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Master Thesis

Phenotypic and Genomic Analysis of Sperm Quality and Male Fertility Traits of Fleckvieh Bulls Used for Artificial Insemination

Submitted by

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Supervisor:
Univ. Prof. DI. Dr Johann Sölkner
Department of Sustainable Agricultural Systems
Division of Livestock Sciences

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Fleckvieh Bull, Credit: Stephan Hauser for Genostar GmbH

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Affidavit

I hereby declare that I have authored this master thesis independently and have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or actual content from unpublished sources or published literature are duly identified and cited, including precise references. This Master's thesis was produced as one of the requirements for the completion of the European Master in Animal Breeding and Genetics at the University of Natural Resources and Life Sciences, Vienna, Austria (BOKU)

Graz, 22 August 2022

Place, date

Signature (Geena Cartick, B.Sc.)

This thesis is dedicated to Cartick-Nadal, Ragoobeer and Dopplinger families

Preface

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Abbreviations and acronyms

AI	Artificial Insemination
Chr	Chromosome
EBV	Estimated Breeding Value
FDR	False Discovery Rate
GCTA	Genome-wide complex trait analysis
GEBVs	Genomic Breeding Values
GRM	Genetic Relationship Matrix
GWAS	Genome Wide Association Studies
LMEM	Linear Mixed Effect Model
LOCO	Leave One Chromosome Out
MLM	Mixed Linear Model
MLMA	Mixed Linear Model Analysis
NRR	Non-Return Rate
RoH	Run of Homozygosity
SCR	Sire Conception Rate
SNP	Single nucleotide polymorphism
TMI	Total Merit Index
ZAR	Rinderzucht Austria-Zuchtwert-Datenbank

Abstract

Artificial Insemination (AI) is an indispensable method for farmers to improve their herds' genetic performance. AI stations are interested in collecting semen of top young bulls. This study investigates the effects of bulls' age and genotype and a range of environmental effects on semen production. We analysed 54,088 ejaculations from 1,136 Fleckvieh bulls from Austrian AI Stations of age 10 to 96 months. Age at ejaculation was analysed, accounting for station, year, season, semen collector and analyst. Number of sperm, semen concentration and volume increased quite linearly until an age of 17 months, then increased at a much lower rate and finally stabilized at older age classes. No deterioration of any of these traits was observed for ages up to 96 months. Percentage of motile sperm seemed to be unaffected by age, except for a few observations from very young bulls with lower percentage of motile sperm. Regions of the genome responsible for differences in sperm quality were also investigated. No significant signals were found for any of the traits, considering the very stringent genome wide Bonferroni threshold. When considering a threshold -log10(p)=4.0, we observed significant relationship between genes that affect sperm quality traits in the additive effect, dominance effect and the effect of ROH GWAS. 16 genes were associated to spermatogenesis and fertility and two genes to growth. Younger bulls (≤17 Monate) show severely reduced sperm volume and concentration, resulting in much lower number of sperm and success of insemination. The results provide grounds that older bulls are beneficial to AI Stations. The study could be used by AI stations to avoid passing down genes that would decrease semen quality. However, including pre-puberty bulls in the insemination process is beneficial to AI station to reduce generation interval and is advantageous for dosing palettes for insemination.

Keywords: Fleckvieh, semen quality, GWAS, additive, dominance, RoH, NRR

Zusammenfassung

Diese Studie untersucht die Auswirkungen von Alter, Genotyp und verschiedenen Umwelteinflüssen auf die Samenproduktion von Fleckvieh-Stieren. Wir analysierten 54.088 Ejakulationen von 1.136 Stieren im Alter von 10 bis 96 Monaten aus österreichischen Besamungsstationen. Berücksichtigt wurden Faktoren wie Station, Jahr, Jahreszeit, spermasammelnde und -analysierende Person. Bis zum Alter von 17 Monaten steigt Spermamenge, -konzentration und -volumen recht linear, später weniger stark und stabilisiert sich schließlich in älteren Altersklassen. Bis zum Alter von 96 Monaten wurde keine Verschlechterung der Eigenschaften beobachtet. Der Anteil motiler Spermien schien vom Alter nicht beeinflusst zu werden, mit Ausnahme einzelner Beobachtungen von sehr jungen Bullen mit wenig motilen Spermien. Die für die Unterschiede in der Spermienqualität verantwortlichen Genomregionen wurden ebenfalls untersucht. Unter Berücksichtigung der sehr strengen genomweiten Bonferroni-Schwelle wurden für keines der Merkmale signifikante Signale gefunden. Bei einem Schwellenwert von -log10(p)=4,0 wurden signifikante Beziehungen zwischen Genen mit Auswirkung auf die Spermienqualität, in Bezug auf den additiven Effekt, den Dominanzeffekt und den Effekt des ROH GWAS beobachtet. 16 Gene wurden mit der Spermatogenese und Fruchtbarkeit und zwei Gene mit dem Wachstum in Verbindung gebracht. Zusammenfassend weisen jüngere Bullen (≤17 Monate) ein stark reduziertes Spermavolumen und geringere Spermakonzentration auf, was zu einer geringeren Gesamtzahl an Spermien und reduziertem Besamungserfolg führt. Die Ergebnisse belegen, dass ältere Bullen vorteilhaft für Besamungsstationen sind. Die Studie kann von Besamungsstationen genutzt werden, um die Weitergabe von Genen zu vermeiden, die die Spermaqualität beeinträchtigen. Die Einbeziehung von vorpubertären Bullen ist jedoch wichtig, um das Generationsintervall zu verkürzen und ist zudem vorteilhaft für die Möglichkeit der Dosierung von Besamungspaletten.

Stichworte: Fleckvieh, Spermaqualität, GWAS, additiv, Dominanz, RoH, NRR

Introduction

Artificial Insemination (AI) is considered an excellent biotechnological process for improving animal performance, particularly in cattle (Robertson, 1954). AI has helped increase the number of services per bull per year from 100 using natural mating to over 1000 (Webb, 1992). AI is the leading method for passing down essential genes in dairy cattle, an indispensable method for farmers worldwide to improve the genetic performance of their herds (Vishwanath, 2003; Manafi, 2011). The benefits of using AI in cattle breeding remain the maximization of superior sires' full potential and detecting the infertile bulls early enough (Webb, 1992; Serrano *et al.*, 2021a).

Generation interval is reduced in the era of genomic selection because accurate estimated breeding values (EBV) are available for very young calves as soon as the SNP-chip genotyping is performed. Therefore, genomic information can be used together with phenotypic information by farmers and breeding technicians to choose mating partners (precision mating) better. AI stations worldwide have different goals depending on their regional needs and demands. An AI station aims to collect the best quality sperm at the earliest possible time for local and international farmers to continue producing high-quality progenies with desired characteristics.

Objectives of the Study

AI stations are interested in collecting semen of top bulls, and sometimes semen collection is performed on bulls as young as ten months old. Limited research is available on the genetic differences in sperm quality, i.e., the volume and concentration of semen, the percentage of motile sperm and the sperm counts, and fertility (e.g., non-return rate) of bulls early in life.

Therefore, in this study we investigated the effects of bull's age, genotype, and a range of environmental effects on semen quality as well as the success of insemination. The study aimed to provide information on sperm quality and fertility of Fleckvieh bulls.

Research Questions

The aim of this research was the phenotypic and genomic analysis of sperm quality and male fertility traits of Fleckvieh bulls used for artificial insemination. The data used in this research was from bulls that produced sperm at three artificial insemination centers in Austria from 2010 to 2020.

Therefore, the following objectives were targeted:

- Do the age of bull, the genotype of bull and environmental effects affect the sperm quantity and quality?
- What are the regions of the genome responsible for sperm quality?
- Does the age of bull at insemination affect the success of insemination?

1. Literature Review

This chapter describes the background of cattle farming and artificial insemination. It explains the importance of Fleckvieh in Austrian farming. It describes analyses done previously on semen quality traits and the success of insemination both in terms of phenotypic and genomic analysis. It outlines the methodology on how to move along from the research questions to the possible results.

1.1. Cattle Breeding in Austria

In Austria, cattle industry is an integral part of agricultural production as consumption of dairy and beef is a part of Austrian culture, with a total of around 1.9 million cattle and over 55,000 cattle keepers (Bundesministerium für Landwirtschaft, Regionen und Tourismus, 2020; Stegfellner and ZAR, 2021). Based on the Federal Association of Austrian Cattle Breeders (ZAR) statistical database, there are 53,310 cattle farmers in Austria and 5% of these farms have herds with more than 100 heads of cattle. It is an economically important sector within the agricultural value chain (Stegfellner and ZAR, 2021). The Bundesministerium für Landwirtschaft, Regionen und Tourismus (2020) also states that Austria is not only self-sufficient in the beef and dairy sector but in fact produces a very considerable surplus of milk and beef. Self-sufficiency also extends to the capability of farmers to produce high-quality meat and milk with the required nutritional content for the locals and for exporting. Therefore, artificial insemination stations play an essential role in livestock production by ensuring one calf/cow/year and also by dissemination of superior genetics through thorough recording (Singh, 2019).

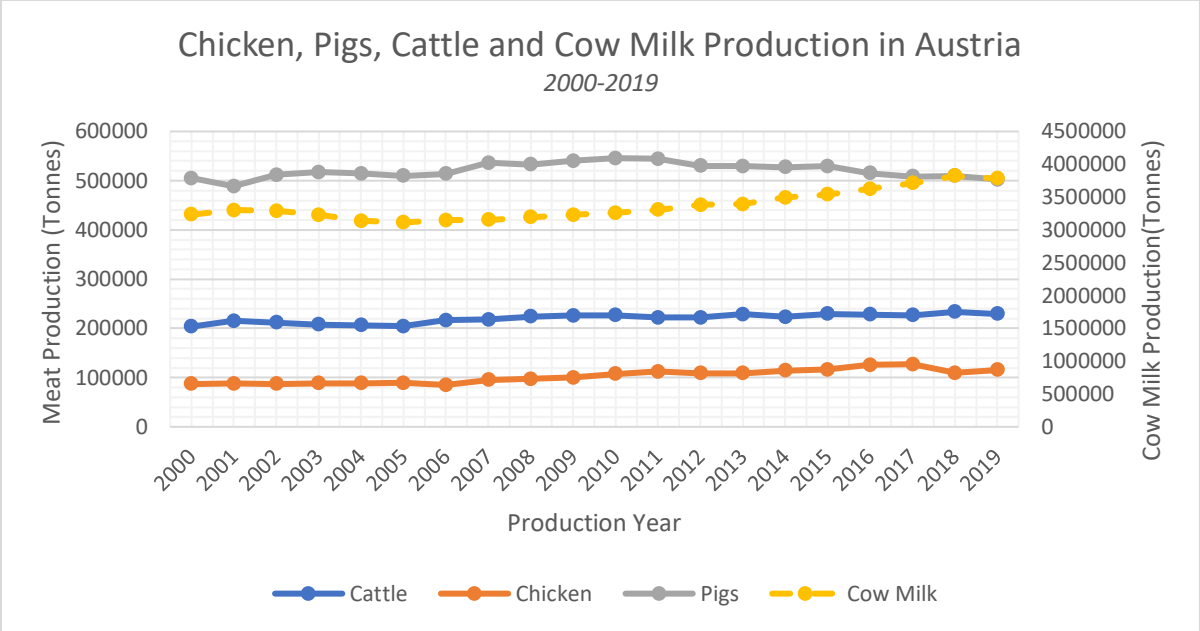

Figure 1 Austria's meat and cow milk production (FAOSTAT, 2021)

In the last 20 years, Austria reached a stable production of cattle meat (approximately 222,000 tonnes/year), it being the second most consumed meat after pork (Figure 1), with chicken production being half of the cattle meat production per year (FAOSTAT, 2021). An increase in dairy milk production in Austria can also be observed within the last decade as the production attained approximately 378,000 tonnes per year in 2019 (Figure 1); cow milk is by far the most produced milk in Austria, with goat and sheep production being under 25,000 tonnes per year (FAOSTAT, 2021). These statistics are a clear sign of the importance of beef and dairy farming in the country.

