THESIS

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Methods of influencing the sex of newborn with special regards to sex sorted semen

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1. List of abbreviations:

AI – artificial insemination

ANOVA – analysis of variance

AuNP – functionalized gold nanoparticles

CASA - computer assisted sperm analyzer

DRMS - Dairy Records Management Systems

DUI – deep uterine insemination

ET – embryo transfer

FACS – Fluorescence activated cell sorting

FISH - Fluorescent *in situ* hybridization

IVF – in vitro fertilization

LAI – laparoscopic inseminations

NRR – non return rate

PIA – percent intact acrosome

PMN - polymorphonuclear leukocyte

TALP - Tyrode´s albumin lactate pyruvate

TRIS - Tris(hydroxymethyl)aminomethane

2. Abstract:

The world's population and its demand for food, including animal products, is growing constantly and is higher than ever before. Besides that, problems like animal welfare and environmental protection are getting more and more important. Further the Sex of Newborn farm animals is one of the most important aspects in animal breeding and genetic progress. All these aspects lead to the desire to be able to influence the sex of newborn animals ideally before artificial insemination. Flow cytometry turned out to be the best suited technology for sex sorting today. During the past decades many advancements and improvements in this technology led to the development of commercially available sexed AI straws used by the cattle industry. Sexed semen products for other species are developed as well. Flow cytometry is currently the most refined and widely used sex sorting technology in existence, but alternatives are being researched as well. In the beginning the reduced fertility and viability of the sperm cells was a big challenge, which led to a limited field of use, which was mainly the insemination of the relatively more fertile nulliparous females. These problems are mostly overcome by the collaboration of many scientists around the world, which led to the development of products like SexedUltra-4M™ and a more widespread use of sorted semen in dairy cattle. Further the next generation of sorted sperm products is currently being researched. Efforts to further establish this technology in other species are promising and might lead to a revolution in their reproduction.

The technology and its improvements, the current state of the art, the economy, future perspectives and the use in other species are described in this thesis.

3. Goals/question:

Is sex sorted semen a viable tool to produce newborn having the desired sex in a reliable and economic sense?

4. History:

Being able to predict the sex of the newborn animal has been one of the highest goals of animal breeding throughout history. For a long time, the basic principles of the biologic causes of sex determination and the probability laws were not understood, which lead to many setbacks in the regards of predetermining the sex of newborn. The chances to get 10 males or females in a row has a probability of 1:1024 assuming a sex ratio of 50:50. This translates to roughly 1000 occurrences of that happening annually in North America alone, based on an estimated 1 million cattle herds. Which means the chances of events like these happening are not unheard of and were in turn often ascribed to certain events like natural phenomena or changes in feeding, "even though the skewed sex ratio is almost always due to chance alone". (Seidel and Garner, 2008) The statistics show that the average sex ratio using unsorted semen for AI or ET is at about 51% males and in IVF at about 53% male calves. (Hasler et al., 1995) It was found that this ratio can be slightly altered by the herd management. Poor management resulted in a ratio of 49% males and very old cows produced 53% male calves (Skjervold and James, 1978)

Advancements in science during the 20th century especially significant breakthroughs in genetics led to the discovery of the X- and Y- chromosomes, which later enabled the differentiation of X- and Y- bearing sperm cells and therefore sex sorting. This realization caused some optimism but did not yet lead to the ability to predetermine the ability to predict the sex of the neonate. (Seidel and Garner, 2008)

L. Johnson from the US Department of Agriculture published "the landmark paper on semen sexing" (Johnson et al., 1989) which portrays a major breakthrough in influencing the sex of unborn animals by proving for the first time that it's possible to introduce an in situ fluorescent signal into the cell without damaging the cell itself. His success was demonstrated by the birth of rabbits having the predetermined sex using his procedures. (Johnson et al., 1989)

5. Flow cytometry:

The most advanced technology available and at the same time the only technology used commercially today is flow cytometry. (Sharpe and Evans, 2009) Dr. George Seidel and his team are credited for the further development, and refinement for the field application of this technology. (R. Vishvanath and Moreno, 2018)

The primary distinguishing feature between the X and Y chromosome is the difference in DNA content (Garner, 2009) In cattle the X-chromosome content is around 4% higher compared to the Y-chromosome. (Garner et al., 2013) The collected semen is prepared for sorting using "carefully cleansed equipment and sterilized media" (Garner et al., 2013) by checking for volume, concentration and motility. The antibiotics Tylosin, Gentamycin and Linco-Spectin are added to protect the cells from unwanted bacterial contamination. The sample is stored for up to 7 hours and diluted to a concentration of 200 $x10^6$/ml. An aliquot of sperm is stained in a staining TALP media (Tyrode´s albumin lactate pyruvate) (Garner et al., 2013) with the "synthetic fluorescent dibenzimidazole dye Hoechst 33342" (Garner, 2009) which can permeate the cell wall and binds selectively to adenine-thymine base pairs. This dye is relatively non-toxic and does not cause more obvious abnormalities in offspring compared to inseminations with unsorted sperm in mammals (Garner, 2009). After the staining process, the stained sperm is filtered using a 50 µm mesh to "remove clumped sperm, media aggregates and seminal debris". (Garner et al., 2013) By adding FD&C #40 food coloring to the TALP solution the Hoechst 33342 dye will be quenched out of cells with a damaged cell membrane therefore identifying nonviable sperm cells. (Garner et al. 2013)

After the sample is stained the sperm will be sorted by a flow cytometer, which can be seen in the picture below (Fig 1). The sperm is surrounded by a "sheath fluid" consisting of a TRIS (Tris(hydroxymethyl)aminomethane) buffer, citric acid monohydrate and fructose. To ensure an accurate reading each sperm cell is oriented in the orienting nozzle so that the flat surface of the sperm nucleus can be used for the evaluation of the DNA content using an electric charge. At the end of the orienting nozzle a piezoelectric vibrator (A) separates the stream into individual droplets, each containing one sperm cell. The droplets are now passed in front of a UV-laser beam (B) with a wavelength of approximately 355 nm. The laser will activate the fluorescent Hoechst 33342 dye and allow the precise measurement of the DNA content of each sperm cell and therefore differentiate between X- or Y-bearing spermatozoa. The DNA content is evaluated by "dual orthogonal photodetectors" (C), positioned at a 90-

degree angle which measures the fluorescence (wavelength ~460nm) emitted by individual cells. Two detectors are used to increase sorting accuracy.

