

3.6 Acknowledgments

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CHAPTER 4: Characteristics of offspring derived from conventional and X-sorted bovine sperm

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4.1 Abstract

The objective of this retrospective study was to compare survival during the first year of life and adult performance of offspring derived from AI with X-sorted or conventional sperm processed from the same ejaculates. The dataset analysed contained records from 5,179 offspring born on 396 farms following inseminations of Holstein Friesian heifers and lactating cows with fresh conventional sperm (3×10^6 sperm per straw), fresh X-sorted sperm (1 or 2×10^6 sperm per straw), or frozen X-sorted sperm (2×10^6 sperm per straw). Offspring were classified as calves born from conventional sperm (CONV) if they were the product of an insemination with fresh conventional sperm, or as calves born from X-sorted sperm (SS) if they were product of any of the three X-sorted sperm treatments. Generalized linear mixed models were used to evaluate the effect of sperm treatment on: (1) survival during the first year of life; (2) reproductive performance, lactation performance, and survival of female offspring; and (3) slaughter characteristics of male offspring. Stillbirth rates and mortality rates during the first 2 months of life were greater for male calves (2.8% and 5.0%, respectively) than for female calves (1.6%, and 2.0%, respectively). No differences between offspring derived from SS and CONV were detected for incidences of stillbirth or mortality during the first 12 months of life within sex of calf. Reproductive performance, milk volume, milk fat and milk protein yields during first, second and third lactations, and survival to third lactation did not differ between female offspring derived from CONV and SS. Across all age groups, CONV steers had heavier carcasses than SS steers (325.3 kg vs. 318.3 kg), but there were no differences in carcass weight between CONV and SS steers within any of the age groups (≤ 24 months, 25 to 27 months, 28 to 30 months, ≥ 30 months of age). The distribution of slaughter age did not differ between CONV and SS steers when the analysis was restricted to herds that had steers of both types. Carcass conformation and fat scores of steers were not affected by sperm treatment. There was no difference in carcass weight between young bulls (≤ 2 yr) derived from CONV or SS. In conclusion, the results provide no evidence of differences in survival during the first year of life between offspring derived from CONV or SS, or for any of the reproductive and lactation performance characteristics studied between female offspring derived from CONV or SS. Herd rearing factors, and not the sex-sorting process, seemed to be involved in

the difference found in overall carcass weight between CONV and SS steers; further investigation is needed to confirm this observation.

4.2 Introduction

Multiple assisted reproduction technologies are available to dairy cattle breeders, ranging from established technologies, such as artificial insemination (AI) and multiple ovulation embryo transfer (MOET), to newer and emerging technologies including *in vitro* embryo production (IVP), sex-sorted sperm, cloning and gene editing. Many of these techniques have been associated with alterations in embryo phenotype and have in some cases been associated with alterations in foetal and offspring development (Farin et al., 2006; Duranthon and Chavatte-Palmer, 2018; Ramos-Ibeas et al., 2019). There is a paucity of data in the literature relating to the health and longevity of bovine offspring produced using these techniques; for example, despite the transfer of > 1 million bovine embryos produced by MOET and IVP annually (Viana et al., 2018), few studies have examined the health and production characteristics of these animals.

In cattle, X-chromosome bearing sperm are larger and carry around 4% more DNA than Y-chromosome bearing sperm (Garner et al., 1983). Currently, bovine sex-sorted sperm is commercially produced using flow cytometric sex-sorting technologies that identify and sort X- and Y-chromosome bearing sperm based on this difference in sperm DNA content (Garner et al., 2013; Vishwanath and Moreno, 2018). Sex-sorted sperm is of particular relevance in dairy cattle breeding, where straws containing around 90% X-chromosome bearing sperm (X-sorted sperm) are used to increase the proportion of female calves born after AI. Despite representing a smaller percentage of the AI market than conventional (i.e., non-sorted) sperm, the use of X-sorted sperm is steadily increasing; for example, the use of X-sorted sperm in US Holstein heifers increased from 9% in 2007 to 31% in 2015 (Hutchison and Bickhart, 2016).

Although there is a growing number of calves derived from X-sorted sperm, few follow-up studies have investigated whether sex-sorting had consequences for calf and adult characteristics; furthermore, these studies have reported contrasting results. While some studies have reported no differences between calves produced after AI with X-sorted sperm and those from conventional sperm (Tubman et al., 2004; Borchersen and Peacock, 2009), other studies have reported a trend for a greater incidence of stillbirths among male calves after AI of nulliparous dams with X-sorted sperm (DeJarnette et al., 2009; Chebel et al., 2010; Norman et al., 2010; Healy et al., 2013). In addition, greater mortality rates (comprised of abortions at ≥ 180 days

of gestation and neonatal death of calves < 7 days of age) have also been reported for male calves derived from insemination with X-sorted sperm after MOET (Mikkola et al., 2015).

More recently, in a study evaluating survival, growth, and production traits of offspring derived from one of four different assisted reproductive technologies on a single large dairy farm in Florida, Siqueira et al. (2017) reported that offspring derived from *in vitro* embryo production with reverse X-sorted sperm had greater cumulative mortality from 90 to 180 d of age, and produced less milk, fat, and protein in their first lactation compared with offspring from AI with conventional sperm. If the previous reports of an effect of X-sorted sperm on male calf mortality are true, it may present a welfare concern but the economic impact is likely to be small, as only 10% of the offspring are male when sex-sorted sperm is used. On the other hand, a detrimental effect on adult performance of offspring derived from X-sorted sperm, especially if female offspring are affected, could negate the advantages of biased offspring sex ratio, and may reduce the adoption of X-sorted sperm by the dairy industry.

We have previously reported the results of a large field trial evaluating the fertility of both conventional and X-sorted sperm processed from the same ejaculates (Maicas et al., 2019; Chapter 2 of this thesis). Here, we present a retrospective study of the offspring that were born following that trial. We tested the hypothesis that calf survival and adult performance was not different between animals that were derived from AI with X-sorted or conventional sperm.

4.3 Material and methods

4.3.1 Data source and handling

The data analysed in this study relate to the calves born following insemination of Holstein Friesian heifers and lactating cows with conventional or X-sorted sperm in a large field trial carried out in Ireland during the 2013 breeding season. Specific details regarding semen collection and processing, distribution of straws and recording of inseminations were reported by Maicas et al. (2019) and described in Chapter 2. Briefly, ejaculates from Holstein bulls were collected and each ejaculate was split into two aliquots; one aliquot was processed to provide straws of fresh conventional sperm, whereas the second aliquot was sex-sorted for X-chromosome bearing sperm (X-sorted sperm) with a 90% bias using SexedULTRA™ sorting technology (Sexing Technologies, Navasota, TX). The fresh conventional straws contained 3×10^6 sperm per straw, and the X-sorted straws were processed as either fresh (1×10^6 or 2×10^6 sperm per straw) or frozen straws (2×10^6 sperm per straw). Straws were used to inseminate heifers and lactating cows on 492 farms from April 15th to May 18th, 2013. Information about the offspring of these inseminations was retrieved from the Irish Cattle Breeding Federation (ICBF) database, which is a repository for animal records, events and performance data for all of the cattle registered in Ireland and the herds in which they reside. Data were merged in a unique dataset and checked for duplicates, outliers and invalid values, which were removed where appropriate. Only calves that were offspring of the conventional and X-sorted AI straws included in the final analysis of the study reported by Maicas et al. (2019) (Chapter 2) were retained for analysis (10 bulls; range 172 to 721 calves per bull). After merging and cleaning steps, the dataset contained records for 5,179 offspring. All calves were born between January 13th and March 6th, 2014. Animals were classified as calves derived from conventional sperm (CONV) if they were the product of an insemination with fresh conventional sperm, or as calves born from X-sorted sperm (SS) if they were product of any of the three X-sorted sperm treatments.

4.3.2 Data handling and statistical analysis

Results are reported as least squares means (LSM) and 95% CI [lower limit, upper limit]. A significant difference or effect was considered to exist when $P < 0.05$.

4.3.2.1 Mortality rates and survival analysis during the first year

The analysis of mortality rates was restricted to the first year of life. Animals with a record for age at death during the first year of life (age at death ≤ 365 d) but without slaughter data were assumed to have died on-farm ($n = 351$), while the remaining animals were considered to have remained alive during that first year. Generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a binary distribution were used to evaluate differences between calves derived from CONV and SS for stillbirth rates (stillborn calves were defined as those born dead or that died within 48 h after calving), mortality rates during the first 2 months (excluding stillborn) and mortality rates during 3 to 12 months of age. Bull, sperm treatment (CONV vs. SS), sex of the calf (female vs. male), and the interaction between sperm treatment and sex of the offspring were included as fixed effects; herd where the offspring was born was included as random effect. Comparisons of LSM were performed using Tukey-Kramer adjustment for multiple comparisons.

The effect of sperm treatment on the interval from birth to death (age at death) was evaluated by the LIFETEST procedure (SAS, 9.4) using Kaplan-Meier analysis for female offspring that died during the first two months of life ($n = 181$) or during their first year ($n = 236$), and for male offspring that died during the first two months of life ($n = 96$) or during their first year ($n = 115$). Kaplan-Meier plots were generated and logrank test was used to compare the survival distributions of offspring derived from SS vs. CONV.

4.3.2.2 Reproductive performance of female offspring

The use of AI in seasonal dairy herds is generally confined to the first 6 weeks of a 12-wk breeding season, which usually starts sometime between late April and early May. For the purposes of the analysis of reproductive traits, mating start date (MSD) was set as 1st of May. For the dichotomous variables (e.g., calved before MSD, calved after MSD), generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a binary distribution were used to evaluate differences between female

offspring derived from CONV and SS. For the continuous variables (i.e., age at 1st calving, calving interval), normality of residuals was checked, data were transformed when necessary and generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a normal distribution were used to evaluate differences between female offspring derived from CONV and SS. Sperm treatment, bull, and the interaction between sperm treatment and bull were included as fixed effects, and herd at calving was included as random effect. Comparisons of LSM were performed using Tukey-Kramer adjustment.