1.2. Bovine Reproduction

The profitability of cattle production across the globe depends largely on the reproductive efficiency. It is essential that the monitoring and management of the bovine reproductive activity is done together with veterinary and cattle experts so as to keep an elite herd (Kramer, 2014). Furthermore, it also impacts the efficiency of sustainable food production and animal welfare as low fertility would increase the window interval between calvings and therefore requiring more inseminations, medical care and treatments, culling and replacement of infertile animals (Boichard, 1990; Roxström and Strandberg, 2002; Kastelic, 2013; Fuerst-Waltl *et al.*, 2016; Shao *et al.*, 2021). In order to maintain this high efficiency, it is important to maintain a good monitoring of the herd, reproductive soundness, physical abnormalities, diseases and importantly the assessment of the bovine reproductive tract (Chastant-Maillard, 2014; Mauchlen, 2020). Managing reproductive traits not only maximise fertility but also helps in genetic diversity and preservation of impactful genes (Dekkers, 1991; Fuerst-Waltl *et al.*, 2016; Shao *et al.*, 2021). In research, a lot has been done regarding cow reproductive management and monitoring, but it is important to address that bulls' fertility monitoring is as equally important. Forty females could be impregnated by one bull through natural service and thousands through artificial insemination (Kastelic, 2013), therefore poor fertility in a single bull could impact a whole production chain in cattle production as it is more impactful than cows in the genetic and production capacity in the flock(Waqas, 2021).

1.2.1. The Male Reproductive System and Bulls' Sexual Development

The bull reproductive tract (Figure 2) consists of three different parts, the production(P), the transport (T) and the transfer (Tr) (Nabors, 2021). Nabors (2021) explains that the production is where the spermatozoa are produced in the testes which is shelled by the scrotum that helps with thermoregulation of the testis to allow production of healthy sperm. The spermatozoa is transported through the tubular transport system where a series of processes takes place including maturation, storage and fluid production to allow the spermatozoa to swim easier towards the ovary (Nabors, 2021; Waqas, 2021).

The development of the bull consists of moving from a young immature bull to a sexually mature bull through the transformation of the reproductive tract due to a spike in reproductive hormones (Barthle and Reiling, 1999). Puberty can be reached at a different age and weight, and this depends on the feed management (Thomas, Patterson and Perry, 2011). Spermatogenesis is an important biological process that marks the beginning of puberty (Waqas,2021). It begins in the transport section of the reproductive tract (Figure 2 more specifically in the seminiferous tubules of the testicles (Barthle and Reiling, 1999; Nabors, 2021; Waqas, 2021). During these sixty days process, the sex cells mature from the germ cells which are then transported and stored while the accessory gland secrets important fluids that helps in triggering the motility of the sperm and provides a nutritious environment for the survival of the sperm cells (Barthle and Reiling, 1999; Waqas, 2021). Barthle and Reiling (1999) explained further that the matured sperm cells are then requires another 14 to 16 weeks of regulation to adjust and for it to be considered fertile. The last stage is when the semen is transferred from the bulls to the cow, and this happens through the elongation of the penis and ejaculation which is done through the muscles that is around this area (Nabors,2021).

A bull reach puberty at 10 months old of age (Staub and Johnson, 2018; Waqas, 2021). Therefore, bulls as young as 10 months old could be used in AI Station.

The possibility of mating animals at a younger age helps decreasing generation interval and increasing genetic gains therefore making puberty an important stage in the bulls' reproductive cycle (Brito, 2021). Brito (2021), states that one disadvantage of using young bull is the lower sperm counts and sperm maturity could negatively impact the herds' genetic superiority.

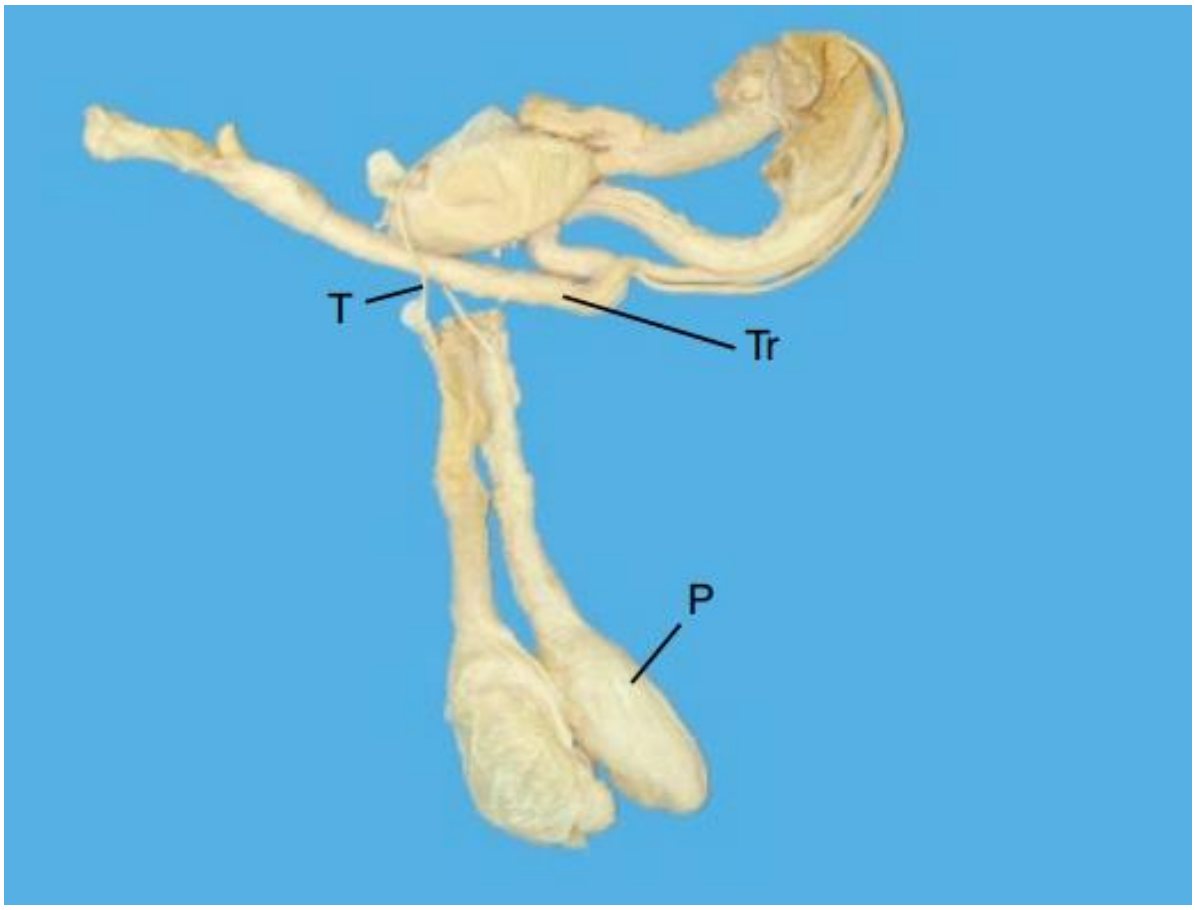

Figure 2 The bull reproductive organ showing the production area (P), the transport (T) and the transfer (Tr) (Nabors,2021)

1.3. History of Artificial Insemination in Animal Breeding

AI is a process that requires a manual introduction of semen in the females' reproductive tract using alternative methods to natural mating(Morrell, 2011; Britannica, T. Editors of Encyclopaedia, 2016). The interest in semen and its potential goes back to 1678 when Antoni van Leeuwenhoek first identified spermatozoa and described it to the Royal Society of London (Foote, 2002; Ombelet and Van Robays, 2015). After this discovery, many scientists got interested in spermatozoa, their structure, and their use. After two centuries, archives report a great number of credible studies on artificial insemination conducted on species like rabbits, dogs, and horses (Foote, 2002; Ombelet and Van Robays, 2015). Ombelet and Van Robays (2015) report that a few years later, significant research was introduced by a Russian scientist, Ivanov, who started to look intensively at the use of AI in livestock species around 1914. He developed essential techniques and tools that helped bring this vital technology to today's level of sophistication.

AI belongs to one of the technologies under the term "Assisted Reproduction Technologies" (ART) that help facilitate reproduction (Figure 3).

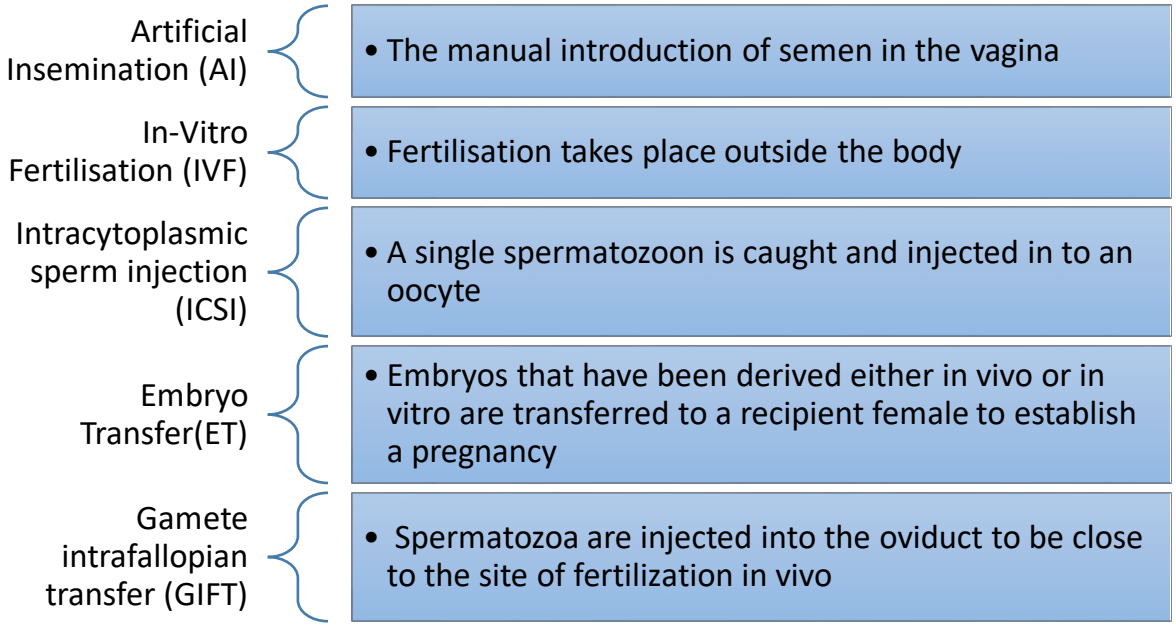

Figure 3 Description of the different types of Assisted Reproduction Technologies (Morrell, 2011)

In the animal breeding and genetic sector, AI has been used for the protection and conservation of endangered species and breeds as well (Morrell, 2011). In Europe and North America, 85% of cattle and pig breeding is done through AI (Morrell, 2011). Additionally, a steady progression of AI use can be observed in other livestock species goat and sheep (Thibier and Wagner, 2002). Advances in the use of AI such as sexing semen have further established it as an essential tool in animal breeding (Thibier and Wagner, 2002; Vishwanath, 2003; Morrell, 2011). AI is seen as an essential technology due to the ease of application and profitability for insemination centres and the breeding industry (Vishwanath, 2003). While the pregnancy rate from artificial insemination is around 10% lower than that of natural service (Galvão, Ribeiro and Santos, 2020), AI remains economically more important as it improves livestock profitability (Baruselli *et al.*, 2018).

1.4. Cattle Artificial Insemination

Cattle keepers uses AI intensively; 94,3% in the case of Austria (ZAR,2020) and different organisations must work together to make this process manageable and efficient. In Austria, the "Rassen Arbeitsgemeinschaften" (Associations based on cattle breeds), breeding associations, "Kontrollverbände" (association responsible for performance and quality control), AI stations and the Chamber of Agriculture work together so that AI is done in a controlled way that works in the interest of both, the animals and farmers (RinderZucht Austria, 2014). In Austria there are currently three big AI stations, with the largest one being GENOSTAR Rinderbesamung GmbH (Genostar Rinderbesamung GmbH, 2021). Austria's AI stations cooperate and follow international standards to achieve the highest quality and maximum breeding progress by selecting young bulls with the highest genomic breeding values (GEBVs). Furthermore, the stations aim for selecting fitness traits that guarantee the efficient production of environmentally friendly milk and meat (RinderZucht Austria, 2014; Genostar Rinderbesamung GmbH, 2021).