“This system can provide sorted subpopulations of X- or Y- bearing sperm at a rate of 8000 sperm/s at a purity of 90%” (Garner et al., 2013)

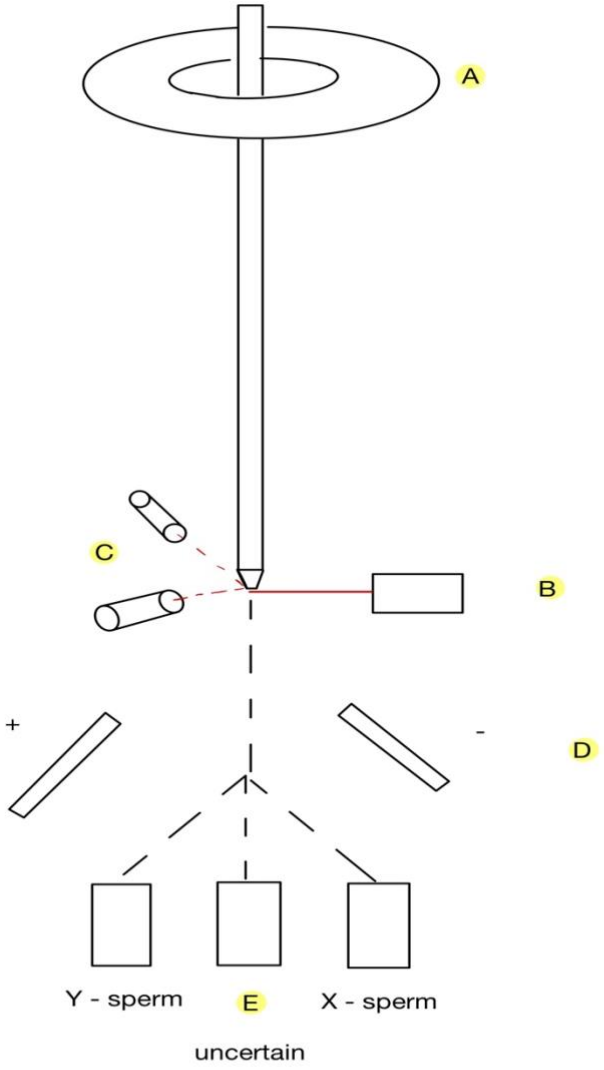
Either a positive or a negative charge is applied to the droplets in accordance with the detected DNA content. This allows the droplets to be separated into three different containers (E) by being deflected by the electrodes. (D) One containing the X- and one containing the Y-chromosome bearing sperm cells. The middle vessel will catch the damaged or improperly stained cells. (Garner et al., 2013)

The industrialization of the process was made by the introduction of the Genesis III sex sorting machines, which were invented by Cytonome ST (Boston, MA, USA) and is represented by the schematic below. (Fig. 1) This sorting system replaced the older MoFlo system, which was slower and required specifically trained personnel. The Genesis III system is a more automated system with dual solid-state lasers oriented at 0° and 90° respectively. It can create X- and Y- chromosome bearing subpopulations at a rate of 8000 cells per second at an input rate of 40.000 cells per second and a purity of about 90%. In practice multiple nozzles are working in parallel (Sharpe and Evans, 2009, Vishvanath et al., 2014) to improve throughput and reduce the cost by using the economy of scale.

Banks of five Genesis III are put in parallel in one pod to improve overall efficiency (Vishwanath and Moreno, 2018)

Modern clusters allow sorting speeds of up to 1 billion cells per hour according to claims by the Cytonome Company. (Boston, MA, USA)

Fig.1: Sorter



After the sorting process the sperm is cooled down to 5°C and then centrifuged at 850g for 20 minutes to reconcentrate the cells to then be examined for motility, integrity and concentration. If at least 70% of the sperm cells in the sample show progressive motility they will be packed in straws and then cryopreserved. (Garner et al., 2013)

This “Sperm Cell Separation System” (patent number US 8,765,365 B2) was patented by John L. Schenk, George E. Seidel and Tae Kwang Suh in July 2014 (patent number US 8,765,365 B2)

6. Problems associated with using sex sorted semen:

In contrast to conventional semen the processing of sex sorted semen involves more than 20 steps (Vishwanath, 2014) and the number of cells per insemination straw is reduced to lower the price per dose due to the high production expenses. This raises the question if the insemination with a dose of sex sorted semen is just as successful as an insemination using unsorted semen and if not, is it still financially sensible? What can be done to improve the fertility of sorted semen dosages?

Studies conducted by DeJarnette et al., published in 2011, concluded that the fertility of sex sorted semen is about 60% in comparison to unsorted semen. This study was conducted on 9172 inseminations of Holstein cows and heifers (DeJarnette, 2011).