4.3.2.3 Lactation performance of female offspring

The normality of residuals was checked, data were transformed when necessary and generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a normal distribution were used to evaluate differences in milk, fat and protein yields for first, second and third lactation between female offspring derived from CONV and SS. Sperm treatment, bull, and the interaction between sperm treatment and bull were included as fixed effects, and herd was included as random effect. The interaction between sperm treatment and bull was removed from the analysis because it was not significant for any of the traits analysed. Comparisons of LSM were performed using Tukey-Kramer adjustment.

4.3.2.4 Survival analysis of female offspring that calved at least once

The effect of sperm treatment on the lifetime survival of female offspring that calved at least once was evaluated by comparing the interval from birth to death (survival age) using the LIFETEST procedure (SAS, 9.4) using Kaplan-Meier analysis. Only heifers that had at least one calving record were included in this analysis (n = 3,472). Survival age was the age at death or slaughter for female offspring that were dead at the time of data retrieval (9/11/2019), while female offspring that were still alive were right-censored and their survival age was their age at time of data retrieval. Kaplan-Meier plots were generated and logrank test was used to compare the survival distributions of heifers derived from SS vs. CONV.

4.3.2.5 Carcass weight of steers and young bulls

Slaughter data were not available from male offspring that died on-farm or that were exported, and not every abattoir provides slaughter data to ICBF. Of the 1,130

male calves that were born, slaughter data was available for 642, and these were classified as bulls (age > 730 d; n = 30), young bulls (age ≤ 730 d; n = 139), or steers (n = 473) at slaughter. Male offspring classified as bulls were not included in further analysis due to the small number of animal records. Young bulls and steers were analysed separately. Steers were grouped by age at slaughter into four categories: ≤ 24 months, 25 to 27 months, 28 to 30 months, ≥ 30 months of age. Young bulls were grouped by age at slaughter into 2 categories: ≤ 20 months, and ≥ 21 months of age. The normality of residuals was checked, and generalized linear mixed models (PROC GLIMMIX, SAS 9.4) for data with a normal distribution was used. Sperm treatment, age category, and the interaction between them were included as fixed effects; the last herd where steers or young bulls were located before being sent to slaughter was included as a random effect. Comparisons of LSM were performed using Tukey-Kramer adjustment. Since not every herd at slaughter had reared both CONV and SS steers, the analysis of carcass weight of steers was re-run with only those herds that had reared both CONV and SS steers (n = 56; total number of steers = 193). Similarly, the analysis of carcass weight of young bulls was re-run with only those herds that had reared both CONV and SS young bulls (n = 13; total number of young bulls = 50).

4.3.2.6 Age at slaughter of steers

The effect of sperm treatment on the interval from birth to slaughter (age at slaughter) of steers (n = 473) was evaluated by the LIFETEST procedure (SAS, 9.4) using Kaplan-Meier analysis. The last herd in which steers were located before being sent to slaughter was considered as herd at slaughter (n = 278). Since not every herd at slaughter had both CONV and SS steers, the analysis was re-run with only those herds that had steers of both types (n = 56; total number of steers = 193).

4.3.2.7 Carcass conformation and fat scores of steers

Carcasses were classified at slaughter based on a visual assessment of carcass conformation and fat content according to EU scales into one of nine conformation classes (P-, P=, P+, O-, O=, O+, R-, R=, R+) that were coded as 1 to 9 from the poorest (P-) to the best (R+), and one of five fat classes that were coded as 1 to 5 from the least fat score (1) to the greatest fat score (5). Multinomial models were used (PROC GLIMMIX, SAS 9.4, with adaptive quadrature method) to evaluate the effect of sperm treatment and age at slaughter on carcass conformation and fat scores.

Carcass conformation scores ≥ 4 were defined as high conformation and those < 4 as low conformation. Similarly, fat scores ≥ 3 were defined as high fat and those < 3 as low fat. Using a generalized linear mixed model (PROC GLIMMIX, SAS 9.4) for data with a binary distribution, with sperm treatment and slaughter weight category as fixed effects, odd ratios for high conformation and high fat scores due to the sperm treatment were calculated. The last herd where the steer was located before being sent to slaughter was included as random effect in both multinomial and binary models.

4.4 Results

4.4.1 Analysis of differences without grouping sex-sorted treatments

To examine the possibility that differences existed between the three X-sorted sperm treatments, all data analyses were initially conducted using all four sperm treatments (fresh conventional vs. fresh X-sorted at 1×10^6 sperm per straw vs. fresh X-sorted at 2×10^6 vs. frozen X-sorted). There was no evidence of differences between offspring derived from each sperm treatment for any of the characteristics analysed (all $P > 0.14$). Hence, the three SS treatments were combined to provide a more strongly powered appraisal of differences between SS and CONV treatments.

4.4.2 Mortality rates and survival analysis during the first year

Stillbirth rates were greater for male offspring than for female offspring (2.8% [1.9, 4.1] vs. 1.6%, [1.2, 2.3]; $P = 0.02$), but no differences were detected between offspring derived from SS and CONV within sex of calf (Table 4.1). Mortality rates during the first 2 months of life (excluding stillbirth) were also greater for male offspring than for female offspring (5.0% [3.8, 6.7] vs. 2.0% [1.5, 2.7]; $P < 0.001$), but no differences were detected between CONV and SS calves within sex of offspring (Table 4.1). Mortality rates from 3 to 12 months of age did not differ between male and female offspring (1.9% [1.2, 3.0] vs. 1.3% [0.9, 1.9], $P = 0.19$), nor was any difference detected between CONV and SS offspring within sex of offspring (Table 4.1). Bull did not affect any of the mortality rates analysed (all $P > 0.22$).

Table 4.1. Mortality rates (LSM [95% CI]) for female and male offspring derived from AI with conventional (CONV) or sex-sorted (SS) sperm.

	CONV	SS	P-value
Females			
N born	846	3,203	
Stillbirth rate ¹ (%)	1.5 [0.9, 2.6]	1.8 [1.3, 2.4]	0.95
Mortality 2 mo ² (%)	1.8 [1.1, 2.9] ^a	2.2 [1.7, 2.9] ^a	0.86
Mortality 3 to 12 mo (%)	1.3 [0.7, 2.3]	1.3 [0.9, 1.9]	1.00
Males			
N born	784	346	
Stillbirth rate ¹ (%)	2.5 [1.6, 3.8]	3.2 [1.8, 5.6]	0.89
Mortality 2 mo ² (%)	4.5 [3.2, 6.3] ^b	5.6 [3.6, 8.5] ^b	0.86
Mortality 3 to 12 mo (%)	1.8 [1.0, 3.0]	2.0 [1.0, 4.0]	0.99

¹Stillborn: dead at or within 48 h of birth

²Excluding stillborn calves

^{a,b} LSM differed ($P < 0.01$) between males and females

There were no differences due to sperm treatment on survival distributions of female offspring that died during the first two months of life (Fig. 4.1a, logrank test: $P = 0.09$) or during their first year (Fig. 4.1b, logrank test: $P = 0.58$), nor on survival distributions of male offspring that died during the first two months of life (Fig. 4.2a, logrank test: $P = 0.66$) or during the first year of life (Fig. 4.2b, logrank test: $P = 0.79$).

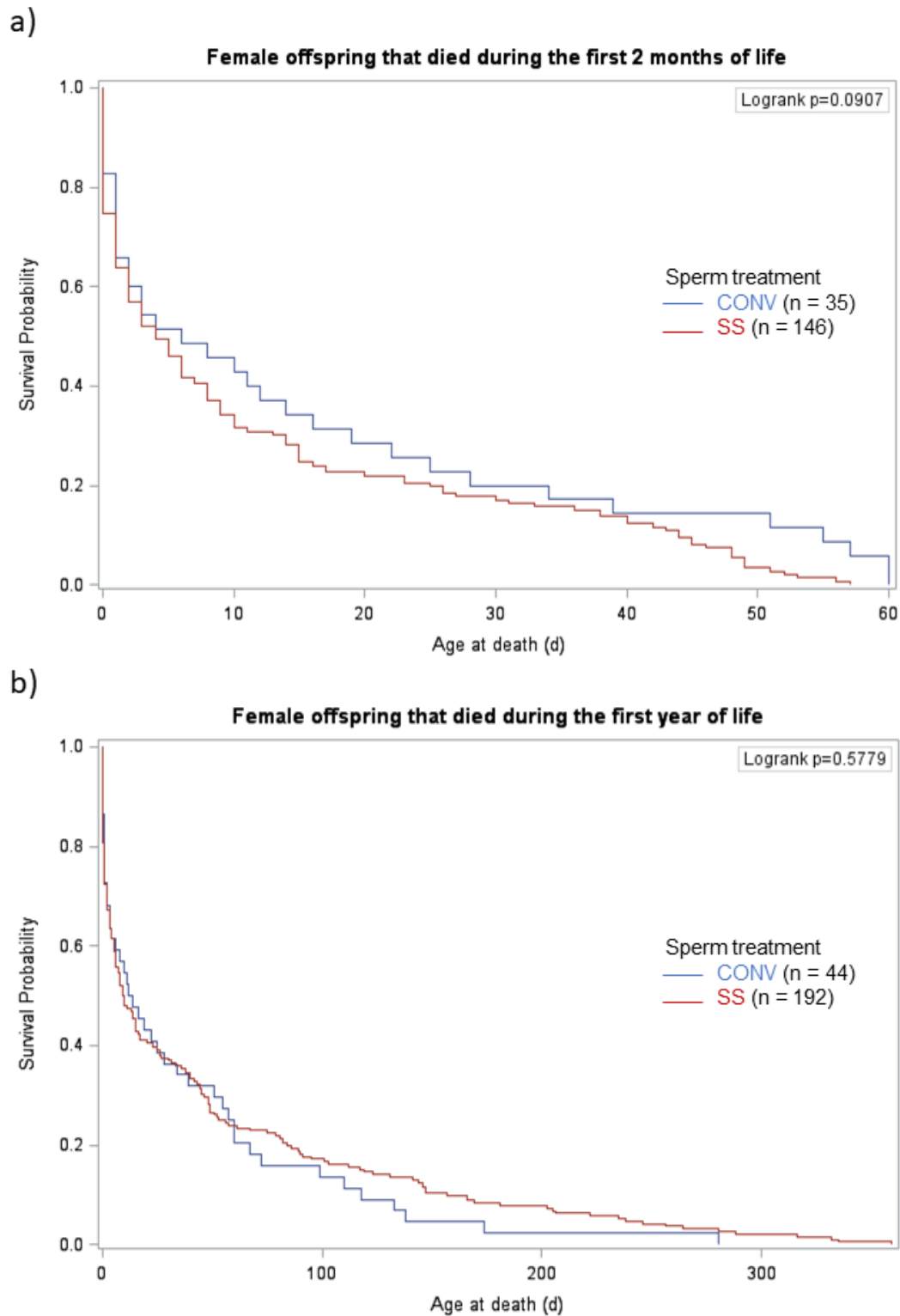


Figure 4.1. Survival analysis (Kaplan-Meier plot and P -value for the Logrank test) for the interval from birth to death on-farm (age at death, in days) of female offspring that died during the first two months of life (a), or during the first year (b), and were derived from AI with conventional (CONV; blue line) or sex-sorted (SS; red line) sperm.