1.4.1. Breeding Soundness

To be able to maximise successful insemination from AI Station a breeding soundness check should be done on the bulls. The breeding soundness consists of three different checks starting as young as 8 months (Sprott, Carpenter and Thrift, 2005; Thomas, Patterson and Perry, 2011; Perry, 2021):

- **Physical Examination**

This examination is a general examination of the physical characteristics of the bulls. It ensures that the bull can mate. Thomas et al. (2011) explain that the test consists of testing all the senses, the ability to move and the overall body condition. This will allow the technician to define whether the bulls are sound for mating.

- **Scrotal Circumference (SC)**

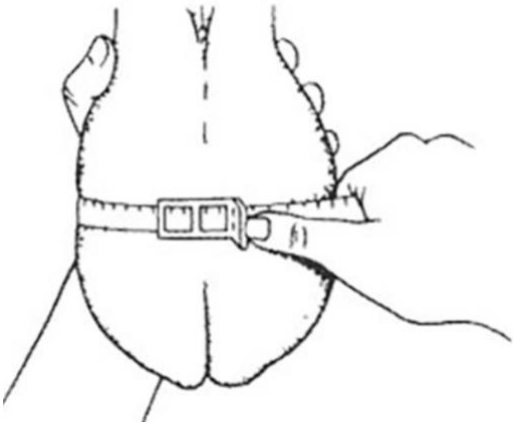

Figure 4 Measuring scrotal circumference(Thomas et al.,2011)

This is an important examination as it can give an estimation of puberty in the bull. Before analysis of sperm to define sexual maturity, puberty was defined by scrotal circumference. A bull was considered to reach puberty if the scrotal circumference was raging between 28 to 32 cm (Taylor, 1994; Barthle and Reiling, 1999). The scrotal insemination is done as shown in figure 4 and the measurement recorded. As shown in figure 5, the scrotal circumference of bulls varies from breed to breed with Simmental having an SC of around 34 cm at 10 months old and Marchigiana around 31 cm at the same age. Literature provides different minimum measurement to pass the breeding soundness examination, 30 to 34 cm (Brito, 2021), an average of 34,5 cm (Eriksson, Lundeheim and Söderquist, 2012) and 28 to 32 cm (Taylor, 1994; Barthle and Reiling, 1999). But it is important to take in consideration age and weight when analysing SC related to puberty.

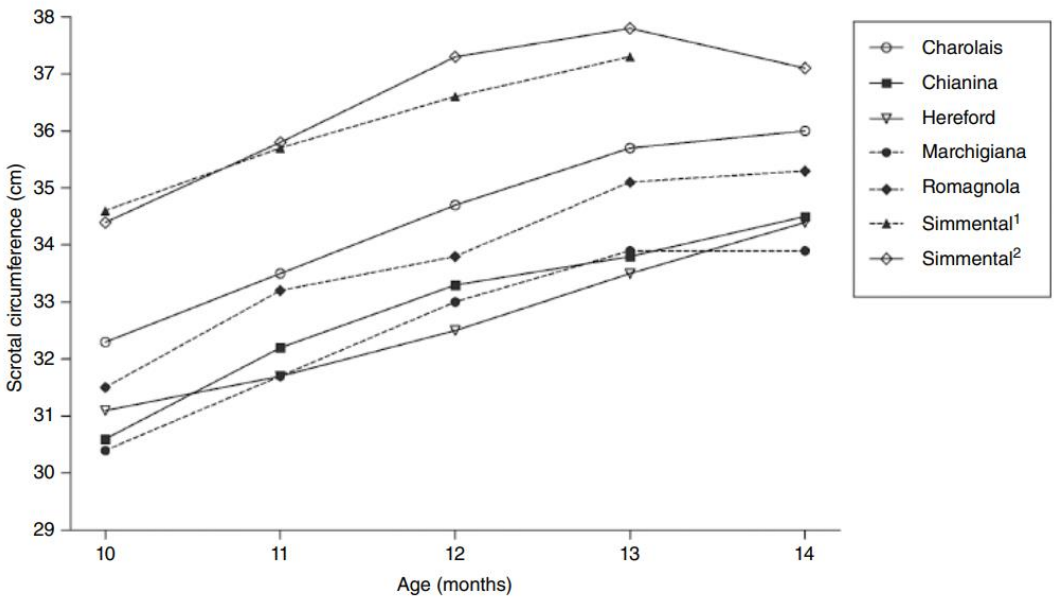

Figure 5 Scrotal Circumference change with age and breed (Brito,2021)

- **Semen Quality**

Semen quality is characterized in different observation, volume, sperm count per ejaculation, motility, and sperm morphology (Figure 6).

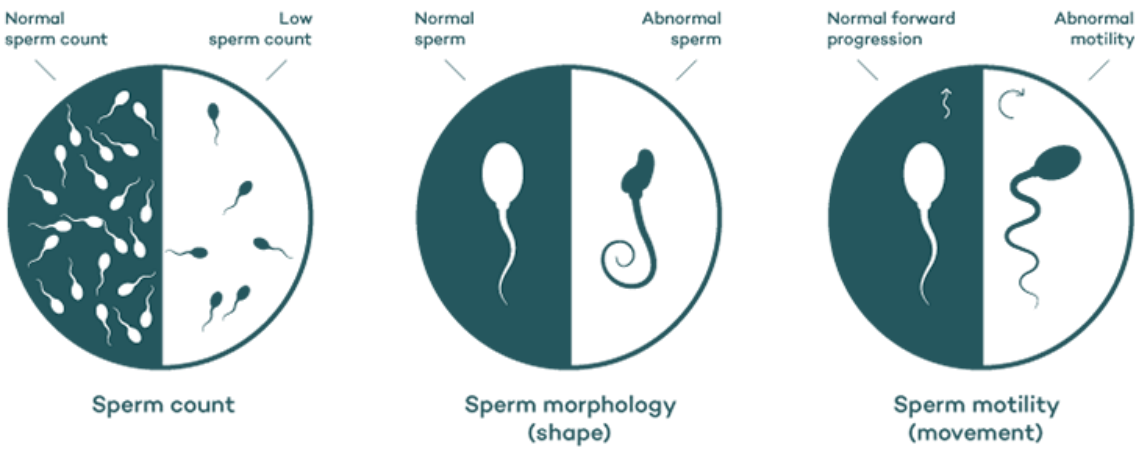

Figure 6 Abnormal and Normal Sperm characteristics

Sperm count and motility are important phenotypic measurements as they can provide information whether bull has attained puberty or not. When analysing motility, one must observe the intensity of the wave motion and grade them in the different category from intense to no motility while the morphology is observed for 100 sperms under the microscope (Bedford-Guaus, 2016). To pass the breeding soundness examination a bull must have at least 30% sperm motility, 70% normal sperm morphology, and a minimum scrotal circumference based on age (Chenoweth, 1983; Perry, 2021). These different traits affect the success of insemination and requires careful analysis specially when done in laboratory. Nowadays, the Computer-Assisted Sperm Analysis (CASA) helps in the sperm quality assessment and to have more accurate results. Furthermore, the CASA system provides more sensitive results including percentage motility, velocities and the track followed by each sperm (Bedford-Guaus , 2016).

1.5. Process of Artificial Insemination of Cows

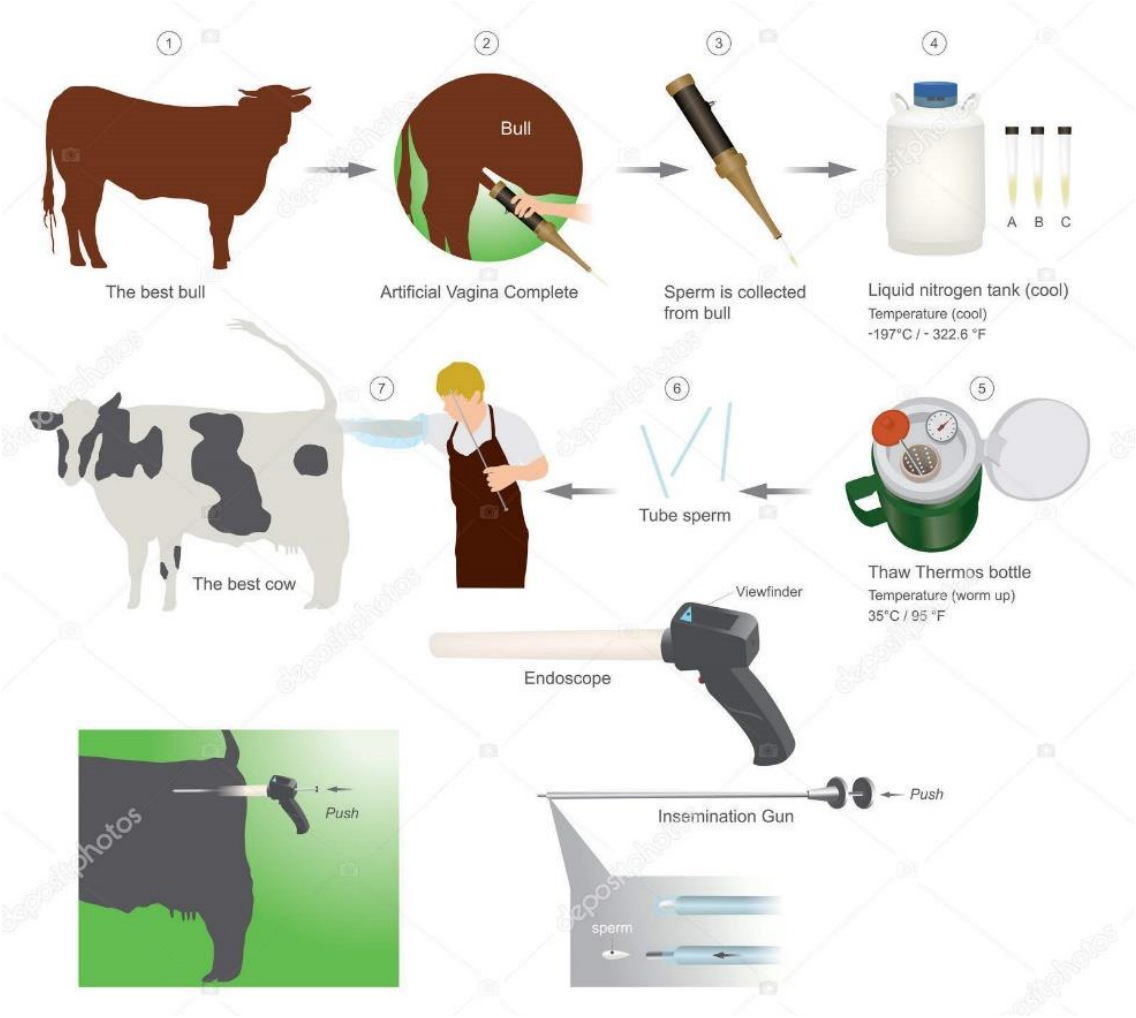

Figure 7 The steps in Artificial Insemination (Credit: Pattar)

The bulls are selected, and an artificial vagina is used for semen collection as shown in Figure 7. The collected semen is analysed again, dosed, and stored in Liquid nitrogen tanks until ready to use. The sperm doses can remain in the tank for years before being used. Farmers can buy semen based on the EBVs and the TMI booklet provided by AI Stations. With the help of the technical officers, they can choose sperm dose that fit their breeding program and which cows fit best for this dose. The cow is then inseminated as soon as the estrus is detected to maximise chances of being impregnated based on the sexual and physical behaviour of its cow.

1.6. Fleckvieh and Artificial Insemination

Fleckvieh (internationally also called Simmental) is a breed – horned or polled - with a relatively medium to large body with spots ranging from yellow, dark brown, to reddish-brown (RoysFarms, 2021; RinderZucht Austria, 2022). Its' origin dates back to the 13th century when it was an already domesticated breed that was also recognised for its extensive and pied cattle in the Simmental valley of the Bernese Oberland region (Alpenvieh, 2019).

Figure 8 Fleckvieh Cattle in Austria (credit: Fleckvieh Austria)

Austrian Fleckvieh (Figure 8) is a dual-purpose breed, producing milk in dairy farms and beef in bull fattening systems, potentially at the same farms. Furthermore, Rinderzucht Austria (2022) states that Fleckvieh is adaptable to environmental impacts and various production systems and has demonstrated good pasture and free-stall housing behaviors. Therefore, encouraging farmers to keep these cattle in intensive as well as in extensive systems is widely suggested (ZAR, 2014).