Due to the complexity of the semen sexing process in comparison to conventional semen the production cost per straw is very high. To make the insemination dose economically viable the concentration of commercially available sex sorted sperm is commonly reduced to about $2{,}1x10^6$ sperm cells per dose. In an attempt to increase the conception rates DeJarnette et al. increased the cell concentration per dose in his above-mentioned study from $2{,}1x10^6$ to $10x10^6$ cells per dose and compared the “semen quality characteristics” (DeJarnette et al., 2011) (Table 1) and conception rates to those of conventional semen at the same concentrations. He observed a “moderate improvement in the fertility potential” of a 5 percent increase in success rate but concluded that the results were “discouraging” since the difference in fertility potential between conventional and sex sorted semen was still significant (17 %). (DeJarnette et al., 2011)

Table 1: Effects of sex sorting and sperm dosage on conception rates

Sperm dose ($x10^6$)	Ejaculates (n)	Motility 0h	Motility 3h	Acrosomal integrity	Prim. abnormalities	Sec. abnormalities
2.1 sorted	21	53 ±0.9	43 ±1.2	72 ±1.3	4.7 ±0.5	4.2 ±0.6
10 sorted	61	50 ±0.5	43 ±0.7	72 ±0.8	4.1 ±0.3	4.5 ±0.3
2.1 conv.*	8	57 ±2.2	45 ±2.3	69 ±2.7	NA	NA
10 conv.*	8	55 ±2.2	43 ±2.3	67 ±2.7	NA	NA

* conv. = conventional semen

(DeJarnette, 2011)

"Descriptive statistics of semen quality characteristics of sex sorted and conventional semen across 8 Holstein sires" (DeJarnette et al., 2011)

7. Sexed ultra – current state of the art:

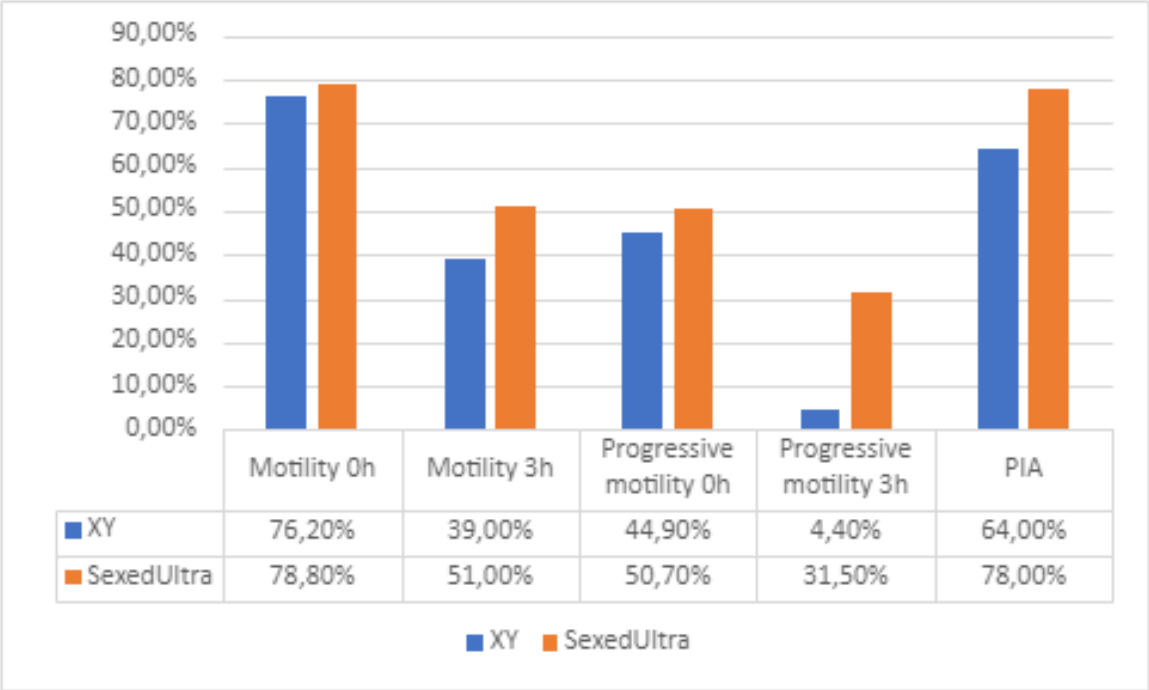
As described above more than 20 steps are involved in the process of sex sorting the sperm cells, including many changes in the environment and the cryopreservation process. These are added burdens for the sperm cells which reduce their integrity and viability. Since just increasing the sperm concentration per insemination dose did not bridge the fertility gap between sex sorted and conventional semen, many steps in the process of sorting were further optimized over the years making the process more benign for the individual cells. These improvements include an optimized media in which the spermatozoa are kept throughout the sexing process. The result of this is the SexedUltra ™ technology by the STgenetics company in Navasota, Texas. SexedUltra ™ promises improved sperm quality after cryopreservation, increased in vitro fertility and better field performance. (STgenetics™)

To analyze the improvement in sperm quality using the SexedUltra ™ technology a laboratory trial was conducted. Ejaculates from 12 bulls from Sexing Technologies, Navasota, Texas were used and divided into two aliquots each. One aliquot was sorted with the old XY method, the other one using the SexedUltra ™ technology. After cryopreservation and thawing, the total and progressive motility as well as the acrosomal integrity was observed at 0 hours and after 3 hours of incubation at 37°C, by a CASA (computer assisted sperm analyzer, Hamilton Thorne IVOS II System). (Gonzalez-Martin at al., 2016). The

results (Diagram 1) show that the SexedUltra ™ technology produced better results in all three characteristics tested. Total motility was improved by 11.6% at 0 hours (78.8% vs 67.2%) and 12% (51.0% vs 39.0%) after 3 hours of incubation. The same trend was observed for progressive motility with an improvement of 5.8% (50.7% vs 44.9%) at 0 hours and 27.1% (31.5% vs 4.4%) after 3 hours of incubation. The percentage of intact acrosomes increased by 14% (78.0% vs 64.0%) using the SexedUltra™ technology. (Gonzalez-Martin at al., 2016)

Further the in vitro fertilization (IVF) was examined as an indicator of "sperm competence" (Gonzalez-Martin at al., 2016). Eight ejaculates from two bulls were used in this test (Sexing technologies, Laceyville, Pennsylvania). More than 5000 oocytes were used for IVF per technology with 5000 motile spermatozoa per oocyte and 5-10 oocytes per IVF drop. The results show a significant improvement in "total and freezable embryo numbers" (Gonzalez-Martin at al., 2016) using the SexedUltra™ technology (Table 2). These trials proved that the better environment during the sexing process provided by the SexedUltra™ procedure has a significant effect on the cell integrity and performance of the sorted semen (Gonzalez-Martin at al., 2016).