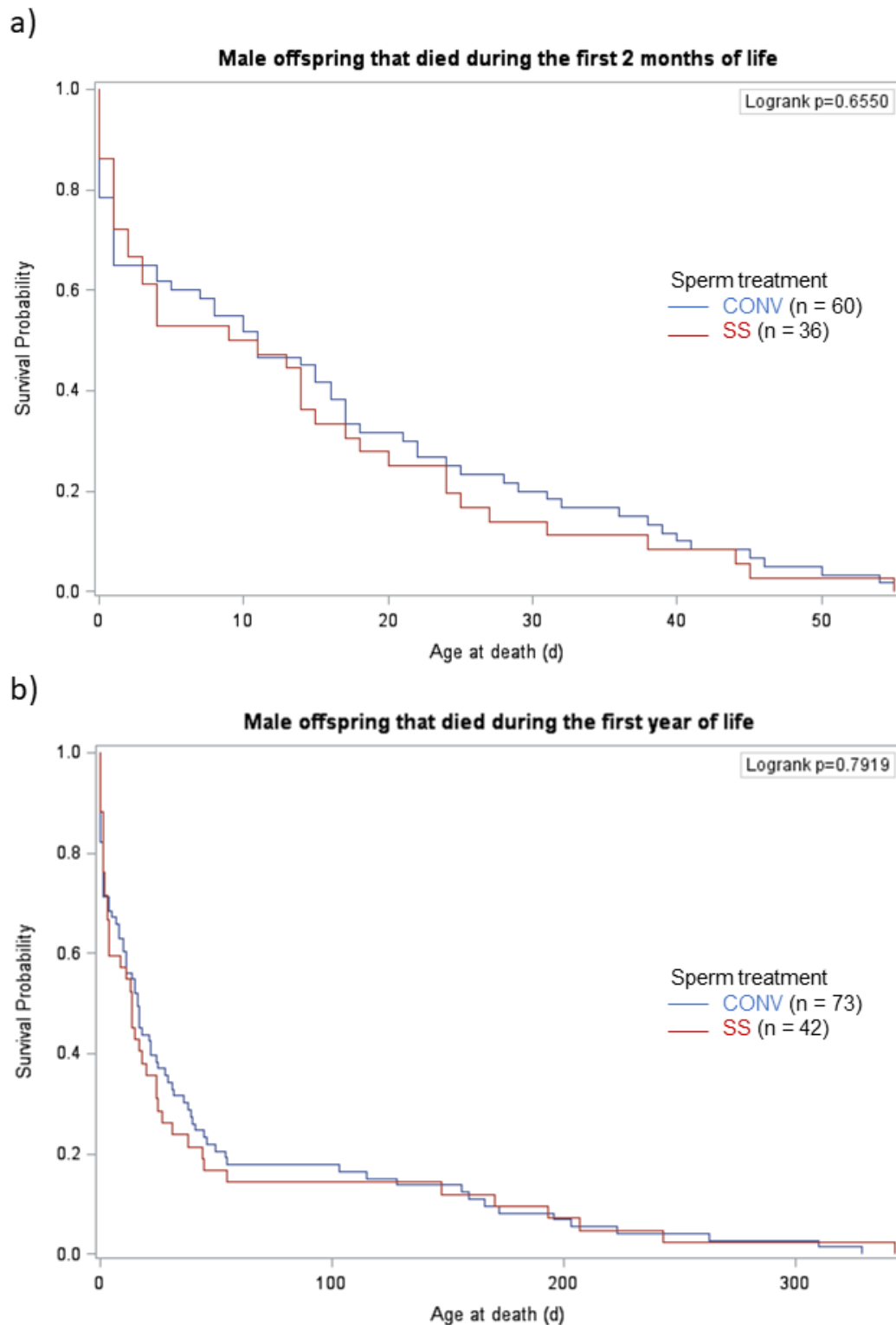


Figure 4.2. Survival analysis (Kaplan-Meier plot and P-value for the Logrank test) for the interval from birth to death on-farm (age at death, in days) of male offspring that died during the first two months of life (a), or during the first year (b), and were derived from AI with conventional (CONV; blue line) or sex-sorted (SS; red line) sperm.

4.4.3 Reproductive performance of female offspring

No differences were found between female offspring derived from CONV and SS for any of the reproductive variables analysed (Table 4.2).

Table 4.2. Reproductive performance (LSM [95% CI]) of female offspring derived from AI with conventional (CONV) or sex-sorted (SS) sperm.

	CONV	SS	P-value
Reached first calving (%)	86.3 [83.4, 88.8]	85.7 [83.9, 87.4]	0.67
Heifers that calved before MSD ¹	96.3 [94.0, 97.7]	97.0 [95.7, 97.9]	0.34
Age at first calving (d)	741.8 [740.2, 744.0]	742.4 [741.3, 744.0]	0.57
Calved between 22 to 26 mo (%)	95.0 [92.6, 96.7]	94.5 [93.0, 95.8]	0.65
Calved at > 26 mo (%)	5.0 [3.3, 7.4]	5.5 [4.3, 7.0]	0.65
Heifers that calved after MSD ¹ (%)	3.7 [2.3, 6.0]	3.0 [2.1, 4.3]	0.34
Reached second calving (%)	75.4 [71.8, 78.7]	73.9 [71.6, 76.1]	0.43
Calving interval (d)	373.3 [370.5, 376.0]	371.2 [369.6, 372.9]	0.20

¹ Mating start date (MSD) set to 1st of May

Female offspring that had a record for a first calving event but not a second calving event (n = 469) included cows that died (n = 65), were slaughtered (n = 291), were exported after the first calving (n = 26) and also cows with no record of slaughter or death (n = 87), which were assumed to have been retained on farm. Of the 469 female offspring that had a single calving event, no differences were found between CONV and SS treatments in the proportion that died (11.6% [6.5, 19.9] vs. 14.4% [11.2, 18.5]; $P = 0.47$), were slaughtered (66.3% [56.1, 76.5] vs. 61.0% [57.2, 68.7]; $P = 0.50$), were exported (6.3% [2.1, 12.1] vs. 5.4% [2.5, 7.0]; $P = 0.66$) or were retained on farm (15.8% [7.4, 22.6] vs. 19.3% [11.1, 20.0]; $P = 0.68$) after the first calving.

For cows that calved more than once, the proportion of second and third calving events that occurred during the interval between January 1st to June 30th was examined to quantify the proportion that remained in a seasonal calving system. For those cows calving for a second time (n = 3,004), there were no differences between CONV and SS treatments in the proportion of those cows whose second calving was between January 1st and June 30th 2017 to start the second lactation (96.4% [94.0, 97.9] vs. 96.3% [94.7, 97.4], $P = 0.90$). For those cows calving for a third time (n = 1,854), there were no differences between CONV and SS treatments in the proportion

of those cows whose third calving was between the January 1st and June 30th 2018 to start the third lactation (91.9% [88.0, 94.5] vs. 94.5% [92.6, 95.9], $P = 0.07$)).

Bull affected age at first calving ($P < 0.001$; range 732.1 d to 745.7 d), and calving interval ($P < 0.01$; range 366.7 d to 378.6 d), but did not affect any of the other reproductive characteristics studied (all $P > 0.27$).

4.4.4 Lactation performance of female offspring

There was no effect of sperm treatment on any of the lactation variables analysed (Table 4.3).

Table 4.3. Projected 305-day lactation performance (LSM [95% CI]) of female offspring derived from AI with conventional (CONV) or sex-sorted (SS) sperm.

	CONV	SS	P-value
First lactation			
N	521	1942	-
Milk yield (kg)	5,256 [5,159, 5,353]	5,195 [5,113, 5,276]	0.10
Fat yield(kg)	228.4 [224.3, 232.5]	226.2 [222.7, 229.7]	0.14
Protein yield (kg)	187.1 [183.8, 190.4]	185.3 [182.4, 188.1]	0.12
Second lactation			
N	455	1670	-
Milk yield (kg)	6,362 [6,246, 6,479]	6,320 [6,223, 6,416]	0.35
Fat yield(kg)	269.4 [264.5, 274.3]	270.0 [266.0, 274.0]	0.76
Protein yield (kg)	229.0 [225.1, 232.9]	228.4 [225.1, 231.7]	0.69
Third lactation			
N	398	1452	-
Milk yield (kg)	6,862 [6,729, 6,996]	6,836 [6,729, 6,943]	0.64
Fat yield(kg)	289.8 [284.2, 295.5]	290.7 [286.1, 295.3]	0.71
Protein yield (kg)	247.5 [243.0, 252.1]	246.5 [242.7, 250.2]	0.58

There was a bull effect ($P < 0.001$) on milk, fat and protein yield during the first lactation (milk yield: range 4,938 to 5,639; fat yield: range 214.8 to 237.2 kg; protein yield: range 180.2 to 198.0 kg), second lactation (milk yield: range 5,974 to 6,676 kg; fat yield: range 260.7 to 282.3 kg; protein yield: 220.5 to 240.6 kg) and third lactation (milk yield: range 6,434 to 7,204 kg; fat yield: range 278.3 to 303.0 kg; protein yield: range 236.4 to 260.2 kg).

4.4.5 Survival of female offspring that calved at least once

The survival distributions of female offspring that calved at least once and were derived from CONV and SS are illustrated in Fig. 4.3; no differences were observed for survival age due to sperm treatment (Logrank test: $P = 0.75$).