Fleckvieh also has the following advantages that make them the most demanded breeds in Austria, i.e., highest food quality, excellent fertility, efficient breeding program, maximum resource efficiency, very good health, excellent animal welfare, exact genomic selection and absolute transparency in its records (Alpenvieh, 2019; Pfleger, 2021, 2021b; RinderZucht Austria, 2022).

In Austria, Fleckvieh is highly important with close to 80% of all cattle in the country belonging to this breed. Therefore, the need to improve the breeding goals is vital, with a target reaching a value of 38% for milk, 18% for meat, and 44% for fitness (RinderZucht Austria, 2022). This ratio is based on a method

that combines these economically important single traits in a Total Merit Index (TMI) and allows breeders to make profitable decisions (Sölkner *et al.*, 2000).

With its current breeding program (Figure 9) and with the help of the different insemination stations, the organisation Fleckvieh Austria was able to produce high-quality Fleckvieh sires (Pfleger, 2021, 2021b; RinderZucht Austria, 2022).

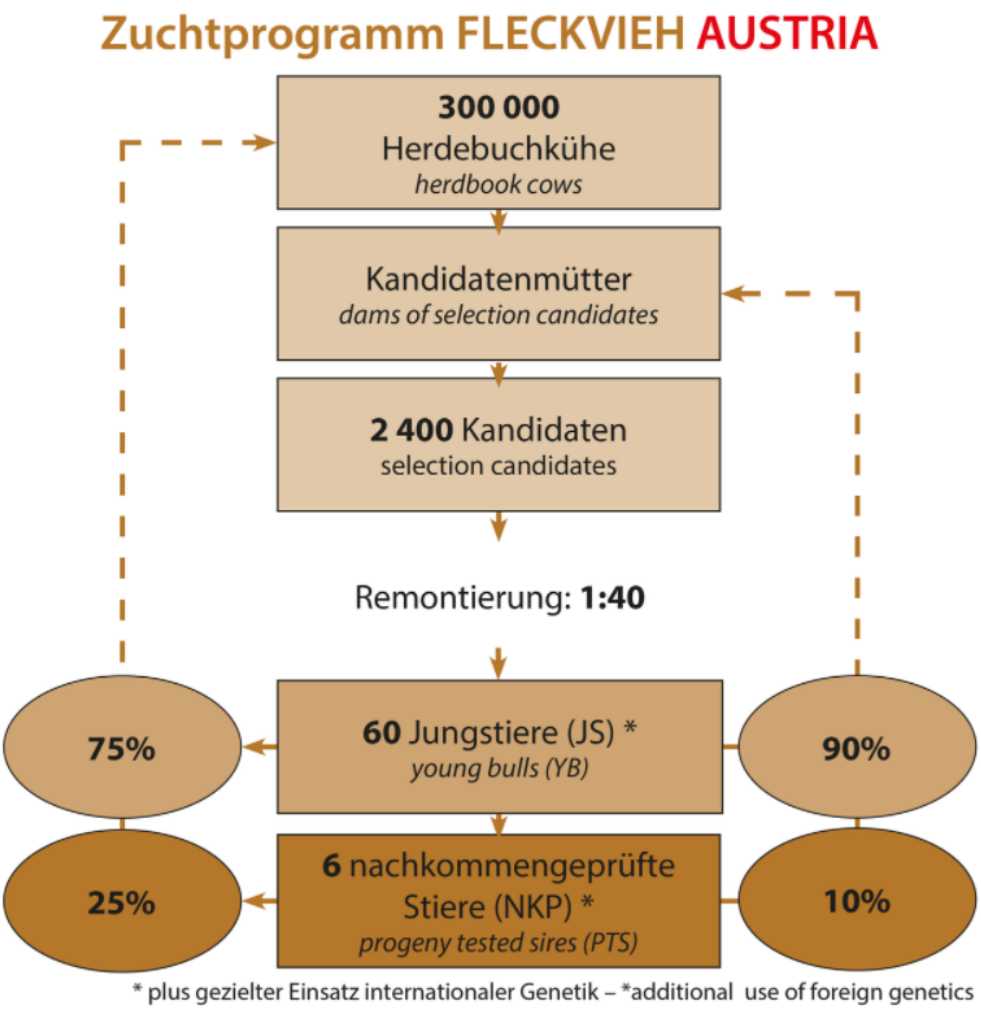

Figure 9: Overview of Fleckvieh Austria Breeding Programme (credit: Fleckvieh Austria)

AI of Fleckvieh has also helped in the agricultural economy of Austria; the high-quality sperm produced by Austrian Insemination Stations has put the Austrian Fleckvieh on the map (Genetic Austria, 2021). AI centres were able to sell sperm all around the globe, putting their bulls on the list of highest performing bulls in the world. The breed is a dual-purpose breed, performant in both sectors, increasing the demand for sperm from top bulls and encouraging Austrian AI centres to improve their techniques, selection process and progeny testing. The continuous progress can be seen through the last report by Fleckvieh Austria from December 2021 (Pfleger, 2021).

1.7. Sperm Quality

Sperm quality is technically defined as the quantitative trait of semen (McGraw-Hill, 2002). The five phenotypic traits that are measurable and therefore, used to elucidate sperm quality are sperm concentration (billion per mL) and sperm volume (mL) per ejaculation, sperm motility, percentage of sperm alive and sperm counts (number of sperm per ejaculations) (McGraw-Hill, 2002; Gredler *et al.*, 2007; Suchocki and Szyda, 2015). The different factors that influence the performance of the mentioned traits are environmental, managerial, and genetic (Beran *et al.*, 2014; Butler *et al.*, 2022).

The environmental impact includes seasonal effect, which is the most significant effect because it includes temperature, relative humidity and hours of sunlight per day (Fuerst-Waltl *et al.*, 2006). Moreover, the environment, including feeding, also impacts on sperm production per year (Mathevon, Buhr, and Dekkers, 1998; Brito *et al.*, 2002; Fuerst-Waltl *et al.*, 2006). Another vital part that influences sperm quality is management, as it includes human factors such as the sperm collectors, the sperm analysts, the number of days between the collection, number of groups per day, and house management (Everett and Bean, 1982; Brito *et al.*, 2002; Fuerst-Waltl *et al.*, 2006). Animal factors like the individual, its breed and its age at sperm collection are important (Mathevon, Buhr and Dekkers, 1998; Fuerst-Waltl *et al.*, 2006; Beran *et al.*, 2014; Argiris *et al.*, 2018). Another factor that can potentially be included in sperm analysis is cryopreservation; the extreme temperatures affect the morphology of sperm and consequently reduce fertility (Ugur *et al.*, 2019).

Studies have shown that sperm quality and production were affected by age (Brito *et al.*, 2002; Igna *et al.*, 2010; Argiris *et al.*, 2018). One of the early studies shows that sperm concentration and sperm counts were the highest when the bulls were at four years of age (Everett and Bean, 1982). In contrast, the most significant result for sperm counts and the number of doses per ejaculations were observed around 5-7 years old a few years later (Igna *et al.*, 2010). Furthermore, the volume of sperm and the sperm counts were found to rise with the age of bulls at collection day (Fuerst-Waltl *et al.*, 2006). Murphy et al. (2017) found that the correlations between the bull's age and volume of sperm, the concentration of sperm and the sperm count were all statistically significant. It was concluded that with the ageing of a bull, the volume per ejaculation was increasing too, and so a sperm count was performed; for sperm concentration, the highest concentration was found between 1 and 2 years old (Murphy *et al.*, 2018a). In Figure 10 based on Murphy et al. (2018) we can observe and increase in production from bulls of less of 1 year old to those of older than 4 years old. The total number of sperm and ejaculation volume(mL) has almost doubled from those two-age range (Murphy et al., 2018).

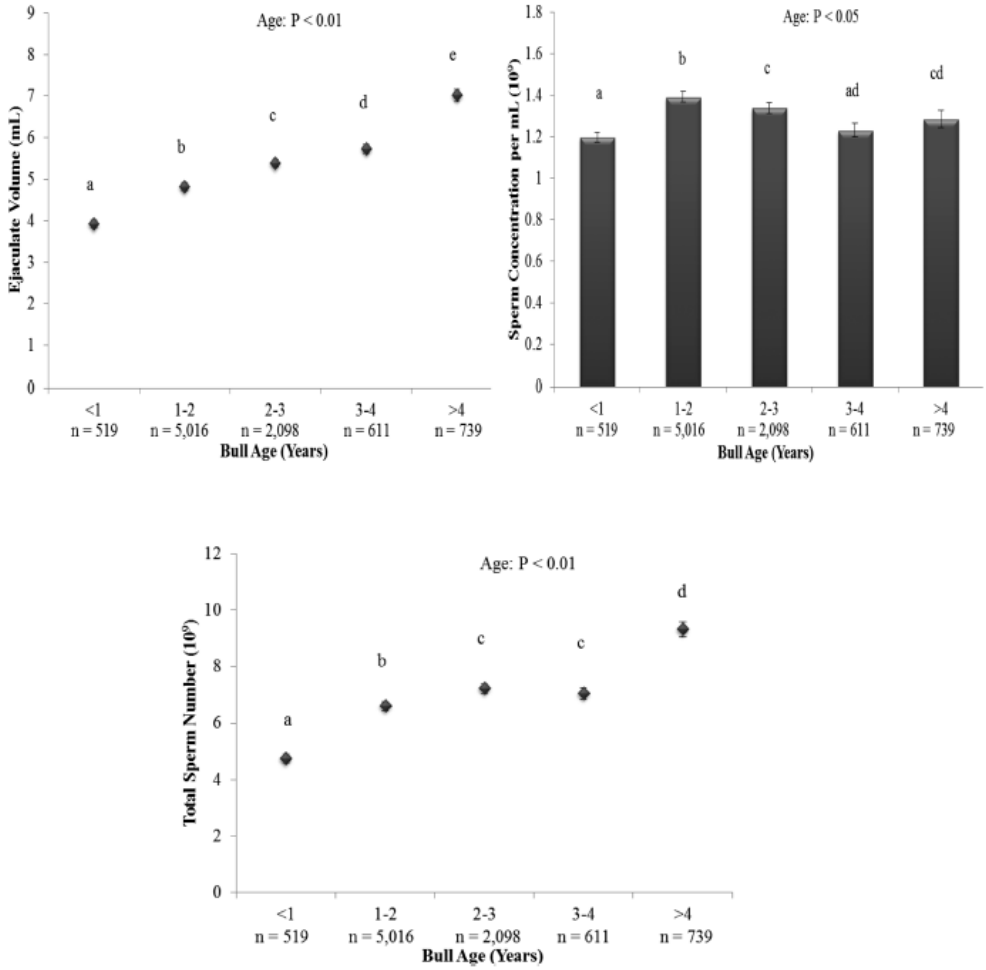

Figure 10: *Effect of bulls age on volume of sperm, the concentration of sperm and sperm counts (Murphy* et al.*, 2018a)*

Fuerst-Waltl et al. (2006) showed that age and collection intervals had the most significant impact on the quality of semen collected due to the high volume of sperm collected per ejaculation and the sperm counts. This finding is supported by other studies, which found the same significant effects on these traits; their collections were conducted in collection intervals of 4 to 5 days (Mathevon, Buhr and Dekkers, 1998). Mathevon and colleagues (1998) also concluded that the sperm collectors had significantly impacted most of the traits of sperm quality in both young and older bulls.

When considering season and age interaction, semen quality significantly improved with younger bulls, whereas no significant results were observed in mature bulls regarding the volume and concentration of sperm (Mathevon, Buhr and Dekkers, 1998). It was observed by other research that Bos Taurus sperm production was less impacted by season effect than Bos Indica (Koivisto *et al.*, 2009; Bhakat *et al.*, 2011; Snoj, Kobal and Majdic, 2013). Furthermore, summer delivered the best results in the Slovenian region, showing that effects of seasons can indeed be observed in the Bos Taurus breeds claiming that the longer day period influenced positively semen production (Snoj, Kobal, and Majdic, 2013). Whereas some studies that summer was not as significant, but spring and winter was positively influencing semen production (Swanson and Herman, 1944; Fuerst-Waltl *et al.*, 2006). Another crucial impact was temperature; high temperature (above 20°C) has negatively affected sperm quality traits (Chemineau, 1994; Igna *et al.*, 2010). Based on the research conclusions above, it is clear that several factors impact sperm quality. Therefore, these factors must be considered within the framework of our research question and must be considered before coming to any conclusions.