Diagram 1: SexedUltra™ vs. XY



PIA = percent intact acrosome

(Gonzalez-Martin at al., 2016)

Table 2: In Vitro Fertilization

Treatmant	Oocytes for IVF (n)	% 8-cell rate (n)	% total embryos (n)	% freezable embryos (n)
XY	5082	32.7% (1664)	18.4% (937)	9.2% (472)
SexedUltra	5081	34.8% (1770)	22.3% (1134)*	13.2% (669)*

"*treatments within IVF endpoint differ (P<0,05)"

(Gonzalez-Martin at al., 2016)

A trial was conducted to determine the actual field performance using ejaculates from eight bulls (Select Siles, Ohio, USA), each was split into two aliquots. One aliquot from each ejaculate was sorted using the XY method, the other one using SexedUltra ™. In total 6930 Holstein heifers in 41 commercial herds located in the USA were inseminated. The collected

data on conception rates were analyzed using a mixed model ANOVA (analysis of variance) by the SAS Institute Inc., Cary, NC, USA. The results of this field trial showed that the heifers inseminated with semen using the SexedUltra ™ method had a 4,5% higher conception rate (45.7% ± 1.7% vs. 41.2% ± 1.6%) than heifers inseminated with semen using the older XY method. (Lenz et al. 2016)

"This is the first report of an improvement in conception rates using sex-sorted bovine semen in a decade since it became commercially available." (Lenz et al., 2016)

8. SexedUltra-4M ™ – current state of the art:

Another investigation was conducted to evaluate the effects of sperm concentrations using SexedUltra ™ sex sorted semen. This trial was carried out on ejaculates from five dairy bulls owned by the German Genetics International GmbH in Cloppenburg Germany. Ejaculates from each bull were split into 4 aliquots. The first one was sorted by the XY method and concentrated at 2.1 million cells per 0.25 ml straw. The other three aliquots were sorted with SexedUltra ™ and concentrated at 2.1 million/straw; 3 million/straw and 4 million cells per straw. Using these insemination straws 7855 heifers were inseminated. 62,398 heifers were inseminated as a control group with conventional unsorted semen from the same bulls with 15 million cells/straw and the 56-day NRR (non-return rate) was recorded. The data was analyzed using a mixed ANOVA model and can be seen in the table below (Table 3) Surprisingly the sorted semen using the SexedUltra ™ technology at a concentration of 4 million spermatozoa per straw had higher 56-day NRR than the conventional semen (66.73% vs 65.66%) (Lenz et al., 2016)

"This is the first time a dose response effect with sex-sorted bovine sperm and parity in conception rates with conventional semen has been demonstrated." (Lenz et al., 2016)

Table 3: SexedUltra™ fertility

Treatment	No. Of inseminations	56-day NRR weighed means (%)	Relative fertility (%)
XY 2.1	1,953	55.89	85
SexedUltra™ 2.1	1,999	59.95	91
SexedUltra™ 3.0	2,013	60.02	91
SexedUltra™ 4.0	1,890	66.73	102
Conventional 15.0	62,398	65.66	

(Lenz et al., 2016)

9. Sex sorted semen for timed artificial insemination:

An experiment was conducted to inspect if SexedUltra ™ semen is suitable for timed artificial insemination. The trial was conducted on beef heifers using the "14-day CIDR-PG" (Thomas et al., 2017) split time protocol. As for the material used for this experiment: Conventional semen with 25 million sperm cells per insemination straw containing 0,5 ml were used as a control. The sex sorted straws were made with the latest SexedUltra ™ Genesis III method, resulting in an accuracy of >90% and measured out at 4 million cells per 0,25ml insemination straw. All straws were cryopreserved. The estrus of 851 heifers were synchronized using a "controlled intravaginal drug release (CIDR) insert" (Thomas et al., 2017), containing 1.38g progesterone, following the "14-day CIDR-PG protocol" (Thomas et al., 2017). The insert was removed after 14 days and 25mg of prostaglandin (PG) F2alpha was administered intramuscularly on day 30. Heifers that expressed estrus 66 hours after the injection were inseminated using AI. Heifers that did not respond to the estrus detection at 66 hours after the prostaglandin administration were inseminated 90 hours after the injection in a split time manner. Those not expressing estrus after 90 hours were injected with 100µg GnRH intramuscularly and artificially inseminated simultaneously. Heifers receiving SexedUltra ™ semen and heifers receiving conventional semen were simultaneously present in all herds taking part in the experiment. The results showed that the "total pregnancy rates at the end of the 60-d breeding season did not differ between heifers that received sex-sorted semen at AI (89%; 376/422) and heifers that received conventional semen at AI (89%; 382/429)." (Thomas et al., 2017) and therefore confirming that sex sorted semen is a viable tool to be used in timed artificial insemination. (Thomas et al., 2017)

"In summary, the pregnancy rates observed suggest that SexedULTRA™ sex-sorted semen can be used effectively for timed AI of beef heifers when split-time AI is performed following the 14-d CIDR-PG protocol." (Thomas et al., 2017)

10. Genetic progress:

Traditionally genetic progress is mainly determined by the selection of a few sires, which are used to produce the AI insemination straws. This limits the possible genetic gain to the selection of males. Having a higher supply of heifer calves allows the farmer to replace a higher proportion of female animals in the herd and therefore sorting out cows with a lower milk yield or health problems, which will in turn increase the overall herd health and average milk production. Consequently, using sexed semen allows for a high potential for selection on both the male and female side. (DeVries, 2019)