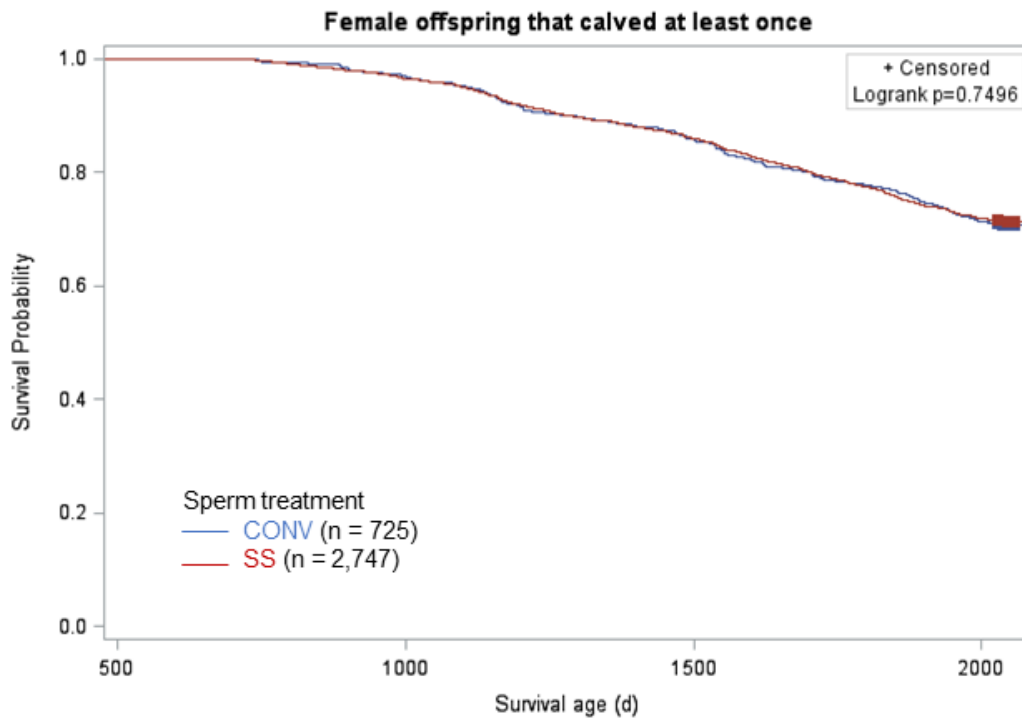


Figure 4.3. Survival analysis (Kaplan-Meier plot and P -value for the Logrank test) for the interval from birth to death or slaughter (survival age, in days) of female offspring that calved at least once and were derived from AI with conventional (CONV; blue line) or sex-sorted (SS; red line) sperm. Females still alive at the time of data retrieval were right-censored.

4.4.6 Carcass weight at slaughter of male offspring

Carcass weight at slaughter of both steers and young bulls was positively correlated with age at slaughter ($R = 0.36$ and 0.44 , respectively, both $P < 0.001$; Fig. 4.4). Across all age classes, steers derived from CONV had heavier carcasses than steers derived from SS (325.3 kg [320.9, 329.6] vs. 318.3 kg [312.3, 324.3]; $P = 0.035$). However, there were no differences in carcass weight between CONV and SS steers within any of the age group categories (Fig. 4.5a). When the analysis was restricted to only herds that reared both CONV and SS steers, steers derived from CONV tended to have heavier carcasses than steers derived from SS (322.6 kg [315.1, 330.1] vs.

314.6 kg [306.3, 323.0]; $P = 0.066$), but there were no differences in carcass weight between CONV and SS steers within any of the age group categories (Fig. 4.5b).

There was no difference in carcass weight between young bulls derived from CONV or SS across age categories (317.1 kg [305.9, 328.3] vs. 307.3 kg [292.5, 322.2]; $P = 0.17$) or within age categories (Fig. 4.6a). Similarly, when the analysis was restricted to only herds that reared both CONV and SS young bulls, there was no difference in carcass weight between young bulls derived from CONV or SS across age categories (318.4 kg [301.5, 335.3] vs. 305.8 kg [286.3, 325.3]; $P = 0.12$) or within age categories (Fig. 4.6b).

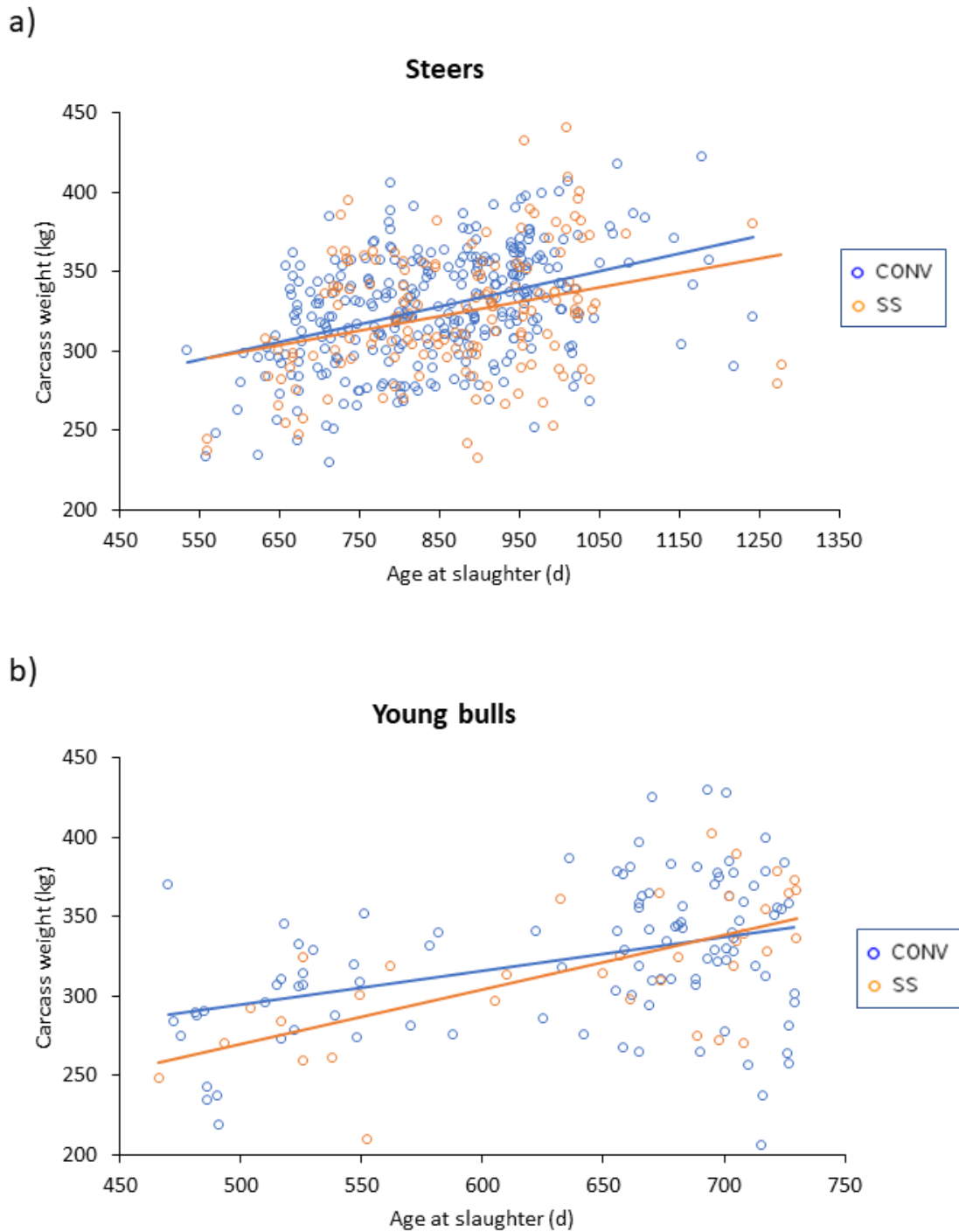


Figure 4.4. Scatter plots and trendlines for carcass weight at slaughter (kg) against age at slaughter (d) for steers (a) and young bulls (b) derived from AI with sex-sorted (SS; orange circles) and with conventional sperm (CONV; blue circles).