1.8. Genome-Wide Association Study and Semen Quality

Genome-wide association studies (GWAS) use single nucleotide polymorphisms (SNPs) as a genetic variant for correlating DNA sequence variants and interesting phenotypes (Donnelly, 2008; Wu *et al.*, 2014). The latter has been used to examine human diseases as it was proven to be a successful approach to detect genetic defects and disease resistance (Wu *et al.*, 2014). This is done by investigating individuals whose phenotypes differ and establishing their genotypes, leading to identifying loci that impact both normal variation and predisposition to diseases, shedding light on the complexity of traits (Donnelly, 2008; Barsh *et al.*, 2012).

Researchers successfully applied GWAS in the animal breeding sector and detected genes and markers of traits that had big economic effect (Barsh *et al.*, 2012; Wu *et al.*, 2014). GWAS of sperm quality using medium-density SNP genotyping arrays in the livestock sector has triggered interest in the scientific community (Serrano *et al.*, 2021a). These studies aimed to identify the genomic regions that affect the phenotypic characteristics of sperm (Hering *et al.*, 2014; Hering, Olenski and Kaminski, 2014; Suchocki and Szyda, 2015; Serrano *et al.*, 2021a).

1.8.1. GWAS: Additive and Dominance Effect of Sperm Quality Traits

Additive effects represent the independent effects of alleles at a locus (Hager, Cheverud and Wolf, 2009; Stöppler, 2021), i.e., when several genes are responsible for the same trait and have the same result for the phenotypic characteristic (Steele, 2016). The data available for this study would allow us to identify polymorphous which could impact the four sperm quality traits and comprehend which genes has an effect on these traits (Costa *et al.*, 2019; Butler *et al.*, 2022). When analysing GWAS for sperm quality the phenotype mostly used were number of sperm, concentration of sperm, volume of sperm and motility of sperm (Serrano *et al.*, 2021) unfortunately not a lot of GWAS was done to assess bull fertility, limiting the amount of literature.

In one study, the additive effect for Holstein-Friesian bull sperm traits was analysed, significant SNPs was identified on the chromosome (Chr) X, 1, 6. 23 and 24 for sperm concentration, 4 SNP were located, and all of them were on Chr X for both volume of sperm and motility score and finally for sperm counts, 12 SNP were found to be significant on chromosome X, 8, 3, 7 and 16 (Suchocki and Szyda, 2015). A previous study, which was also done on a Holstein-Friesian population, identified significant SNPs on Chr 22 for sperm counts and semen volume, which were neighbored by different genes called DCP1A (decapping mRNA 1A), SFMBT1 (Scm-like with four mbt domains 1) and TMEM110 (transmembrane protein 110), one significant SNP on Chr 25 for sperm count and another on Chr 10 for the volume of sperm (Hering *et al.*, 2014). Both studies were carried out in Poland but concluded with different results. A recent study was carried out on Angus bulls' fertility traits (Figure 11). In this specific research, the volume of sperm had five significant SNP (Chr 2, 3, 6 and 27), the concentration of sperm three significant SNP (Chr 1, 3 and 5) and sperm count six significant SNP (Chr 1, 6, 8, 9, 11, 17 and 24). Genes linked to bulls' fertility were located on the SNP or neighboring the significant SNP (Butler *et al.*, 2022). NR5A2(nuclear receptor subfamily 5 group A member 2), NPC1(intracellular cholesterol transporter 1), and DMRT1(double-sex and mab-3 related transcription factor 1) were suggested as promising genes related to semen traits in the Chinese Holstein bull (Yin *et al.*, 2019). Studies show that the sperm quality analysis differs, but some chromosomes seem to be found repetitively among populations.

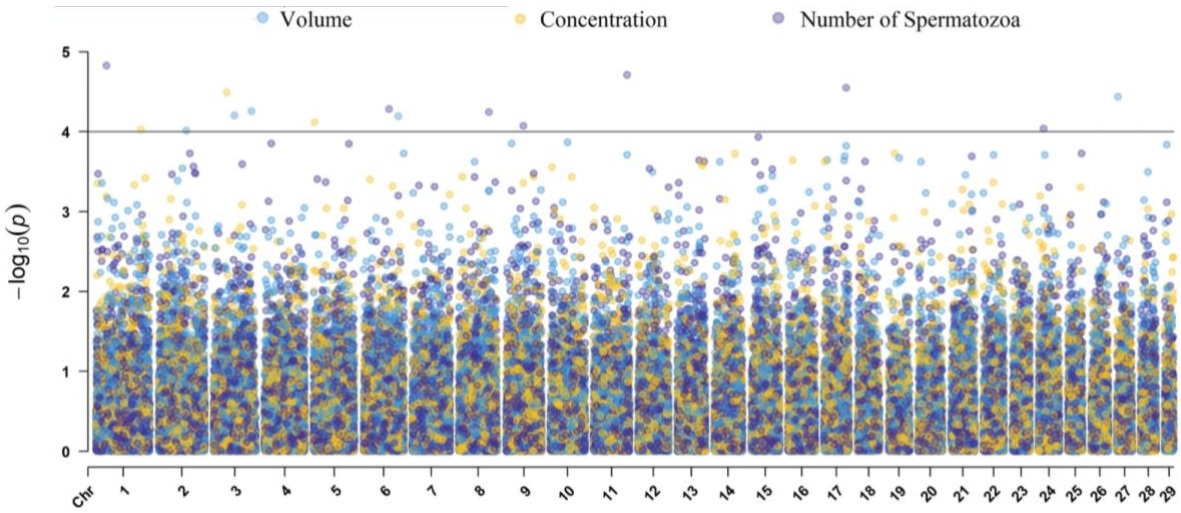

Figure 11: Manhattan plot from Butler et al. (2022) analysis for the three different bulls' sperm traits: Volume of sperm, the concentration of sperm and sperm count with a significant threshold of 4.0 (Butler et al.*, 2022)*

In contrast to the GWAS for additive effects which may be done using EBV, the dominance effects analysis requires the phenotypic records for each genotyped animal (Mao *et al.*, 2020). Dominance effects play an essential role in the non-additive genetic effects representing the connections between alleles at the same locus (Falconer and Mackay, 1996; Hager, Cheverud and Wolf, 2009; Mao *et al.*, 2020). Limited research was done with GWAS on the dominance effect of the different sperm quality traits. Most studies involving bulls and male fertility tend to focus on the sire conception rate (SCR), and genes associated with dominance effect in male fertility were on Chr 8, Chr 9, Chr 13, Chr 17, and Chr 27(Nani, Rezende and Peñagaricano, 2019). Another study associated Chr 9, Chr 11, Chr 19 and Chr 28 with additive and dominance effects in male fertility of Danish Holstein Cattle (Mao *et al.*, 2020). Unfortunately, these genes involve male fertility and SCR and not the four primary traits associated with cattle sperm quality. Butler et al. (2022) and Yin et al. (2019) used the BLUPF90 software (Masuda, 2019) for their statistical analysis and the GWAS. Butler used a stringent threshold (-log10(p) value = 4.0), arguing that the False Discovery Rate (FDR) threshold, was too strict, allowing to do investigation of the biological potential of the significant SNP at that threshold (Butler *et al.*, 2022)

1.8.2. GWAS: Run of Homozygosity and Sperm Quality Traits

Runs of homozygosity (ROH) are continuous parts of the genome where no heterozygosity is observed in the diploid state (Sölkner *et al.*, 2010; Ferencakovic *et al.*, 2011; Ferenčaković *et al.*, 2017). ROH occur when the parents pass on identical and hereditary haplotypes from common ancestors (Ceballos *et al.*, 2018; Zhao *et al.*, 2021). Long ROH suggest recent inbreeding and a lower chance of recombination, whereas short ROH suggest older inbreeding (Broman and Weber, 1999; Zhao *et al.*, 2021). Inbreeding depression has affected measurable traits like sperm quality, but studies kept focusing on how pedigree and genome-wide autozygosity affect quantitative phenotypes (Ferenčaković *et al.*, 2017).

Mapping inbreeding depression using ROH related to the sperm quality traits can better comprehend the relationship between phenotypic traits of sperm quality and genome homozygosity (Zhao *et al.*, 2021). A study by Ferenčaković et al. (2017) showcased that the model revealed genomic regions significantly associated with ROH status (Figure 12). The sperm count had four significant regions with the genes shown in Figure 12B, and the percentage of live spermatozoa had five regions (Figure 12D).

The genes shown in Figure 12 influenced spermatogenesis and sperm morphology (Ferenčaković *et al.*, 2017).

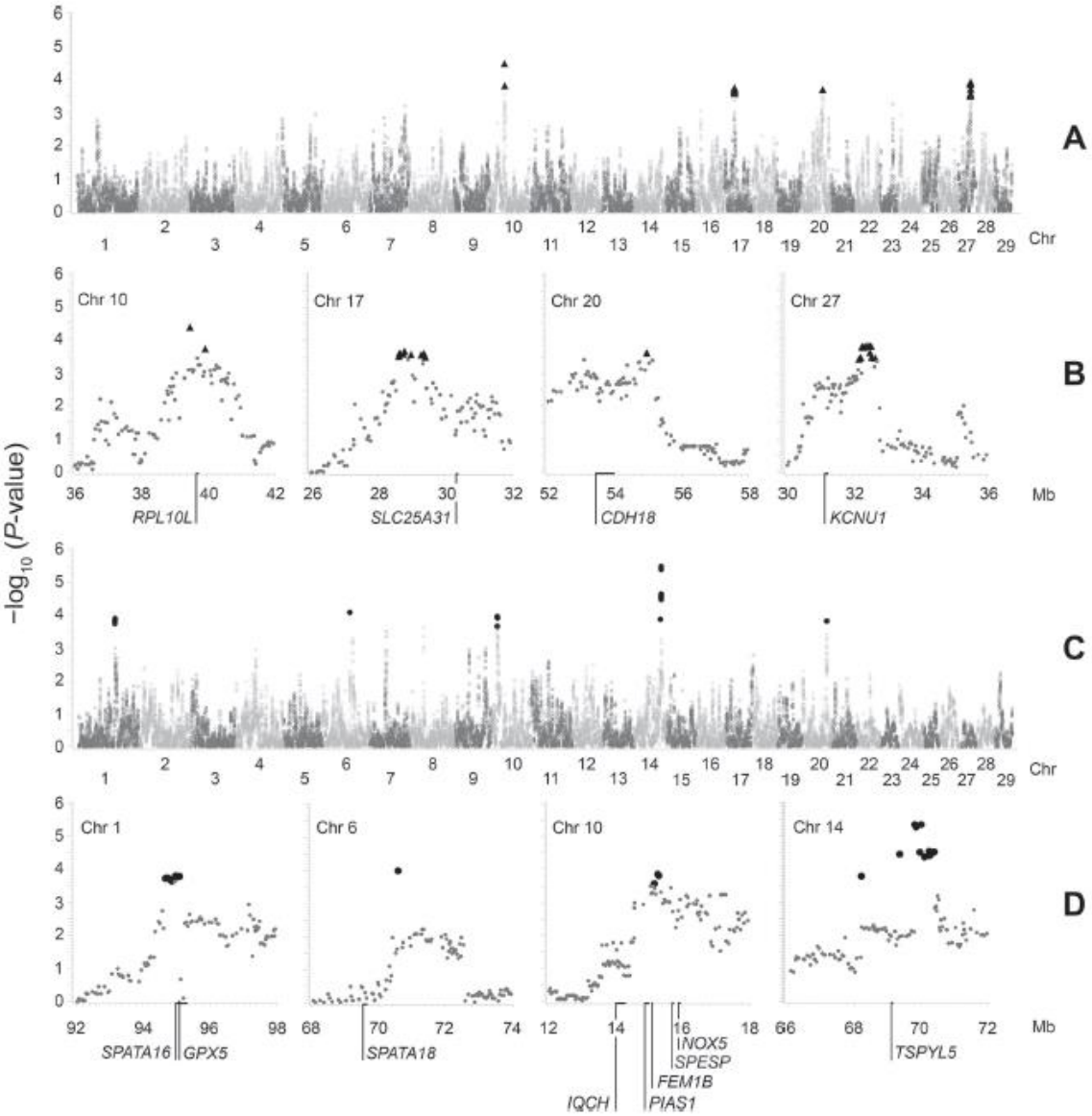

Figure 12: Significance of estimated ROH effects for male fertility traits according to genome location. Significance of estimated results for the total number of spermatozoa and percentage of live spermatozoa are illustrated in a Manhattan plot in panels A and C for the whole genome, whereas significant estimates with the location of suspected genes associated with function in the male reproductive system are presented in panels B and D, respectively) (Ferenčaković et al.*, 2017).*

Another study that performed a whole-genome homozygosity mapping in the US Holstein breed located three significant genes on the Chr 10, which included the gene named ARID4A, which holds an essential role in sperm quality as it has impacts spermatogenesis (Wu *et al.*, 2013; Nani and Peñagaricano, 2020). In another research, no significant results were obtained when mapping the ROH effect in the Dutch Holstein Friesian population (Doekes *et al.*, 2020). Research on these two effects was mainly done on male fertility and conception rate, and essential genes were found to be significant, which helped in mating decision.