Sources differ in their findings on how much the genetic gain can be improved using sexed semen. Estimates range from up to 15% genetic gain if sorted semen is used on a large scale (Van Vleck, 1981) to not really being relevant if sexed semen is used in elite cattle. (Baker et al., 1990) DeVries concluded that "The value of increased genetic gain from the female side can often be ignored in practice." (DeVries, 2019)

11. Economy:

The usage of sex sorted semen is gaining in popularity but has been held back by factors like fertility, sexing accuracy and increased cost in comparison to conventional semen. Improvements in the sexing procedure led to sexing accuracy of more than 90% and conception rates comparable to conventional semen as seen in SexedUltra™ and UltraPlus™ sorted semen. (De Jarnette et al., 2008) Improvements in technology brought the production cost of sexed semen down but can still be up to 4 times more expensive than a conventional insemination straw. This leads to the important question if sex sorted semen is an economically viable solution to be used in everyday artificial insemination on the cattle farm. Due to the reduced conception risk in heifers, the extra expense of sex sorted semen is mostly accepted for use in the first pregnancy, other farms use sexed semen for both dairy cows and

heifers. To evaluate the cost efficiency of sex sorted AI it´s usage was analyzed and it´s economy assessed. (DeVries, 2019)

11.1. Economic considerations:

11.1.1. Calves:

The financial advantage of using sorted semen results from the higher value of a female calf at an estimated 400$ to 540$ in comparison to a male calf at an estimated 50$ per individual. (DairyProfit Weekly, 2008) As long as the heifer calf is not sold the value of a female calf results from the difference between the cost of rearing a female calf to become a useable heifer and the purchase cost of buying an already reared heifer of the same quality "(genetics, biosecurity, age at first calving, value of the calf in the freshening heifer, etc.)". (DeVries, 2019) "Bred heifers (seven to nine months pregnant) are worth anywhere from $1,600 to $2,275." (DeVries, 2019) It must be kept in mind that the value of the female calf might change if the calf is sold, and a heifer of equal quality is purchased for replacement depending on the momentary changes of its market values. (DeVries, 2019)

11.1.2. Semen prices:

Due to its more excessive processing procedure sexed semen is on average about $30 more expensive per straw than conventional semen, varying depending on the sire used. (Fetrow et al., 2007)

11.1.3. Fertility:

Another crucial factor to consider is the difference in conception rates between sorted and conventional AI. The theoretical conception rate of sorted semen is at about 90% of that of conventional semen due to Sexing technologies own claims. "Communications with dairy producers revealed that they typically observe a 10 to 15 percentage point drop in conception risk (say from 55 to 45%, a reduction of 18%, or from 55 to 40%, a reduction of 28%)." (DeVries, 2019) Reports suggest that a total conception rate of about 45% can be expected in the first AI in virgin Heifers. In comparison the conception risk can be similar for sorted and conventional semen for dairy cows as shown by DeJarnette et al. (2008) if the cows are

cherry picked for their fertility, which might be impractical in everyday work on the farm. (DeVries, 2019) All in all a certain variance in conception rate must be expected.

11.1.4. Age of first calving:

Another factor that needs to be considered is that the reduced conception rate for heifers in the first service might affect the age of the first calving. Considering that raising a heifer cost about $2 a day (Kohlman, 2008) the expenses of raising a heifer until its entry into the lactating period might be increased. A later entry into the lactation period could result in a higher milk yield but older heifers can experience a higher rate of dystocia (Ettema, Santos, 2004), additionally the chance of dystocia is about 10% higher in bull calves. (Fetrow et al., 2017) Since the age of the first calving depends mostly on the growth of the heifer and not on its age it is "not likely that extended age of first calving necessarily reduces profitability of lactating cows." (DeVries, 2019)

11.2. Heifers:

To calculate the cost-effectiveness of AI using sexed semen in heifers the following data is assumed. (Table 4) The table is based on data documented by Fetrow et al. (2007) and DeVries (2019). It is assumed that the sexed semen used provides a 15 percentage points lower conception rate in comparison to conventional semen resulting in a rate of about 75% of that of unsorted semen or a total concentration of 45%. Heifers not getting pregnant after 8 breeding cycles are culled, which creates revenue (Ettema et Santos, 2014) and then replaced. (DeVries, 2019)

Table 4: Assumed cost

Age at first breeding	400 days
21-day service rate	65%
Conception risk (conventional semen)	60% (first service; - 5,75 percentage points per later service)
Conception risk (sexed semen)	45% (75% of conventional)
Cost to raise heifers	2$ / day
Maximum numbers of breedings	8
Number of sexed semen breedings	0-8 remainder with convectional semen

Annual discount rate	8%
Market value raised but culled open heifers	≤ 980$
Market value calving heifer (wo. calf)	1800$
Semen cost	sexed: 40$ conventional: 10$
Value heifer calf	450$
Value bull calf	50$
Death loss per calving	10%
Heifer calves from sexed semen	90%
Heifer calves from convectional semen	48%
Extra dystocia cost	14.70$ per bull calf

(DeVries, 2019)

The calculations show that sexed semen can be profitable up to the fourth service given the assumptions listed above. The highest increase in profit margin is achieved up to the second service at $10.35. “The profit is the sum of the cull revenue, value of the calf, and value of the heifer to the dairy and the costs for raising, breeding, and dystocia.” (DeVries, 2019) Additionally it was proven again that the use of sexed semen AI increased the birth rate of female calves. Since the maximum increase in revenue is relatively small at $10.35 per calf born the revenue depends highly on the market value of heifers. The calculations show that a heifer price of 500$ almost always results in a profitable scenario, whereas at a heifer price of 300$ “few scenarios make sexed semen a profitable choice” (DeVries, 2019) Other authors (Fetrow et al., 2007; Olynk et Wolf, 2007; Seidel, 2003) came to similar conclusions mentioning there is an increase in profit if the insemination is successful in the first few services but highly depends on the market value of heifers and semen prices. (DeVries, 2019)