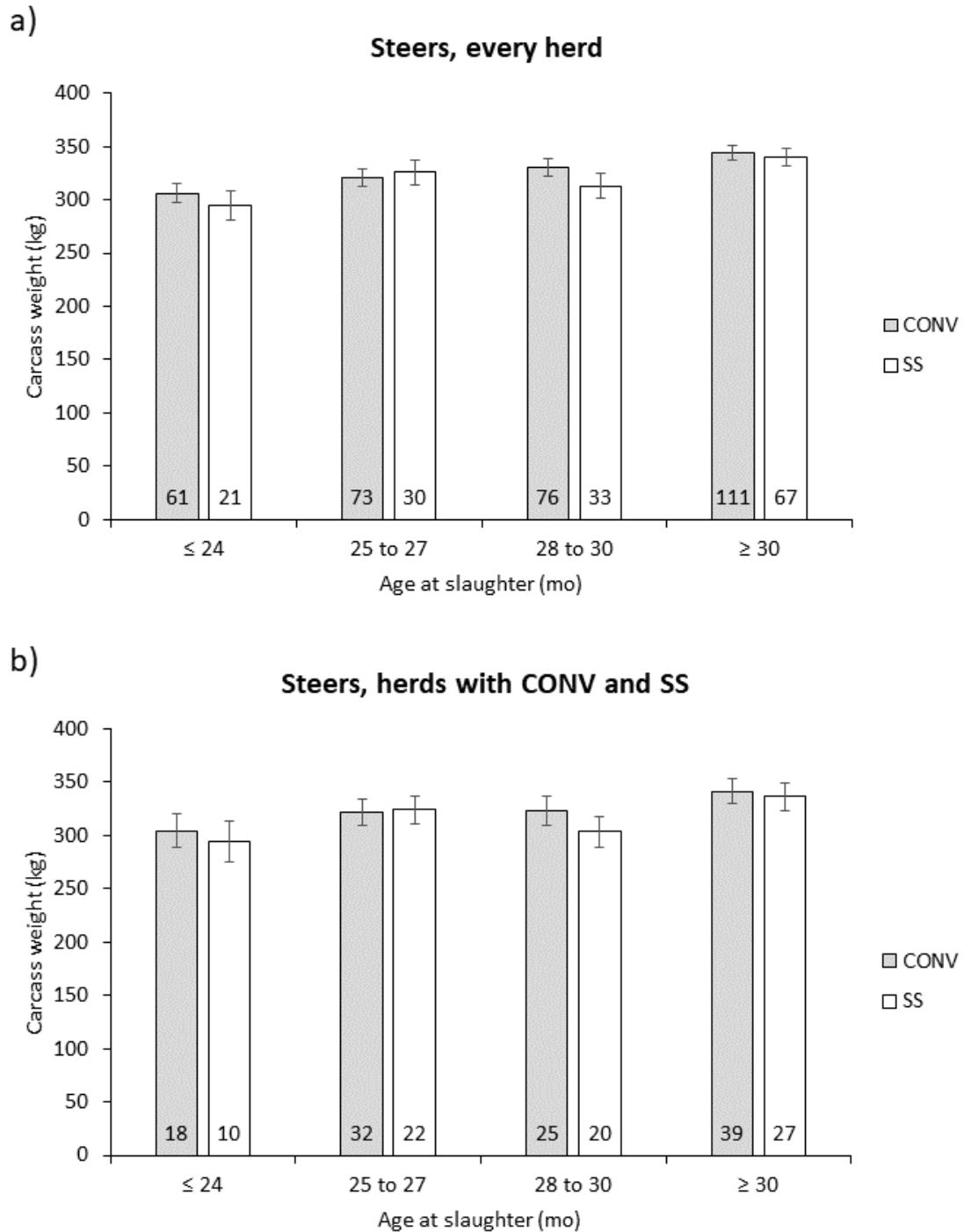


Figure 4.5. Mean carcass weight (LSM) at slaughter for steers derived from conventional (CONV) or sex-sorted (SS) sperm and grouped by age at slaughter: (a) when every herd at slaughter (i.e., the last herd where steers were located before being sent to slaughter, $n = 278$) was included in the analysis, or (b) when the analysis was restricted to those herds at slaughter ($n = 56$) that reared both CONV and SS steers. Mean carcass weight did not differ ($P > 0.13$) between sperm treatments within any age group. Sample sizes are displayed at the base of the bars, and 95% CI of the means as error bars.

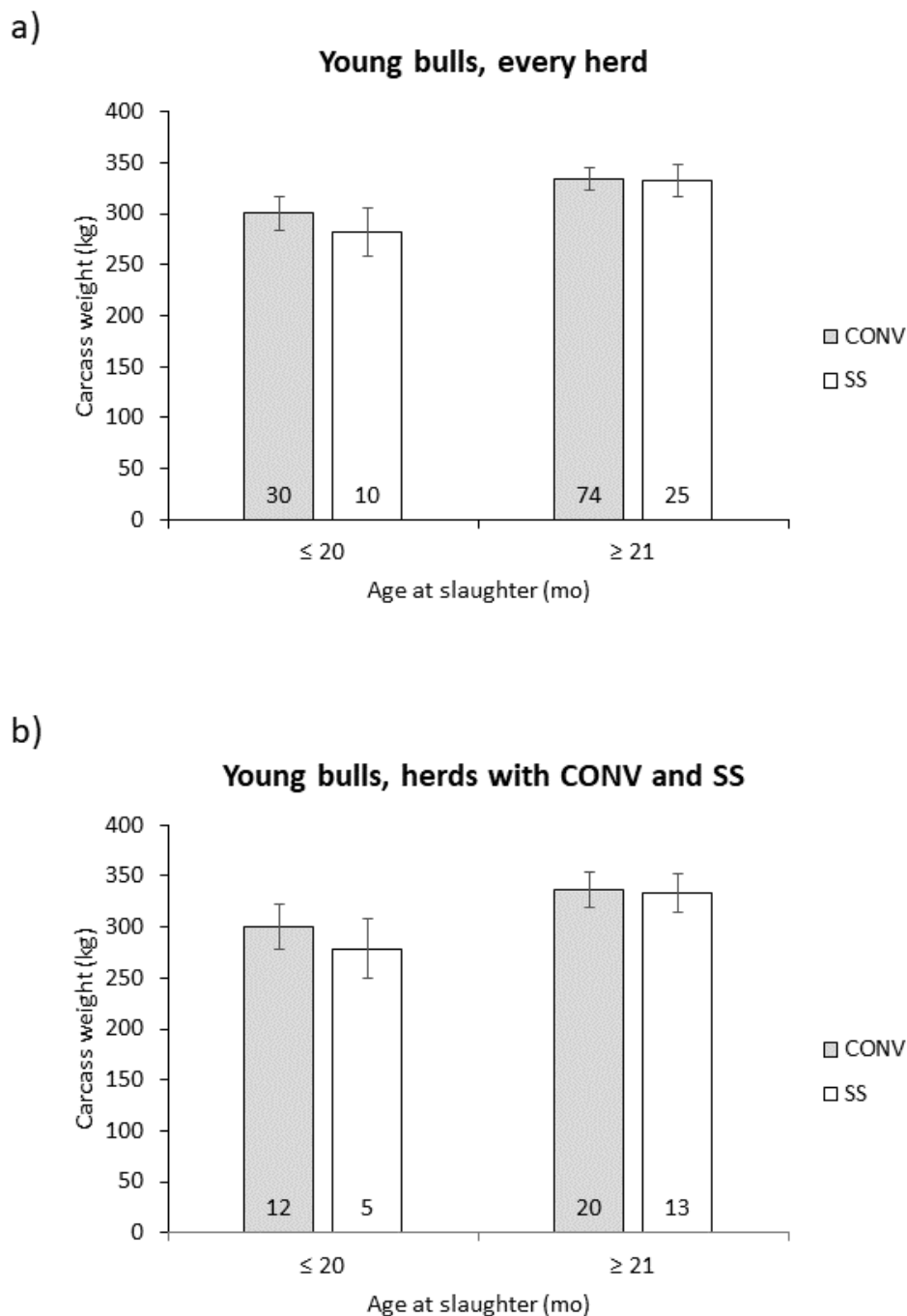


Figure 4.6. Mean carcass weight (LSM) at slaughter for young bulls derived from conventional (CONV) or sex-sorted (SS) sperm and grouped by age at slaughter: (a) when every herd at slaughter (i.e., the last herd where young bulls were located before being sent to slaughter, $n = 74$) was included in the analysis, or (b) when the analysis was restricted to those herds at slaughter ($n = 13$) that reared both CONV and SS young bulls. Mean carcass weight did not differ ($P > 0.38$) between sperm treatments within any age group. Sample sizes are displayed at the base of the bars, and 95% CI of the means as error bars.

4.4.7 Age at slaughter of steers

The distribution of slaughter age differed (Logrank test: $P = 0.03$) between CONV and SS steers when every herd was included in the analysis, with CONV steers being culled earlier than SS steers (Fig. 4.7a), but it did not differ (Logrank test: $P = 0.45$) when the analysis was restricted to herds that had steers of both types (Fig. 4.7b).

4.4.8 Carcass conformation and fat scores of steers

Carcass conformation and fat scores were both affected by slaughter weight (both $P < 0.001$) but not by sperm treatment ($P = 0.46$ and $P = 0.79$, respectively; Table 4.4). The use of SS did not affect the odds of carcass conformation score ≥ 4 (OR: 1.17 [0.78, 1.75]) or fat score ≥ 3 (OR: 0.94 [0.58, 1.51]).

Table 4.4. Carcass conformation and fat scores of steers derived from AI with conventional (CONV) or sex-sorted (SS) sperm.

	N	Mean [95% CI]	Median	Minimum	Maximum
Conformation score ¹					
CONV	322	4.0 [3.9, 4.1]	4	1	9
SS	151	3.9 [3.7, 4.1]	4	1	7
Fat score ²					
CONV	322	3.0 [2.9, 3.1]	3	1	4
SS	151	3.0 [2.8, 3.1]	3	1	5