1.9. Success of Insemination and Bull Fertility

Bull fertility represents an essential focus as one bull can naturally mate with a maximum of 40 cows, and through artificial insemination, more than 100,000 cows could be inseminated (Kastelic, 2013). The sperm quality cannot solely measure bull fertility as it cannot be defined before mating has occurred; it is therefore measured by "the percentage of cycling females exposed to the bull and impregnated during a specific period" (Nadarajah, Burnside and Schaeffer, 1988; Hamilton, 2015), also known as the non-return rate (NRR). The non-return is recorded for each cow as 1 and 0 where 1 is pregnant and 0 is not pregnant (Reurink, Den Daas and Wilmink, 1990). It could be considered a reliable indicator of fertility if the data collected is accurate (Varotto *et al.*, 2016). Typical NRR values, also used in this study are those after 25 days, 56 days, and 90 days.

The fertility of bull is also impacted by different factors, mainly genetic ability and environmental impact involving feed management, diseases, and cattle and mating management (Hamilton, 2015). To take the right decision related to selecting the best fertile bull, there must be continuous recording, monitoring, and analysis of the NRR over the years (Stålhammar, Janson, and Philipsson, 1994). NRR, as mentioned before, is affected by several environmental effects which were all significant, but the factor with the highest significance is the age of cows at insemination, where a 20% difference was observed between heifers and mature cows (Miglior, Pizzi and Guaita, 1998).

Early studies stated that there is a decrease in conception rate as bulls get older, with optimum age varying between 1 and 6 years old (Bowling, Putnam and Ross, 1940; Hilder, Fohrman and Graves, 1944; Tanabe and Salisbury, 1946; McCullough, Seath and Olds, 1951). A study in Namibia (2004 to 2017) on different beef breeds of bulls aged 4 to 14 years old used for mating with cows of maximum of 17 years old showed an overall 71.7% conception rate. In the same study, the age of bulls were analyses based on conception rate. There were no significant results between the different age groups but a slightly higher pregnancy rate with semen from bulls of less than 7 years old (Table 2) (Samkange *et al.*, 2019). In contrast, there were studies where the conception rate had a positive effect when the age of bulls was between 1.3 to 5.5 years old and decreased after that (Norman, Wright and Dürr, 2015; McWhorter *et al.*, 2020) based on a 70 days NRR (Meland, 2016). One of the studies made a general conclusion: with younger cows (2 to 6 age-old), pregnancy rate increased and decreased from 7 to 11 age independently of the culling strategy (Shorten, Morris and Cullen, 2015).

Table 1: Breed and age of sires and relationship to conception rate in a Namibian farm (Samkange et al., 2019)

Category	Non-pregnant	Pregnant	Total animals	Pregnancy rate (%)
Breed				
Afrikaner	429	1047	1476	70.9[a]
Nguni	55	199	254	78.3[b]
Simmental	26	48	74	64.9[a]
Sire age				
< 7 years	355	919	1274	72.1[a]
7 to ≤ 11 years	139	336	475	70.7[a]
> 11 years	16	39	55	70.9[a]
Overall	510	1294	1804	71.7

Values sharing the same suffix [a,b] within each category were not different since $p > 0.05$

2. Data & Methods

This chapter covers the material and methods used in this chapter. It provides insight on what methods were used and how the analysis was conducted. It covers the source of data, the data manipulation and the software used to ensure that we meet the research objectives.

2.1. Data Collection

The data used in the analysis was provided by ZuchtData and was collected in a 12-year window within Austria. The data collected was by technical staffs on different stations. The data collected was important for both phenotypic data and genomic data analysis.

2.1.1. Phenotypic Data

The phenotypic data was provided by three different Austrian AI stations (Figure 13): Hohenzell (Upper Austria), Wieselburg (Lower Austria) and Gleisdorf (Styria). The insemination stations located in Wieselburg and Gleisdorf are owned by Genostar Rinderbesamung GmbH and the one located in Hohenzell by Oberösterreichische Besamungsstation GmbH.

Figure 13: Map of Austria with the locations of the three different AI Stations, Gleisdorf (Styria), Hohenzell (Upper Austria), and Wieselburg (Lower Austria)

The stations operate similarly with a tie-stall barn system and semen being collected two to three times a day except in Gleisdorf, where it is done once a day. The total number of ejaculations recorded between 2000 and 2021 was over 130,000 ejaculations with 15 different breeds. The three AI stations recorded the following sperm quality traits: the volume of sperm, the concentration of sperm, and the percentage of motile spermatozoa. The recordings also included the date of birth of bulls, semen collector and analyst, as well as the date of collection. From this data, we funneled out the breeds to one breed, namely Fleckvieh, with ejaculations records from 2008 to 2021, leaving us with 1136 Fleckvieh bulls with around 80,000 ejaculates.

To assess sperm fertility, ZuchtData EDV - Dienstleistungen GmbH (Vienna, Austria) provided us with NRR (25, 56 and 90 days) of the cows inseminated by the selected bulls for further analysis. The records included age of cows at first insemination, parity of cows, age of bulls at the first insemination with its respective cow (3,125,362 records from 2010 to 2020).

2.1.2. Genotypic Data

A total of 1,136 bulls were genotyped, and quality controlled by ZuchtData EDV - Dienstleistungen GmbH using Bovine SNP50 BeadChip (Illumina, San Diego, CA), which contained 40,884 SNP after quality control performed by the company. The genotypic data included 5-generation pedigree information for the bulls.

2.2. Quality Control

The genotypic data had already been controlled; no further change was required. The phenotypic data was funneled to keep only Fleckvieh as a breed due to the highest density of data compared to other breeds.

The further analysis consisted of ejaculations from bulls of 10 to 96 months of age with intervals between 2 ejaculations ranging from 2 days to 21 days. Ejaculation records with the volume of semen (mL) varying from 1.0 to 25.0 mL and concentration of sperm between 0.1 and 4.0 billion per mL was kept. Everything else was excluded, including missing records for the percentage of motile spermatozoa. The total number of spermatozoa (x 10^6) was calculated by the volume and concentration of sperm at collection. The total number of spermatozoa was transformed by using the TRANS REG procedure with the Box-Cox Transformation with equation defined by *Ferenčaković et al. (2017), i.e.* **(Total Number of Spermatozoa$^{0.3}$ – 1) / 0.3** so that the trait follows a normal distribution (Ferenčaković *et al.*, 2017). Table 3 shows an overview of the characteristics used for statistical analysis. The data for further analysis consisted of 54,088 ejaculations from 1136 Fleckvieh bulls of 10 to 96 months old.

Table 2: Descriptive statistics summary of quality-controlled records of Fleckvieh bulls aged 10 to 96 months old collected from the three different stations

Descriptive statistics : Fleckvieh of 10 to 96 months of Age

Statistic	N	Mean	St. Dev.	Min	Pctl(25)	Median	Pctl(75)	Max
Bulls_age	54,088	32.6	22.9	10.6	17.4	20.7	39.0	96.0
Number_sperm	54,088	6.8	3.5	0.1	4.3	6.2	8.7	36.0
Number_Sperm_TR	54,088	2.4	0.9	-1.6	1.8	2.4	3.0	6.4
Collection_Interval	54,088	4.1	2.1	2	3	4	5	21
Volume_ml	54,088	5.8	2.3	1.0	4.0	5.5	7.0	19.0
Concentration	54,088	1.2	0.5	0.1	0.9	1.1	1.5	4.0
Percentage_Alive	54,088	67.5	8.7	0	65	70	70	95

3. Statistical Analysis

The descriptive statistical analysis for semen production with age, year, and season was done using R-Studio (R Core Team, 2015) and the tidyverse packages (Wickham and RStudio, 2021), using dplyr data manipulation and ggplot2 package for data visualization. The R package "stargazer" (Hlavac, 2018) was used to generate the descriptive statistics. Descriptive statistics are presented as summary tables.

3.1. Mixed Linear Model

R was to run a linear mixed effect model (LMEM) using the lme4 package and using the lmer package (R Core Team, 2015) and the car package for the summary and anova of the models run . The package allows to fit and analyse mixed linear models which involve fixed- and random-effects from which the overall mean of the observations can be assessed (Bates *et al.*, 2015).

To analyse the environmental effects on the different semen quality traits, the following statistical ***model (A)*** was used:

$$\gamma_{ijklmn} = \mu + age_i + collection\ interval_j + semen\ collector_k + semen\ analyst_l + (year\ x\ season\ x\ station)_m + \alpha_n + \varepsilon_{ijklmn'}$$

Where γ_{ijklmn} is the individual observation, μ is the overall mean, *age* is the fixed effect of age(rounded to nearest whole number), $collection\ interval_j$ is the fixed effect of interval in days between each semen collection, $semen\ collector_k$ is the fixed effect of semen collector, $semen\ analyst_l$ is the fixed effect of semen analyst, $(year\ x\ season\ x\ station)_m$ is the fixed effect of the interaction of year, season and station at the time of semen collection, α_n is the random effect of the bulls and $\varepsilon_{ijklmn'}$ is the unexpected error due to each effect(Ferenčaković *et al.*, 2017). The models were repeated for each trait, i.e., the volume of semen, the concentration of sperm, the transformed total number of spermatozoa, and the percentage of motile spermatozoa.

3.2. Genome-Wide Association Studies

3.2.1. Genome-wide complex trait analysis Software Tool for GWAS analysis

Genome-wide complex trait analysis (GCTA) software tool is a comprehensible tool that analyses a chromosome or a whole genome by estimating the variance explained by all the SNPs along with it for a complex trait (Yang *et al.*, 2011). The software requires binary files coded as 0, 1 and 2, showing homozygous or heterozygous alleles.

The software includes an option widely used nowadays: mixed linear model association studies (MLMA). Yang et al. (2014) explains that the MLMA avoids false positives due to population or relatedness structure. The mixed linear model (MLM) leaving-one-chromosome-out (LOCO) option was used for our analysis. The latter works with an MLM based associated analysis where the candidates' SNP chromosome is excluded from the Genetic Relationship Matrix (GRM) calculation coded as --mlma-loco (Yang *et al.*, 2011, 2013, 2014).

The --mlma-loco option has the following ***model (B)***:

$$Y = a + bx + g- + e$$

y is the phenotype, a is the mean term, b is the additive effect (fixed effect) of the candidate SNP to be tested for association, x is the SNP genotype indicator variable coded as 0, 1 or 2 and g- is the accumulated effect of all SNPs except those on the chromosome where the candidate SNP is located, and e is the residual, an essential step with the $-mlma\text{-}loco$ is that the genetic variance, var(g-), is re-estimated every time a chromosome is left out from the GRM calculation(Yang *et al.*, 2011, 2014). Yang et al. (2014) state that this method is less efficient but more powerful than the MLM analysis that only uses the –mlma code.