11.3. Cows:

The analysis of the economy of using sexed semen in cows is based on the following data collected by DeVries, 2019. (Table 5)

Table 5: Assumptions

	Convectional semen	Sexed semen
Conception risk	35%	25%
Semen cost	$10	$40
% heifers at 450$ per calf	49%	90%
% bull calves at 50$ per calf	51%	10%
% Abortions and dead calves	10%	10%
Value of new pregnancy	$200	$200

(DeVries, 2019)

The Results of his calculations are shown in Table 6. The calf's revenue is based on the cost of semen, conception rate, expected sex and therefore its value plus the rate of abortions and stillbirths. The value of a new pregnancy is based on the costs associated with a failed insemination, consisting of the consequences of days open, cost of the next insemination, higher culling rate etc. "Nonpregnant cow costs were calculated as the percentage that failed to get pregnant times the value of a new pregnancy based on conventional semen." (DeVries, 2019) Possible increased genetic progress was not considered since its controversial whether it has a decisive role. The calculations show that a conception risk of 31% must be achieved to make the cost of sexed semen break-even with conventional AI. Furthermore, it was found that "reasonable variations in the price of sexed semen, the value of a heifer calf, and the value of a new pregnancy" (DeVries, 2019) didn´t influence the break-even point of the sexed versus conventional semen. This shows that if the reduction in conception risk of sexed semen is minor using sexed semen is an economically viable option. (DeVries, 2019)

Table 6: Results

	Conventional semen	Sexed semen
Expected calf value per pregnancy	$218	$369
Cows not pregnant after service	65%	75%
Calf revenue	$76	$92
Semen cost	$10	$40
Non-pregnant cow cost	$130	$150
Net return	($64)	($98)
Gain (loss) of sexed semen breeding		($34)

(DeVries, 2019)

Accepting the above-mentioned data, it is financially beneficial to use sexed semen if the conception risk using sexed semen is not significantly lower than using conventional semen. (DeVries, 2019) Other factors however can further increase the profit margin using sexed semen for example the value of the increased genetic gain, which is estimated at about a value of $33 per heifer used for breeding if sexed semen is used. (Fetrow et al., 2007) In addition having more female calves available enables the farmer to replace a greater percentage of cows having a low milk yield, which can result in a higher average milk production of the herd, increasing the overall profit. (Fetrow et al., 2007) Furthermore profitability depends highly on the current market situation which makes it subject to significant fluctuations. (DeVries, 2019)

12. Usage today:

Two years after sexed semen became available in 2016 the usage was at 17.8% of inseminations in Holstein heifers and at 0.4% of Heifer cow inseminations (Norman et al., 2010)

Sex sorted semen was not used on a broad scale for a long time, since the increased costs and reduced conception rate often outweighed the benefits and only made sense in animals with excellent reproductive performance (Cabrera, 2022) and for heifers, because of their naturally intact fertility in comparison to dairy cows. (Norman et al., 2010) Animals with a lower reproductive performance couldn't create a "positive income from calves over semen costs" (Cabrera, 2022)

Lauber et al, (2023) analyzed data from the Dairy Records Management Systems (DRMS) which included a total of 9,338,862 inseminations of heifers and lactating cows between 2019 and 2023 conducted within the continental United States. Included in this dataset are 8,244,653 inseminations of 4,880,752 Holstein Friesian (HF) cows and heifers from 9,155 herds and 435,267 inseminations of 266,058 Jersey cows and heifers from 2,759 herds. The dataset was divided into breed, service number, year and whether it's a cow or a heifer. (Lauber et al., 2023) The usage of sexed semen can be seen in the table below. (Table 7, 8)

Table 7: Usage of sex sorted semen in Holstein females.

2019	Nulliparous	Primiparous	Secundiparous	Multiparous
conv.*	67.20 ± 0.12	80.11 ± 0.07	79.81 ± 0.07	75.65 ± 0.07
sexed	20.83 ± 0.12	5.69 ± 0.04	3.67 ± 0.03	2.51 ± 0.02
2020				
conv.*	61.62 ± 0.07	74.76 ± 0.05	73.75 ± 0.05	70.81 ± 0.05
sexed	25.58 ± 0.07	8.03 ± 0.03	5.20 ± 0.03	3.38 ± 0.02
2021				
conv.*	51.36 ± 0.08	70.27 ± 0.06	69.62 ± 0.07	65.21 ± 0.06
sexed	33.24 ± 0.09	8.48 ± 0.04	5.26 ± 0.03	3.83 ± 0.02

*conv. = conventional

(Lauber et al., 2023)

Table 8: Usage of sex sorted semen in Jersey females.

2019	Nulliparous	Primiparous	Secundiparous	Multiparous
conv.*	54.40 ± 0.57	54.40 ± 0.57	62.90 ± 0.41	65.40 ± 0.35
sexed	29.20 ± 0.50	20.40 ± 0.29	19.80 ± 0.33	17.00 ± 0.26
2020				
Conv.*	49.70 ± 0.30	54.70 ± 0.22	55.70 ± 0.25	57.20 ± 0.22
sexed	34.60 ± 0.29	25.10 ± 0.19	23.20 ± 0.21	20.20 ± 0.17
2021				
conv.*	35.20 ± 0.34	48.50 ± 0.28	50.70 ± 0.31	50.30 ± 0.26
sexed	47.70 ± 0.35	27.40 ± 0.24	24.20 ± 0.26	21.10 ± 0.21

*conv. = conventional

(Lauber et al., 2023)

The usage of sexed semen has increased across both breeds and in heifer and cows respectively. During the three years represented in this study the usage increased from 20.83% to 33.24% in Holstein heifers and from 3.38% to 3.83% in multiparous Holstein dairy cows and from 29.20 to 47.70% in Jersey heifers and from 17.0% in Jersey cows to 21.10%. It must be noted that the usage in nulliparous heifers increased the most (Lauber et al., 2023)