¹ Coded as 1 to 9 from the poorest (1) to the best (9) conformation score

² Coded as 1 to 5 from the lowest (1) to the greatest (5) fat score

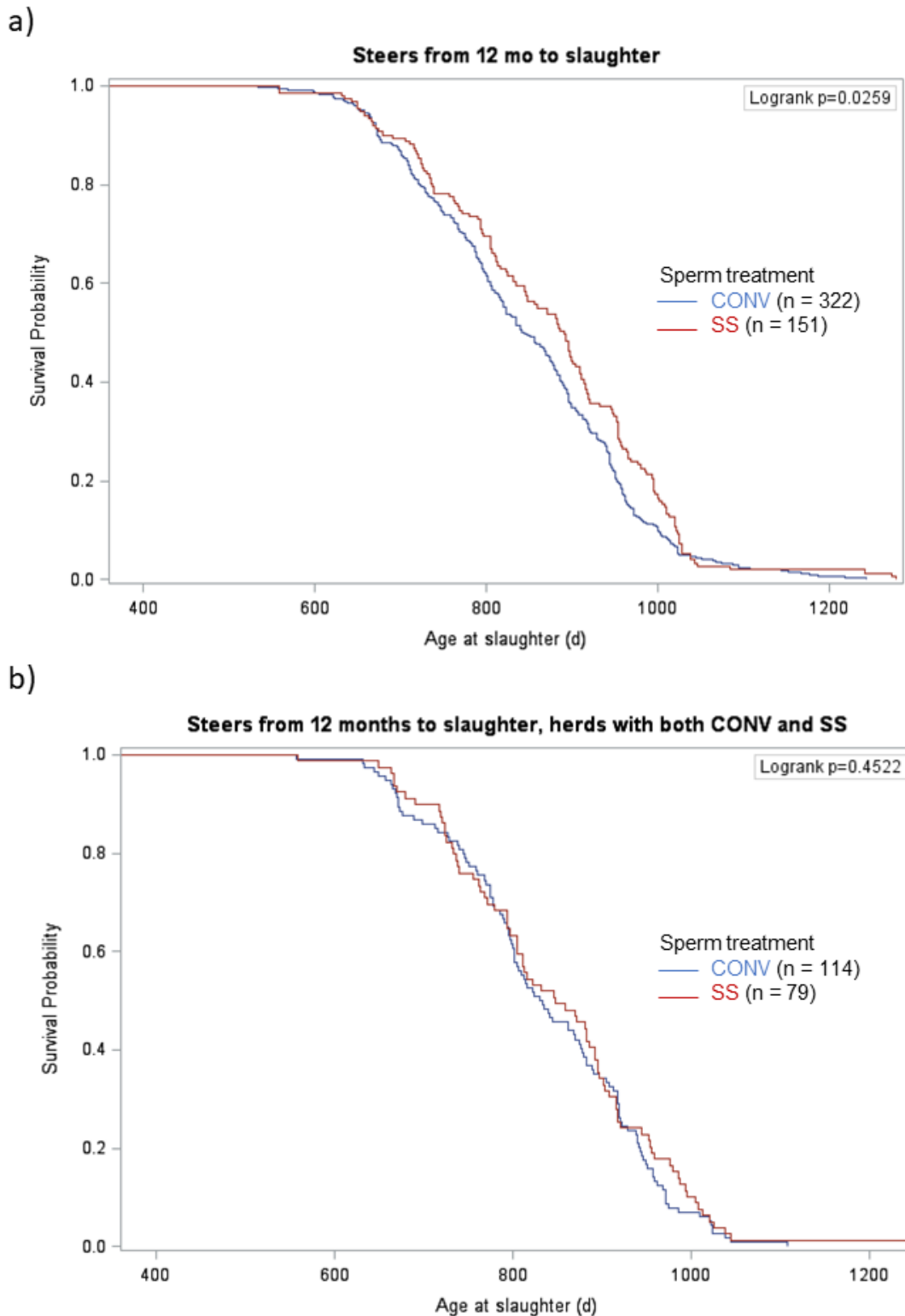


Figure 4.7. Survival analysis (Kaplan-Meier plot and P-value for the Logrank test) for the interval from birth to slaughter (age at slaughter, in days) of steers derived from AI with conventional (CONV; blue line) or sex-sorted (SS; red line)sperm: (a) when every herd at slaughter (i.e., the last herd where steers were located before being sent to slaughter, $n = 278$) was included in the analysis, or (b) when the analysis was restricted to those herds at slaughter ($n = 56$) that had both CONV and SS steers.

4.5 Discussion

This retrospective study compared calf survival during the first year of life and adult performance of offspring derived from AI with X-sorted or conventional sperm processed from the same ejaculate. The main findings of this study are: (1) survival during the first year of life was not different between animals that were derived from AI with X-sorted or conventional sperm; (2) male calves had higher mortality rates during the first 2 months of life than female calves independent of sperm treatment; (3) no differences existed due to sex-sorting in reproductive and lactation performance of female offspring; and (4) steers derived from AI with conventional sperm were slaughtered earlier and had greater carcass weight than those steers derived from sex-sorted sperm, but this finding seemed to be explained by differences in herd rearing and not due to the sex-sorting process.

No differences were observed in stillbirth rates, mortality rates or survival during the first year of life between calves produced after AI with conventional or X-sorted sperm derived from the same ejaculate, in agreement with Tubman et al. (2004) and Borchersen and Peacock (2009). Tubman et al. (2004) compared birth and weaning weight, calving ease score, calf vigour, neonatal (calves born dead or that died within 24 h of birth) and preweaning mortality rates of calves with different dam breeds (Holstein heifers and Angus heifers and cows), and found no difference between calves derived from X-sorted sperm and conventional sperm for any of the characteristics studied. Of note, although the data analysed by Tubman et al. (2004) included data from calves born in 13 different field trials in 13 herds with various experimental designs (mostly concerning numbers of sperm per insemination and site of insemination), conventional and X-sorted sperm doses were processed from the same ejaculates. Similarly, stillbirth rates were not different when Holstein, Jersey, and Danish Red heifers were inseminated with sex-sorted or conventional sperm doses from the same ejaculates (Borchersen and Peacock, 2009). In a study conducted on two dairy commercial herds in California, Chebel et al. (2010) observed that, among Holstein heifers that conceived to the first AI, the proportion of calves born dead was greater for those heifers that had been inseminated with X-sorted sperm than for those inseminated with conventional sperm (8.8% vs. 3.4%, respectively), independent of calf sex. A tendency for a greater incidence of stillbirths was also reported among singleton calves from Holstein heifers that had been inseminated with X-sorted sperm

(X-sorted: 13.3% vs. conventional: 11.7%; $P = 0.051$) in a single commercial dairy herd in Australia (Healy et al., 2013). However, these two studies were limited to either one or two herds, which limits the ability to extrapolate the results to all herds. In a large study exploring the use of X-sorted sperm in US Holstein herds, DeJarnette et al. (2009) reported a greater incidence of stillbirths among male calves when the pregnancy was established following insemination of heifers with X-sorted sperm compared with conventional sperm (X-sorted male = 19.9% vs. conventional male = 12.9%); this effect was partially explained by differences in dam age at calving. In a subsequent larger study, Norman et al. (2010) also observed the highest incidence of stillbirths among singleton male calves from heifers that had been inseminated with X-sorted sperm compared with conventional sperm (X-sorted male = 15.5% vs. conventional male = 10.8%); however, this difference was not statistically significant, and no difference in stillbirth rates of male calves to sperm treatment was observed in multiparous cows. A common characteristic of these four studies (DeJarnette et al., 2009; Chebel et al., 2010; Norman et al., 2010; Healy et al., 2013) is that X-sorted and conventional sperm were not derived from the same sires. The sire used during AI can influence the occurrence of perinatal calf mortality in dairy herds (reviewed by Mee et al., 2014). Furthermore, results from previous studies indicate that the decrease in field fertility of X-sorted sperm when compared with conventional sperm varied depending on the sire used, suggesting that the damaging effect of sex-sorting on sperm is sire-dependent (Frijters et al., 2009; DeJarnette et al., 2010; Healy et al., 2013; Maicas et al., 2019; Thomas et al., 2019). Therefore, comparison of X-sorted and conventional sperm produced from the same ejaculates from the same bulls, as in the present study, allows a more accurate evaluation of the effect of sex-sorting than studies that compared sex-sorted and conventional sperm from different bulls, where a sire effect cannot be discounted.

We observed greater mortality rates during the first 2 months of life for male calves than for female calves independent of sperm treatment. This may be related to a greater incidence of dystocia in male calves and its long-term consequences for calf health as male calves are, on average, heavier at birth than female calves (Sieber et al., 1989; Johanson and Berger, 2003; Tubman et al., 2004), which potentially causes a mismatch between the size of the foetus and the birth canal. Johanson and Berger (2003) found birth weight to be a strong predictor of dystocia, with a 1 kg increase in birth weight resulting in a 13% increase in the odds of dystocia. Greater incidence of

dystocia has been reported for male calves (Tubman et al., 2004; Norman et al., 2010). Dystocia can have a negative impact on calf health beyond the first 48 h after parturition and affect calf survival during the following month (Lombard et al., 2007; Barrier et al., 2013). In addition, male dairy calves are at risk of receiving less attention and care than female calves during this crucial period of rearing because they have a lower economic value (Renaud et al., 2017; Shivley et al., 2019).

None of the aforementioned studies evaluating offspring characteristics after using X-sorted sperm for AI (Tubman et al., 2004; Borchersen and Peacock, 2009; DeJarnette et al., 2009; Chebel et al., 2010; Norman et al., 2010; Healy et al., 2013) or IVP (Mikkola et al., 2015) investigated whether sex-sorting of sperm had effects on adult performance. Siqueira et al. (2017) reported adverse effects on lactation performance during the first lactation for heifers born after IVP when reverse X-sorted sperm was used, but not when conventional sperm was used. Unlike the current study, however, the data analysed by Siqueira et al. (2017) made it difficult to draw conclusions related only to the effect of sperm sex-sorting. For example, neither dams nor sires were randomly assigned to reproductive technique, thus parental effects and bias due to the preferential use of any of the reproductive techniques cannot not be discounted. Moreover, sperm was sex-sorted after cryopreservation (reverse X-sorted sperm), and was always used in association with IVP, which by itself has been associated with loss of genomic imprinting (Chen et al., 2015), abnormal fetoplacental development (Miles et al., 2004; Farin et al., 2006; Bloise et al., 2014), and increased neonatal mortality (Bonilla et al., 2014). In the present study, we evaluated adult performance of offspring born after AI with conventional or X-sorted sperm processed from the same ejaculate, which had been randomly used to inseminate dams in a large cohort of herds. We found no differences due to sex-sorting in survival and reproductive and lactation performance during the first, second or third lactation of female offspring. Overall, steers derived from AI with CONV sperm were slaughtered earlier and had greater carcass weight than SS steers, but it should be noted that this finding may be an artefact of the relatively small total number of steers and the relatively large number of rearing herds. Hence, there were differences in steer husbandry and nutritional management depending on the rearing herd. This was supported by the observation that the distribution of slaughter age did not differ between CONV and SS steers when the analysis was restricted to herds that had steers derived from both sperm types. An experimental study in which steers derived

from conventional or sex-sorted sperm from split ejaculates share herd management and body weight measurements at the same ages would be helpful to further clarify whether there is a true difference in body weight gain due to the sex-sorting process.

In conclusion, the results provide no evidence of differences in survival during the first year of life between offspring derived from CONV or SS, or for any of the reproductive and lactation performance characteristics studied between female offspring derived from CONV or SS. Herd rearing factors, and not the sex-sorting process, seemed to be involved in the difference found in overall carcass weight between CONV and SS steers; further investigation is needed to confirm this observation.

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CHAPTER 5: General discussion

The objectives of the work described in this thesis were (1) to investigate the phenotypic fertility of dairy heifers and lactating cows inseminated with sex-sorted sperm in Irish seasonal-calving pasture-based dairy herds, and (2) to assess the survival and the productivity of offspring derived from sex-sorted vs. conventional sperm.