3.2.2. GWAS: Additive, Dominance, and Run of Homozygosity Effect

For the additive effect, the SNP genotypes were coded as 0, 1 and 2, with 0 being AA, 1 being AB and 2 being BB. While analysing the dominance effect, the SNP genotypes were coded as 0 and 1, being 0 being AA and BB and 1 being AB. No quality control was required since the genotype file was cleaned by ZuchtData beforehand.

Only autosomal SNP assigned to an autosome chromosome were used for the GWAS analysis for the ROH effect. SNP for which more than 10% of genotypes were missing and bulls with more than 5% of their genotypes missing were excluded from further analysis using PLINK (Purcell *et al.*, 2007). The ROH homozygosity analysis was carried out using the cgaTOH software (Zhang *et al.*, 2013). The ROH were called if 15 or more consecutive homozygous SNP were present, with a minimum length of 2 Mb, with gaps of no more than 1,000 kb between them and without allowing any heterozygous calls (Ferenčaković, Sölkner and Curik, 2013; Ferenčaković *et al.*, 2017). We used the ROH output (ROH >2Mb) generated from the software to produce a genotype file. The genotypic matrix was generated using R to have every SNP run for every bull. The SNP genotypes were coded as 0 and 1, for SNP in a run and SNP not in a run, respectively. This genotype file was then used in the GCTA software for GWAS analysis.

3.2.3. GWAS Analysis

The three genotype files generated above with the binary coding were used in GCTA using the process described above.

$$"gcta64 \;\; --mlma-loco \;\; --bfile\ file \;\; --pheno\ phenoFL.txt \;\; --mpheno\ x \\ --autosome-num\ 29 \;\; --out\ Gwas\_Residual\_FL\ "$$

Each binary file recorded above for the additive, dominant and ROH effect analysis was introduced in the GCTA program using the –bfile code. The –pheno file is the text file that contains the corrected phenotype for all the traits; it corresponds to the residuals calculated from model A (see chapter 3.3.1). Each column after the ID column in the phenotype text files corresponds to each trait assessed in this paper. For each desired quality, the column is designated accordingly from the phenotype text file using the code –mpheno.

An essential aspect of GCTA is that it automatically generates output files using human autosome pair number, therefore for GCTA software to recognise that the analysis is for another species, we must add to the code line –autosome-num, which corresponds to the number of autosome pairs in cattle, i.e., 29 pairs. The code runs the following model B described above with the GRM calculation integrated into the software analysis. The output contains the Bonferroni threshold of -log(p) value of 5.91 and

the p-value for each SNP with a significant level of -log(p) value of 4.0. The gene names were referenced using NCBI Bos Taurus ARS-UCD1.2 Assembly.

3.3. Success of Insemination with Bulls Age

To analyse sperm quality, the insemination success was measured using the cow's non-return rate at 25, 56 and 90 days after the first insemination service. The 3,125,362 records from 2010 to 2020 were cleaned and processed into the format required using SAS Software (SAS Institute Inc., 2015). Briefly, only records that matched the phenotypic data of bulls used for the quantitative and genomic analysis of semen quality were retained for further analysis.

The bull's age was kept from 9 to 96 months old, bulls of 9 months old to 12 months were merged and categorised as “12 months old” due to low number of records. The records with parity of 0, i.e., insemination of females that were never pregnant previously was removed and the records with no cow ID. Age of bulls at insemination was calculated by the difference between the date on insemination and date of birth of bulls.

Further analysis was done using the 1,158,670 records left after cleaning the original set of data. SAS Software (SAS Institute Inc., 2015) was used to generate a mean value of NNR at 25, 56 and 90 days against the simple model of age effect on the success of insemination, the error bars were included which were 2 times standard error. A frequency table was also generated for each non-return rate group and age group.

4. Results and Discussion

This chapter covers the results and discussion. It includes the quantitative and genomic analysis results. The two chapters were merged to achieve a better understanding of results. The discussion contains the conclusion based on research papers discussed in literature review but also on my personal point of view and understanding.

4.1. Environmental and Management Effects on Semen Quality Traits

This subchapter is based on the results from the mixed-linear model A discussed above and how each of the effects; bulls' age, collection interval, sperm collector, sperm analyst, year, season, and station, affect sperm quality traits, namely number of sperm, volume of sperm, concentration of sperm and percentage of motile spermatozoa. The summary (car package) from the LMEM generated by lme4 R Studio package showed that all the effects were significant for the number of sperm ($p < 0.05$) and concentration of sperm ($p < 0.05$). Every effect was statistically significant for the volume of sperm ($p < 0.05$) except for the effect of the three stations ($p > 0.05$). The percentage of motile spermatozoa was significantly affected by semen collector, semen analyst, season, year, and station ($p < 0.05$) but not by bulls' age and the collection interval ($p > 0.05$).

4.1.1. Effect of Season on Sperm Quality Traits

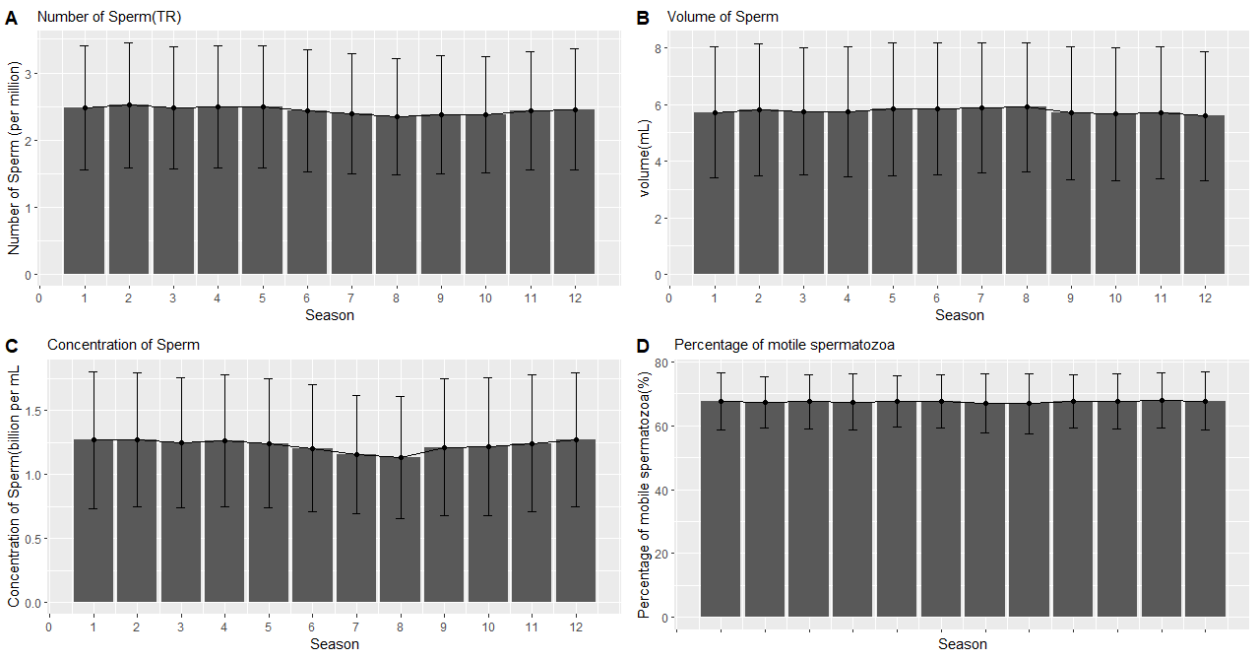

Figure 14: Season production for the for Number of Sperm (TR) (A), Volume of Sperm (B), Concentration of Sperm (C) and Percentage of motile spermatozoa (D)

Figure 14 shows a drop in the mean number of sperm and concentration of sperm from June and slightly increasing as from October. In Austria, this period corresponds to the beginning and end of summer. As mentioned by Chemineau (1994), Igna (2010) and Fuerst-Waltl et al. (2006), the season has an impact on sperm traits and, therefore, its production. A slight increase is observed in the same period for the mean volume of sperm collected. The difference between the summer and winter production based on Figure 15 does not vary immensely, reflecting how Fleckvieh has adapted to the

seasonal effect through the years. The background that the change between summer and winter, could be that in winter the temperature is in the range to maximise semen production (Morrell, 2020) and it is then compensated by the longer days help to increase production of semen (Snoj, Kobal and Majdic, 2013). The percentage of live spermatozoa quantitively seems stable through the season, ranging between 60% to 70%, this could be due to how the bulls were kept in the stations.

4.1.2. Effect of Year on Sperm Quality Traits

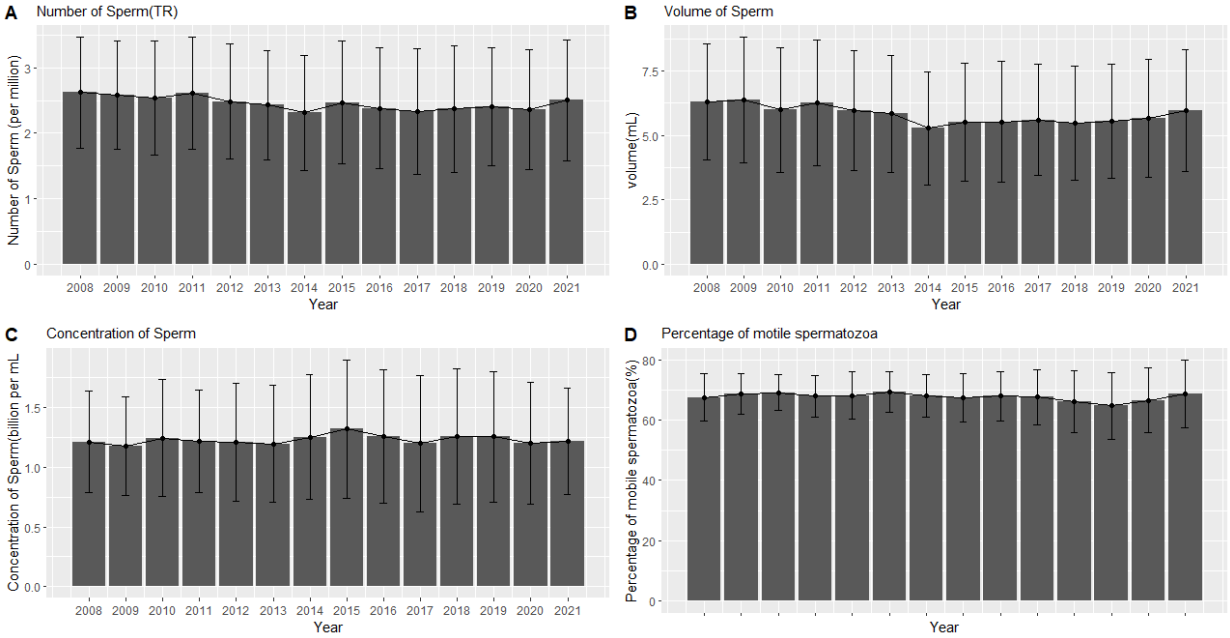

Figure 15: Yearly production for the Number of Sperm (TR) (A), Volume of Sperm (B), Concentration of Sperm (C) and Percentage of motile spermatozoa (D)

The average number of sperm, the concentration of sperm and volume of sperm fluctuated a lot from 2008 to 2021 (Figure 15), with a decrease in sperm counts until 2020. The same was observed in the volume of sperm and concentration of sperm, which has been relatively similar except with a peak in 2015. With the current situation where global warming has become a big topic and Austrian temperature change through the year is thoroughly reported, we can see an increase in mean temperature per year through the last 20 years (Figure 16).