Over the years multiple improvements in herd management, such as optimizations in the periparturient period and the introduction of fertility programs, for example the double OvSynch protocol. (Fricke and Wiltbank, 2022; Carvalho et al., 2018) This led to an overall improvement in fertility, especially in lactating dairy cows, making the use of sex sorted semen more feasible, (Lauber et al., 2023) which was previously almost exclusively used for the naturally more fertile heifers. (Norman et al., 2010)

“The concurrent increase in beef and sexed semen inseminations over the past 3 [years] suggests that dairy farmers are using both semen types as a precision management tool to regulate herd inventory and increase the value of calves.” (Laubner et al., 2023)

13. Future

UltraPlus ™

The newest generation of SexedUltra ™ semen was announced on April 7th, 2022, by Sexing Technologies ®. UltraPlus ™ is supposed to set the newest standards in sexed semen. According to their own statistics this "most innovative improvement to the sorting process" (STgenetics Marketing, 2022) developed during the past three years led to a product with more than 90% gender purity and is supposed "to bring the highest Conception Rates in the history of this product to help our customers meet their profitability and sustainability goals." (Juan Moreno, Sexing Technologies Chief Executive officer, 2022) Sexing technologies conducted a field trial to assess the effectiveness of UltraPlus ™. Sorted semen from 52 sires were used on 72 farms for over 15.000 inseminations. The data collected in this experiment showed a "14% rate gain over the initial XY marketable semen sorting technology available since 2005 from Sexing Technologies®" (STgenetics marketing, 2022) This in turn means that the newest product UltraPlus ™ has "an additional 3 percent improvement in conception rate" over the SexedUltra ™ 4M semen, which makes it the world's highest fertility sex sorted semen commercially available. (STgenetics marketing, 2022)

14. Alternative Technologies:

Flow cytometry has been the only commercially available technology for decades, but some alternative methods have been explored throughout the years. Fluorescent *in situ* hybridization (FISH)" has been successfully used to differentiate X- and Y- bearing sperm by detecting the differences in their DNA sequence, unlike the Flow cytometry, which focuses on the amount of DNA inside the cell. (Vishwanath and Moreno, 2018) The downside of this approach is that it requires the disintegration of the sperm head (Kawarasaki et al., 1998) in turn damaging the cell. Rath et al. (2013) used this principle employing "functionalized gold nanoparticles (AuNPs)" (Rath et al., 2013) which enter the sperm head through the intact membrane and bind to the marker locations within the chromosome. While this procedure was effective in the sense that the binding was successful, actual fertility data was not presented. (Rath et al., 2013) Further efforts are being made to find alternative technologies but failed to produce a functionally intact and economically viable sexed semen to be used commercially. Some of the more promising papers provide evidence about

successfully using immunology (Rosenfeld, 2012) or using a swim-up method. The later was proven to be functional by SYBR green real-time-PCR using “Nili Ravi buffalo bull sperm” (Ul-Husna et al., 2017) In this process the lower fraction contained significantly more X-chromosomes, while the concentration of Y-chromosomes was higher in the upper fraction. The quality of the sperm cells was still not compromised after sorting and even after freezing/thawing. These results are promising, since the modified swim-up method is cheaper and less complex than flow cytometry, making it more feasible for developing countries to employ. (Ul-Husna et al., 2017)

15. Other species

15.1. Ovine:

To evaluate the efficacy of sex-sorted semen in sheep fertility, a field trial was conducted. Merino ewes were inseminated with sex-sorted semen (MoFlo® SX) sourced from three distinct rams. The semen was sorted using flow cytometry and then cryopreserved/thawed. In total 360 ewes were estrus synchronized using “progestagen-impregnated pessaries, PMSG and GnRH treatment” (De Graaf at al., 2017) and then inseminated with sorted or non-sorted semen at concentrations of 1 million, 5 million or 15 million motile frozen-thawed sperm cells per inseminate. Another group of ewes were inseminated with commercially available non-sorted frozen/thawed inseminates at a concentration of 50 million motile cells per dose as a control group. The lambing rates were similar for all groups of ewes but lower for those inseminated with unsorted semen at concentrations of 1 million and 2 million motile cells per insemination. “This study demonstrates sorted ram spermatozoa are equally fertile to non-sorted spermatozoa even when inseminated at 2% of the dose.” (De Graaf at al., 2017) Additionally it was demonstrated that “at very low artificial insemination doses (1 or 5 million motile) the fertility of sorted ram spermatozoa is superior to non-sorted spermatozoa inseminated in equal numbers.” (De Graaf at al., 2017) These results are very promising and have “significance for the future commercialization of sex-preselection technology in sheep” (De Graaf at al., 2017) since the global marked for sheep is experiencing a “resurgence” (R. Vishwanath, J.F. Moreno 2018). Since the inseminations were performed using LAI it was suspected that this technique mitigates the adverse effects of the sex sorting process. (R. Vishwanath and J.F. Moreno 2018)

15.2. Caprine:

A field trial using sex sorted caprine sperm from 2 males, was conducted using frozen-thawed sperm cells to inseminate Saanen does per LAI insemination. While the overall fertility of sex sorted insemination was lower at 38% kidding rate versus 50% in does inseminate with unsorted semen, successful insemination and fertility was demonstrated. (R. Bathgate, 2013) Advancements in the SexedUltra^{TM} technology led to the commercial availability of sorted caprine semen. (R. Vishwanath and J.F. Moreno 2018)

15.3. Equine:

To evaluate the field usability of sex sorted stallion sperm aliquots from 4 stallions were "either cryopreserved directly, sex-sorted and cryopreserved, or sex-sorted and returned to liquid storage until insemination". 23 mares were inseminated using "low dose hysteroscopic insemination" (Z. Gibb et al., 2016). The conception rates of the sex sorted semen were comparable to those using conventional semen (R. Vishwanath, J.F. Moreno, 2018) but the "high incidence of pregnancy loss suggests that the development of the resulting embryos was significantly impaired by the sperm processing treatments" (Z. Gibb et al., 2016) presently making the sex –sorted semen impractical for field use.