The skewed sex ratio in favour of female calves obtained after insemination with X-sorted sperm offers multiple potential advantages to the dairy farmer (Butler et al., 2014a; Holden and Butler, 2018). However, studies conducted before 2013 indicated that the field fertility of frozen sex-sorted sperm was lower compared with that of frozen conventional sperm in both heifers and lactating cows (DeJarnette et al., 2009; Chebel et al., 2010; DeJarnette et al., 2010; Norman et al., 2010; DeJarnette et al., 2011; Sales et al., 2011; Healy et al., 2013). This is an obstacle to the widespread adoption of the technology, especially in seasonal pasture-based systems such as that operated in Ireland, which are highly dependent on an excellent fertility during the breeding season to match herd pasture feed requirements with the pasture growth profile (Butler, 2014).

In 2013, Sexing Technologies began to introduce changes to the original XY technology (Vishwanath and Moreno, 2018), which are collectively called SexedULTRA™, in an effort to alleviate the negative impact that the sex-sorting process could have on sperm lifespan and functionality, one of the main factors thought to be responsible of the reduced fertility of sex-sorted sperm (Frijters et al., 2009). In addition, results obtained in a study conducted with fresh sex-sorted sperm in seasonal pasture-based dairy herds in New Zealand (Xu, 2014) suggested that fresh sex-sorted sperm may have the potential to yield higher fertility than frozen sex-sorted sperm (Xu, 2014). These two facts motivated our group to conduct the large field study described in Chapter 2. In that study, a temporary sex-sorting laboratory was set in Ireland. We used split ejaculates in order to mitigate against ejaculate-to-ejaculate variability (Utt, 2016; Harstine et al., 2018), and we compared P/AI of seasonal-calving pasture-based dairy heifers and lactating cows inseminated after spontaneous oestrous with fresh sex-sorted sperm (at two different sperm doses), frozen sex-sorted sperm or fresh conventional sperm. The main findings of this first study were:

- Sex-sorted sperm achieved relative P/AI ranging from 78.4 to 88.9% of those achieved with conventional sperm.

- In lactating cows, both fresh sex-sorted sperm treatments achieved lower P/AI than conventional sperm, and in heifers, although P/AI achieved by both fresh sex-sorted sperm treatments were not statistically different from conventional sperm, there was a tendency to be lower ($P < 0.10$). Moreover, P/AI achieved with fresh sex-sorted sperm did not differ from that achieved with frozen sex-sorted sperm, and doubling the concentration of sperm in fresh sex-sorted sperm straws did not improve P/AI.
- Frozen sex-sorted sperm, which was packaged at 2×10^6 sperm per dose, had lower P/AI than conventional sperm in both heifers and lactating cows, which agrees with many other studies conducted with a similar dose (DeJarnette et al., 2009; Chebel et al., 2010; DeJarnette et al., 2010; Norman et al., 2010; DeJarnette et al., 2011; Sales et al., 2011; Healy et al., 2013; Djedović et al., 2016; Lenz et al., 2016).
- There was a significant bull effect on P/AI in both lactating cows and heifers. Additionally, the P/AI of lactating cows inseminated with fresh sperm (conventional and sex-sorted) varied between bulls as dispatch-to-AI interval increased.
- Heifers had greater P/AI than lactating cows, and P/AI in lactating cows decreased with increasing parity. Among young cows (parity ≤ 2), the following cow characteristics were associated with greater likelihood of pregnancy establishment: BCS ≥ 3 , DIM > 60 d, and fertility sub-index of the EBI $> €100$.

In that first study, despite having lower P/AI than conventional sperm, frozen sex-sorted sperm had greater P/AI relative to conventional sperm (84.7%) than reported in previous studies with lactating cows (DeJarnette et al., 2010; Norman et al., 2010). This, coupled with the commercialisation of SexedULTRA-4M™ in 2017, motivated our group to conduct the large field study described in Chapter 3. In that study, once again, we used a split-ejaculate technique, and we evaluated the reproductive performance at spontaneous oestrus of frozen sex-sorted sperm at 4×10^6 sperm per dose (SexedULTRA-4M™) relative to frozen conventional sperm at 15×10^6 sperm in seasonal-calving pasture-based lactating dairy cows. The difference in sperm concentration between the control and the sex-sorted treatment reflects the commercial reality. In the absence of a sex-sorting laboratory in Ireland at this time, while the ejaculates of 4 bulls were collected near sex-sorting laboratories (resident

bulls), the ejaculates from the other 6 bulls were shipped by air to the sex-sorting laboratory. The main findings of this second study were:

- Overall, sex-sorted sperm achieved a mean relative P/AI of 76.0% compared to CONV. Although P/AI results for both conventional (59.9%) and sex-sorted (45.5%) sperm were better than those achieved in our previous study (48.0% vs. 40.6%, respectively; Chapter 2), the relative P/AI was not improved either across all bulls, or when the analysis was restricted to resident bulls alone.
- The effect of sex-sorting on P/AI was variable between bulls, with greater variation amongst bulls whose ejaculates were shipped to the sorting centre compared with ejaculates from resident bulls.
- Relative P/AI was greater in lactating cows with high or average fertility potential (defined by parity ≤ 2 , DIM at AI ≥ 70 , EBI fertility sub-index $> €60$) than in those with low fertility potential (58.1%).
- Around one third of the enrolled herds had a relative P/AI $> 90\%$, explained by the fact that most of those herds had an excellent performance with sex-sorted sperm but moderate or poor performance with conventional sperm.

The results of these two field studies would suggest that the gap in field fertility between sex-sorted sperm and conventional sperm still exists for both heifers and lactating cows inseminated after spontaneous oestrus, at least under the Irish seasonal-calving pasture-based system.

Although in both studies the P/AI achieved with sex-sorted sperm was lower than that achieved by conventional sperm, it was highly dependent on the bull, the fertility potential of the female, and the herd where the AI took place. Even though all ejaculates passed minimum pre-sorting and post-thaw quality control criteria to be released from the sex-sorting laboratory, we observed variation between bulls in field fertility, in agreement with previous studies (Frijters et al., 2009; DeJarnette et al., 2010; Thomas et al., 2019). It seems that the tests used during quality controls – which were limited to subjective assessment of sperm motility and acrosome integrity – were not sufficient to detect changes in sperm characteristics due to sex-sorting that could affect sperm lifespan or functionality *in vivo* (e.g., survival during its transit through the uterus and oviduct, capacity to interact with the oviductal reservoir and with the oocyte). More objective and powerful analyses are currently available and continually evolving, such as motility assessment by CASA and multiparametric flow cytometric analysis of

sperm characteristics as well as testing sperm function with *in vitro* fertilisation. The inclusion of such assays during these quality controls may increase the ability to detect and discard affected ejaculates. Moreover, premature sperm capacitation-like modifications could be responsible for the detrimental effect that sex-sorting induces in some ejaculates (Lu and Seidel, 2004; Bucci et al., 2012; Umezu et al., 2017; Carvalho et al., 2018). Therefore, further research should be focused on the detection of early capacitation-like modifications due to the sex-sorting and freezing, and to the development of *in vitro* assays that allow to differentiate between bulls of different susceptibility.

The results of our studies show that the use of sex-sorted sperm does not have to be limited to heifers as traditionally advised, and that breeding strategies can include heifers and cows of high fertility potential. We defined cow characteristics that increase the likelihood of successful pregnancy establishment (parity ≤ 2 , BCS ≥ 3 , DIM > 60 d, and fertility sub-index of the EBI $> \text{€}100$ during the first study; parity ≤ 2 , DIM at AI ≥ 70 , EBI fertility sub-index $> \text{€}60$ during the second study), and confirm that cows with those characteristics (i.e. elite cows) had greater P/AI with sex-sorted sperm than non-elite cows. Therefore, when using sex-sorted sperm in lactating cows, P/AI can be increased by targeting its use to early calving, young cows with the greatest fertility potential.

Caution should be taken when comparing the fertility rates for fresh sex-sorted sperm reported by Xu (2014) with those of our study because of the differences in the nature of the fertility measurements between both studies. Whereas P/AI in our study were calculated based on a combination of ultrasound scanning at 55 to 80 days post AI and calving rates, Xu (2014) used a 24-d NRR. Non-return rates are influenced by many factors not associated with conception (e.g., poor oestrus detection, culling, death, breeding management decisions). Moreover, 24-d NRR is a very early fertility measure, which may not reflect embryo losses occurring after maternal recognition of pregnancy (Humblot, 2001), which occurs around Day 16, because of the consequent delayed luteolysis (Northey and French, 1980; Humblot and Dalla Porta, 1984). Consequently, any possible difference between P/AI achieved by sex-sorted and conventional sperm will be more evident for later and more accurate fertility measures such as those used in our studies. Therefore, a bigger gap between the fertility of sex-sorted and conventional sperm than of that reported by Xu (2014) was, to a certain

extent, expected. Nevertheless, as pointed out in Chapter 2, a lack of optimization of the media used to extend the fresh sex-sorted treatments used in the first study — which differed from that used by Xu (2014) and from that used with frozen sex-sorted sperm in both of our studies — could have negatively influenced P/AI achieved by those fresh treatments, since high levels of sperm agglutination and lower progressive motility were reported when fresh sex-sorted straws used in this trial were analysed (Holden et al., 2016). No sperm quality problems were reported for fresh sex-sorted sperm processed with the latest sex-sorting technology in a more recent *in vitro* study (González-Marín et al., 2018). Furthermore, according to an article published on the website of the New Zealand company LIC (<https://www.lic.co.nz/news/sexed-semen-make-it-liquid-make-it-fresh/>), the fertility of their fresh sex-sorted product is only 1 to 5% behind that of their fresh conventional product. The demand of fresh sperm increases dramatically during the peak of the Irish breeding season, and dairy farmers would be interested in using fresh sex-sorted sperm from high genetic merit sires. Large field studies evaluating the fertility of fresh sex-sorted sperm processed with the latest sex-sorting technology should be useful to confirm that the gap in fertility between fresh sex-sorted and conventional sperm has been reduced.

With regard to the fertility of frozen sex-sorted sperm in lactating cows during our studies, our results agree with other two recent studies conducted with lactating cows (Utt et al., 2017; Xu et al., 2018) that also used a split-ejaculate technique, demonstrating that lower P/AI achieved with SexedULTRA-4M™ vs. conventional sperm was due to the sex-sorting process and (or) to the lower final sperm dose, but not to ejaculate-to-ejaculate variation. In order to further clarify how much of the decrease in fertility is due to these factors, it would be interesting to conduct a trial with SexedULTRA-4M™ and conventional sperm processed from the same ejaculates and extended at equivalent sperm doses (e.g. SexedULTRA-4M vs. conventional sperm at a 4 million dose). This trial should be ideally conducted inseminating heifers or cows of high fertility potential in well-managed herds and included ejaculates from several bulls. If a low sperm dose is selected for both sperm treatments, some factors should be kept in mind: (1) the low sperm dose could be sufficient to compensate the negative effect that sex-sorting may have on the function and longevity of sperm from certain bulls but not for other bulls; and (2) it is possible that lowering the sperm dose in conventional straws could also affect P/AI of certain bulls whose sperm suffer from compensable defects that are not evident when commercial sperm doses are used. In

order to get the maximum amount of information possible that allows to delimit which sperm characteristics and functions are impaired by sex-sorting, the trial could be accompanied by the assessment of sperm from representative straws by (1) a battery of CASA and flow-cytometric tests that evaluate sperm at different time-points after thawing; (2) *in vitro* tests evaluating the capacity of sperm to interact with the oocyte vestments; and (3) evaluation of embryo development (including zygote formation) and embryo quality after *in vitro* fertilisation.

That around one third of the enrolled herds had a relative P/AI > 90% in the second study, and this was explained by most of those herds having an excellent performance with sex-sorted sperm but moderate or poor with conventional sperm, was intriguing. We discounted that the cows in those herds had better reproductive characteristics, which would have benefited P/AI of sex-sorted sperm, but also of conventional sperm. Farmers were responsible for the identification of cows ready for AI, so the accuracy of heat detection and the decision to identify the best time of AI varied widely between farms. Therefore, one possible explanation for this observation was that those particular farms had decision rules for the timing of AI that were particularly suited to sex-sorted sperm (i.e., delayed AI relative to heat onset). In fact, improved P/AI has been reported for delayed AI, therefore, closer to ovulation, when sex-sorted sperm was used (Sá Filho et al., 2010; Sales et al., 2011; Thomas et al., 2014; Bombardelli et al., 2016). An experiment was conducted by our group during the 2019 breeding season (Drake et al., manuscript submitted for publication) to test this hypothesis, in which approximately 2,250 early-calving (DIM > 50) younger cows (parity 1 to 4) on 24 farms were synchronized with a Progesterone-Ovsynch fixed-time AI protocol and inseminated with frozen conventional sperm 16 h after the second GnRH injection (CONV-16), frozen sex-sorted sperm 16 h after the second GnRH injection (SEXED-16), or frozen sex-sorted sperm 22 h after the second GnRH injection (SEXED-22). Pregnancy status was assessed by ultrasonography 35-40 days after the timed AI. The greatest P/AI was achieved by the CONV-16 treatment (61%), but, contrary to hypothesized, P/AI of sex-sorted sperm did not increased when the AI was delayed (P/AI for SEXED-16 = 49.3%; P/AI for SEXED-22 = 51.2%). Similar to what happened in our second study, marked variation in relative P/AI existed between herds: when herds were ranked by relative P/AI and divided in thirds, relative P/AI of herds in the best third ranged from 93.9% to 116.8%, relative P/AI of herds in the average third ranged from 77.7% to 90.9%, and relative P/AI of herds in the poorest

third ranged from 50.9% to 76.9%. Ejaculate and bull effects were discarded as contributors that could explain this variation in fertility between sperm treatments observed between herds, since sperm treatments were derived from split ejaculates, and the proportion of conventional and sex-sorted straws from each bull was identical for each herd. Within each herd, treatments were balanced for parity and DIM, and cows within each herd were exposed to the same nutrition and herd management conditions. However, not every AI technician contributed to every herd, with 16 of the 18 AI technicians being assigned to a single herd each. Moreover, there was an effect of AI technician on P/AI achieved by sex-sorted sperm. Therefore, P/AI achieved with sex-sorted could have been influenced, perhaps, by differences between AI technicians in any of the procedures involved during insemination (e.g. handling of straws, temperature and duration of thawing, interval from thawing to deposition of sperm in the uterus, site of sperm deposition). In our studies, both conventional and sex-sorted sperm were packaged in 0.25-mL straws, which have a large surface-to-volume ratio that makes them vulnerable to rapid temperature fluctuations. Diskin (2018) reviewed the impact that straw handling and insemination technique have on P/AI, and recommended, among other things, that straws are thawed to a maximum speed by placing them in a water bath at 35°C for 45 seconds and then protected from either cold or warm shock, that not more than six straws are thawed simultaneously, and that all six straws are inseminated within 15 min. Slightly variations in the way that AI technicians performed these steps could have had an impact on P/AI during our trials. Early studies with sex-sorted sperm found no advantage to uterine horn compared to uterine body insemination (Seidel et al., 1999; Seidel Jr and Schenk, 2008). However, Diskin (2018) observed that switching to uterine horn insemination appeared to be beneficial mostly when AI technicians were achieving low P/AI with uterine body insemination, and suggested that this could be due to the avoidance of cervical insemination. Differences in the competence of the AI technician to correctly place the sperm in the uterine body or beyond could have a more pronounced effect on P/AI when sex-sorted sperm is used, since sex-sorted sperm is packaged in lower sperm doses than conventional sperm. Further research could be focused on the development of procedures that reduce this variation among AI technicians when sex-sorted sperm is used.

Although there is a growing number of calves derived from sex-sorted sperm, only a few follow-up studies have investigated whether sex-sorting had subsequent

effects on calf survival, and they reported contrasting results (Tubman et al., 2004; Borchersen and Peacock, 2009; DeJarnette et al., 2009; Chebel et al., 2010; Norman et al., 2010; Healy et al., 2013; Mikkola et al., 2015). The question of whether sperm sex-sorting could have an effect on the adult phenotype of the offspring was highlighted by Hansen and Siqueira (2017) who reported that IVP was associated with adverse lactational outcomes during the first lactation when reverse sex-sorted sperm was used, but not when conventional sperm was used (Siqueira et al., 2017). A detrimental effect on health and productivity of offspring derived from sex-sorted sperm, especially if female offspring are affected, could negate the advantages of biased offspring sex ratio. In the third study (Chapter 4), we compared perinatal and postnatal mortality as well as productive and reproductive performance of offspring derived from AI with sex-sorted or conventional sperm processed from the same ejaculates. The main results of that study were:

- Calf survival during the first year of life was not different between animals that were derived from AI with sex-sorted or conventional sperm.
- Male calves had higher mortality rates during the first 2 months of life than female calves independent of sperm treatment.
- No differences existed due to sex-sorting in survival, and reproductive and lactation performance of female offspring during the first, second or third lactation.
- Overall, steers derived from conventional sperm were slaughtered earlier and had greater carcass weight than steers derived from sex-sorted sperm. However, the distribution of slaughter age did not differ when the analysis was restricted to herds that had steers derived from both sperm types.

The results of our retrospective study do not provide any evidence of impaired health and productivity among offspring derived from AI with sex-sorting, at least in the case of the female offspring. Herd rearing factors, and not the sex-sorting process, were likely involved in the difference in overall carcass weight found between steers derived from X-sorted or conventional sperm. However, the characteristics of the study make difficult to fully attribute the difference to only the herd effect, since the total number of steers was relatively small but the number of rearing herds large, and steers could have been reared in more than one herd before being sent to the slaughter, each of those rearing herds likely having different nutritional and management practices. Therefore, controlled studies are needed to clarify whether there is a true negative

effect on body weight gain of steers due to the sex-sorting process, and the economic impact that this effect, may have, if any.

Sperm have long been considered to carry only the paternal genetic component, but accumulating evidence suggests that sperm biomolecules influence epigenetic reprogramming during early embryonic development and affect long-term development up to adulthood (Donkin and Barrès, 2018). To what extent the sperm epigenome may be transmitted to the embryo and impact the subsequent development and performance of the progeny is currently unknown. The common assumption is that DNA methylation changes in sperm occur exclusively prior to ejaculation, thus this mechanism would not be relevant for sex-sorted sperm. However, DNA methylation-modifying enzymes have been found in mature sperm (Marques et al., 2011; Ni et al., 2016), dynamic DNA methylation can occur in non-dividing cells (Pattamaprapanont et al., 2016), and somatic cells may deliver small RNAs directly to the mature sperm (Sharma et al., 2016). Thus, as highlighted by Donkin and Barrès (2018), mature sperm may be epigenetically remodelled even after ejaculation. Thus, new knowledge on the role of the sperm epigenome in shaping the offspring phenotype and regulating molecular mechanisms governing embryo development in cattle is essential.

One other area that requires research is the application of sex-sorted sperm to multiple ovulation embryo transfer. Many studies have reported impaired reproductive performance when sex-sorted sperm was used in conjunction with *in vitro* embryo production, or during superovulation programs, particularly in cows. *In vitro* fertilisation with sex-sorted sperm resulted in delayed cleavage and (or) blastocyst formation rate (Wilson et al., 2006; Bermejo-Álvarez et al., 2008; Palma et al., 2008; Bermejo-Alvarez et al., 2010; Trigel et al., 2012). Nonetheless, use for *in vitro* fertilisation means more oocytes can be fertilised per sperm dose than would be achievable in AI. Superovulated donors inseminated with sex-sorted sperm yielded a smaller proportion of transferable embryos and larger proportions of unfertilised oocytes and (or) degenerate embryos (Peippo et al., 2009; Monteiro Jr et al., 2016; Mikkola and Taponen, 2017), and a lower proportion of grade 1 embryos (Larson et al., 2010; Soares et al., 2011; Mikkola and Taponen, 2017) than donors inseminated with conventional sperm. However, data from these studies originated from sex-sorted sperm produced before the most recent improvements in sex-sorting technology. SexedULTRA™ sperm was used in a recent study (Dell'Eva et al., 2019) investigating

different superovulation protocols for dairy lactating cows with sex-sorted sperm; however, this study did not include a control treatment (conventional sperm). Thus, it remains to be clarified if the latest technical improvements in sex-sorting technology lead to an increase in the efficacy of embryo production with sex-sorted sperm.

CHAPTER 6: References

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