15.4. Porcine:

Sex sorted sperm would be highly advantageous for pig farms since female piglets are often preferred to be able to select more breeding sows. Further boars are not desired for meat production since meat from uncastrated boars is not favored by the customer and the castration of male piglets is subject to increased regulations, which in turn increases production cost. There is no commercially available sex sorted semen for pigs yet because there are some technical challenges that need to be dealt with before the profitable production of sorted insemination doses is possible. Fluorescence activated cell sorting (FACS) is the most used technology today, but it has some limitations concerning boar sperm. One challenge to overcome is the "increased susceptibility of sexed boar spermatozoa to injuries induced by liquid storage and cryopreservation that, in turn, impairs sperm quality leading to unsatisfactory results *in vivo*." (Spinaci et al., 2016) Another challenge is the limitation of sorting speed of FACS since a commercial insemination dose of boar semen contains "2.5 to 3.0 billion motile sperm in 75 to 100 mL of extender" (Knox, 2016), even though sorting speed was improved to 20 million cells per hour it would take about 100 hours to produce

one single dose for artificial insemination. (Spinaci et al., 2016) Such a high sperm concentration is required since the sperm and the seminal plasma provoke an inflammatory reaction within the uterus which activates the polymorphonuclear leukocyte (PMN) phagocytosis causing the death and removal of many spermatozoa. Further the sperm cells get repelled by neutrophils entering the lumen further reducing their likelihood of reaching the oocyte. These mechanisms cause the "loss of billions of spermatozoa" (Vazquez et al., 2008)

To overcome this challenge field trials were conducted using alternative methods of AI which require a lower sperm concentration, i.e. by placing the sperm higher up in the reproductive tract of the sow. Del Olmo et al. (2014) used LAI to inseminate sows with 1 million sperm in each oviduct and 2 million sperm in each uterine horn which resulted in a farrowing rate of 80,7 % and an average litter size of 10,5 making this a potentially viable method. Alternatively, a study using DUI (deep uterine insemination), which promised a up to 20-fold reduction in fresh semen concentration, was conducted. For DUI a 1,5m catheter is used which can bypass the uterine folds therefore making it a non-surgical procedure (Vazquez et al., 2008), therefore being a way more practical procedure in the everyday work on the breeding farms. Vazquez et al. conducted field trials with unsorted semen on estrus synchronized sows, which showed conception rates and litter sizes comparable to traditional artificial insemination techniques, even though the sperm number was reduced twentyfold. (Vazquez et al., 2008) Trials using sexed semen for DUI conducted by Rath et al. 2003 and Vazquez et al. 2003 with concentrations ranging between 50 up to 140 million cells per inseminate produced inconsistent results (Vishwanath, 2018), proving that the use of sex sorted semen is possible in pigs but not yet ready for commercial marketing.

16. Summary

After many years of research, flow cytometry stands out as the most effective technology in the commercial sorting of X- and Y-bearing spermatozoa, revolutionizing the field of reproduction and animal breeding. Improved and refined by G. Seidel and his team, this technology relies on precise staining techniques and sophisticated machinery to sort sperm based on its DNA content, which is about 4% higher in X-chromosomes. Through staining with Hoechst 33342 dye and filtration, the sperm sample is prepared for sorting by a flow cytometer, such as the Genesis III system. This automated system, with its dual solid-state lasers, achieves remarkable sorting rates with high purity levels, of about 90%, enhancing efficiency and scalability in sperm sexing operations. Furthermore, advancements like modern clusters increased sorting speeds up to 1 billion cells per hour demonstrating the continuous evolution of this technology. While the sexing process is damaging to the sperm cell itself, advancements in the sorting technology made the procedure much more benign and lead to products like SexedUltra™ and SexedUltra-4M™ increasing the fertility to almost the same level as conventional semen. By having more females available more selection can take place on the female side of animal breeding, increasing the potential for genetic progress. While the slightly reduced conception risk and higher cost of sexed semen led to the almost exclusive use of sexed semen in heifers or selected highly fertile dairy cows. Sexed semen insemination is used on a broader scale nowadays and will most likely become even more widely used with further advancements such as UltraPlus™. Considering the economic aspect, it showed that the price of sorted sperm has come down significantly, making its use increasingly sensible in an economical way but is still highly dependent on the current market conditions. Today the use of sex sorted semen for artificial insemination is almost reserved for cattle but recent experiments using sorted semen in other species such as goats, sheep and swine produced promising results and can potentially lead to a revolution in reproduction of species other than cows.

In conclusion, predetermining the sex of newborn using sex sorted semen can help to make modern farming more efficient and is a viable tool to satisfy the worlds needs for animal products in an economical and potentially more ecological way.

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Appendix 1. Thesis topic declaration form

University of Veterinary Medicine Student name: Schifferings, Till

THESIS TOPIC DECLARATION FORM

I hereby request approval from the Head of Department of the Department of/and.....
Obstetrics and food animal medicine
to prepare a thesis based on a topic announced and supervised by said Department as follows.

Date: Budapest, 09/10/22 T. Schifferings
Student signature

Thesis topic:

Title of Thesis (English title as well):
Methods of influencing the sex of newborn with special regards to sex sorted semen

Supervisor signature:
Zsolt Kátai

Approved by:

Head of Department signature

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HuVetA relies on the latest technology in order to provide easy searchability (by search engines, as well) and access to the full text document, whenever possible.
Based on the above, HuVetA aims to:

- *increase awareness of Hungarian veterinary science not only in Hungary, but also internationally;*
- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*