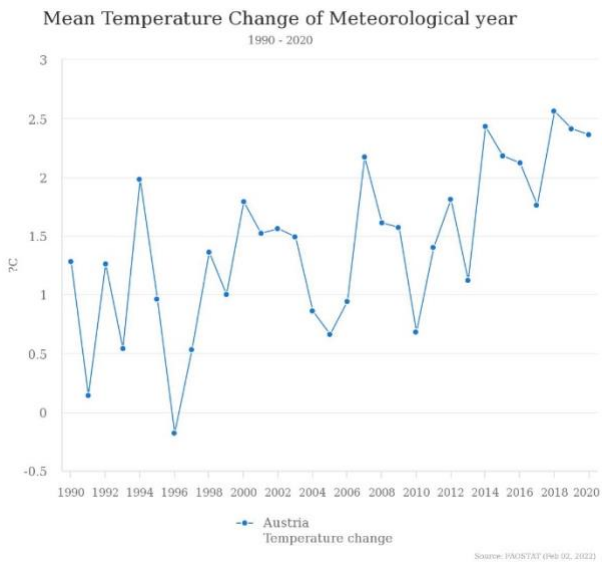

Figure 16: Mean temperature change from 1990 to 2020 in Austria (FAOSTAT, 2022)

Since heat and humidity impact spermatogenesis, they hence could affect the volume of sperm, sperm counts and volume of sperm (Mathevon, Buhr and Dekkers, 1998; Fuerst-Waltl *et al.*, 2006; Igna *et al.*, 2010; Snoj, Kobal and Majdic, 2013). The change of +1.2°C in the mean temperature during the last ten years could

be critical as optimal temperature for spermatogenesis was assessed to be 15-18°C, i.e., for 65–70 days before collection (Morrell, 2020), and in the last years, summers have been warmer and winters colder.

The technology used in AI stations and the use of good breeding programs may have prevented a drastic decrease in sperm quality traits. The adaptation of breeds with time might have also had an impact on the stabilisation of semen output through the years.

4.1.3. Effect of Age on Sperm Quality Traits

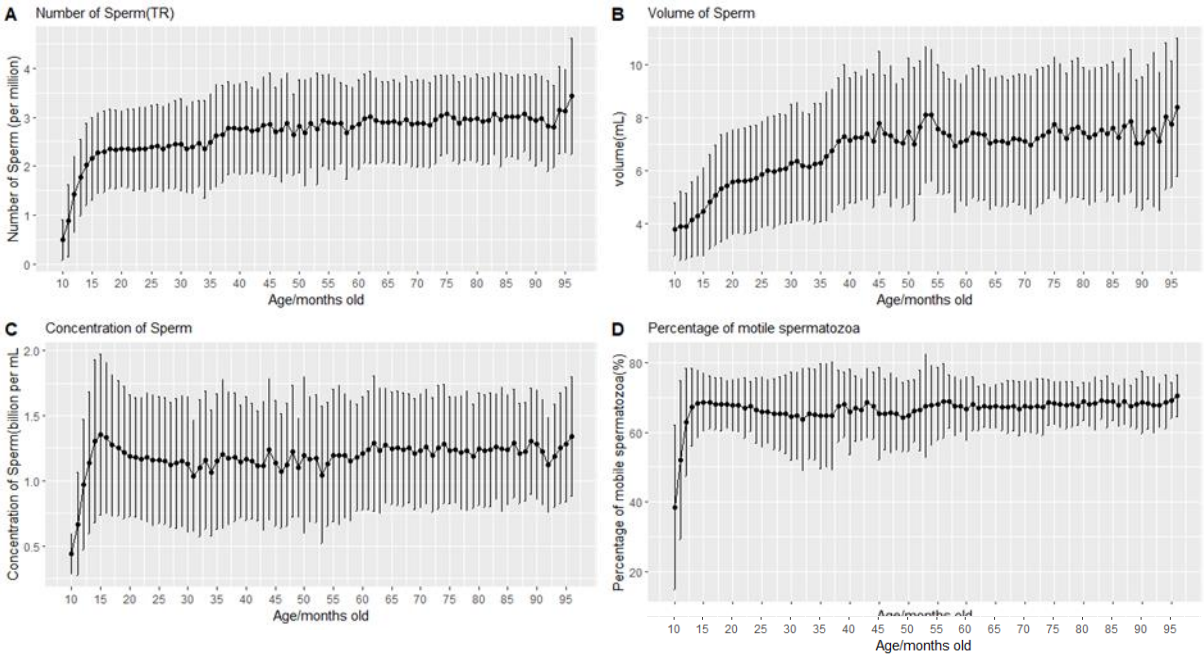

Figure 17: Effect of bulls' age on the Number of Sperm (TR) (A), Volume of Sperm (B), Concentration of Sperm (C) and Percentage of motile spermatozoa(D), note that the number of individuals are relatively low for ten months old (n=3), 11 months old (n =87) and 96 months old (n=10)

The effect of the age of bulls on sperm traits was significant for all traits ($p < 0.05$) except for the percentage of motile spermatozoa. The trend observed in Figure 17 for all traits was an increase from 10 to around 15 months old, followed by a decrease, and then an increase again at 55 months old and another drop at 93 months old with another boost. However, it should be noted that the percentage of alive spermatozoa after 15 months stabilised to around 65% and experienced a slight increase at 55 months old to about 75%. The group of 10 months old (n=3), 11 months old (n=87) and 96 months (n=10) are a small group, and therefore the results might not be as representative as necessary.

Bulls are considered to have passed puberty if they have reached an ejaculation containing 50 million spermatozoa per ejaculation with a minimum of 10% motility (Killian and Amann, 1972); based on this definition, the bulls who are 12 months old or younger (≤ 37 million per spermatozoa per ejaculation) were in the pre-puberty period - explaining the slow increase in sperm traits until around this age. After reaching puberty, the semen should take two weeks to mature and get better quality after that period (Barthle and Reiling, 1999). Even if the sperm motility shows a mean percentage motility of less than 60% for bulls of 15 months age or less, the pass the breeding soundness threshold of minimum 30% motility for semen quality (Thomas, Patterson and Perry, 2011), therefore we could consider the viability of the sperm of bulls older than 12 months years old to be of really good quality at puberty. The increase in scrotal circumference also impacts sperm production; literature suggests that the highest growth happens until 12 months, then only a gradual increase can be observed (Beitelscpacher, 1998), it would be an interesting further analysis to back up our analysis of sperm quality.

Studies have confirmed that sperm production increases significantly after 12 months, as several development pre-puberties happen in the next five years to increase sperm production (Murphy *et al.*, 2018b). A peak was observed at four years old (48 months old) (Everett and Bean, 1982). This could result from bulls reaching their full sexual maturity at that age, or they are managed to adapt to the weather and other environmental impacts. It could also be due to younger bulls' sperm being affected more by heat than older bulls (Balic *et al.*, 2019). The drops at 30 and 55 months old could be due to the resting period and the feed management. Feed management changes with age and period of life, the five period are defined as followed: Pre-weaning nutrition, post-weaning nutrition, conditioning prior to breeding season, breeding season, and post breeding season (Walker *et al.*, 2009). As bull mature, spermatogenesis stabilises and the quality of the traits improves significantly (Morrell, 2020). These two factors are essential in bull management. An important point that needs to be reiterated in this chapter is that proper recordings help and are essential in bull selection; further study of scrotum circumference and feed management could be analysed in the future to assess sperm production.

4.2. Genome Wide Association Studies

The chapter discuss the different results for the GWAS of the additive effect, the dominance effect and the ROH effect on the four different traits for semen quality, volume of sperm, concentration of sperm, percentage of motile sperm and number of sperms per ejaculations. The results were all insignificant when taking the stringent Bonferroni Threshold of -log(p) value of 5,91. The significant signals were analysed based on the p-value = 4,0 which were used in Ferenčaković studies which used the same data. The author applied a simple M method to calculate the threshold. The exact threshold with –log (p) value of 4.0 across the chromosome was used for sperm traits GWAS by Butler et al. (2022) (Figure 11) as no significant SNP was found using the strict FDR threshold.

4.2.1. GWAS: The Additive Effect on Semen Quality

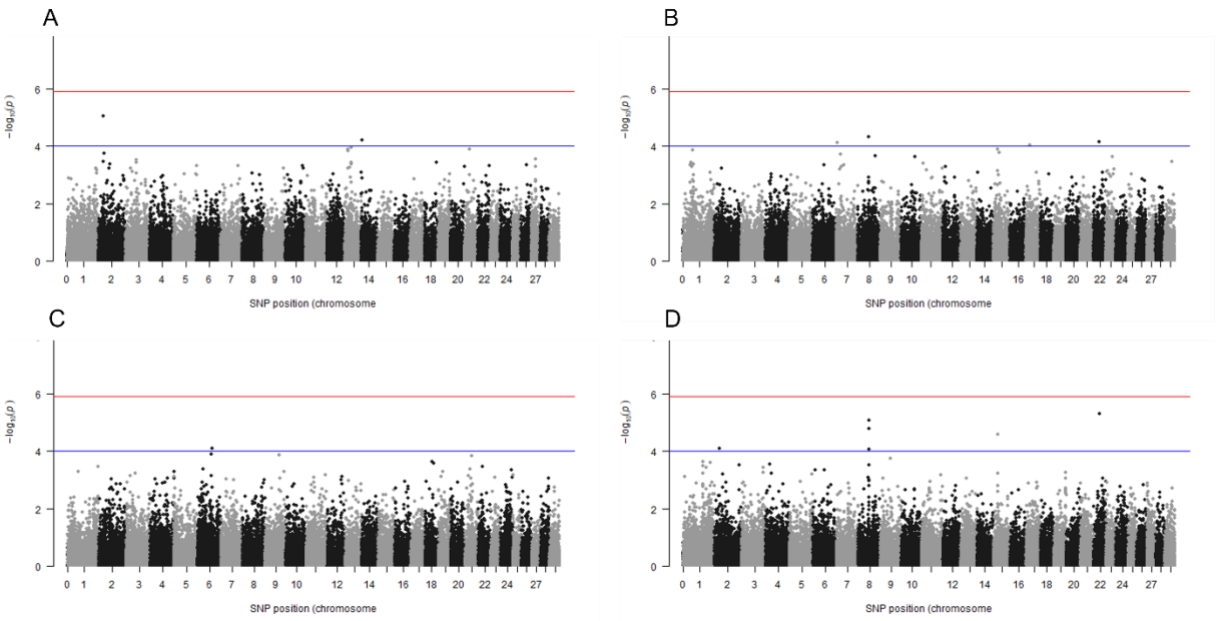

Figure 18: GWAS for the additive effect for sperm quality traits: Volume of Sperm(A), Concentration of Sperm(B), Percentage of mobile Spermatozoa (C) and Number of Sperms (D), and the significant threshold 4.0 (blue line)

The GWAS analysis (Figure 18) has had two SNPs signal for the volume of semen (A), four SNPs for the concentration of sperm (B), one SNP signal for the percentage of alive spermatozoa (C) and six SNPs for the number of sperms. For the volume of sperm, we found a gene of interest on BTA2 called GAD1(glutamate decarboxylase 1), the expression of GAD1 is associated with another enzyme. Together, they affect testosterone production and spermatogenesis in humans and rats (Kaewman, Nudmamud-Thanoi and Thanoi, 2018). Three SNPs out of four for the additive effect of concentration sperm were associated with male fertility. BTA7, BTA8 and BTA22 had significant SNP signals for the CALR3 (calreticulin 3), CEP78 (centrosomal protein 78) and SUCLG2 (succinate-CoA ligase GDP-forming subunit beta), respectively. The genes were connected to an indispensable role in the development of sperm fertilising ability (Ikawa *et al.*, 2011), reduction of male fertility (Ascari *et al.*, 2020) and low motility of buffalo sperm (He *et al.*, 2019). The analysis of the additive effect on the percentage of alive spermatozoa SNP signal was on BTA6 with THEGL (theg spermatid protein like) gene. The latter is associated with male fertility in mice and German warmblood horse testis (Blake *et al.*, 2017; Nolte, Thaller and Kuehn, 2019). BTA8 and BTA22 had significant SNP signals for the sperm count, with BTA 8

having a significant peak. Two genes on BTA8 were associated with lower fertility and sperm motility, CEP78 and PSAT1(phosphoserine aminotransferase 1) (Kosova *et al.*, 2012; Ascari *et al.*, 2020). Furthermore, PROK2 (prokineticin 2) and RYBP (RING1 and YY1 binding protein) were close to the BTA22 SNP signal; they are intertwined with motility, sperm production and infertility, respectively (Tian *et al.*, 2020; Wang *et al.*, 2021). In general, we observed that the genes that had significant signals were related to both semen traits quality and fertility of bull. These genes were not previously observed in cattle but are significant for AI stations as some of them (CEP78, SUCLG2, PSAT1 and RYBP) could cause a reduction in sperm production and fertility if it was expressed.

4.2.2. GWAS: The Dominance effect on Semen Quality

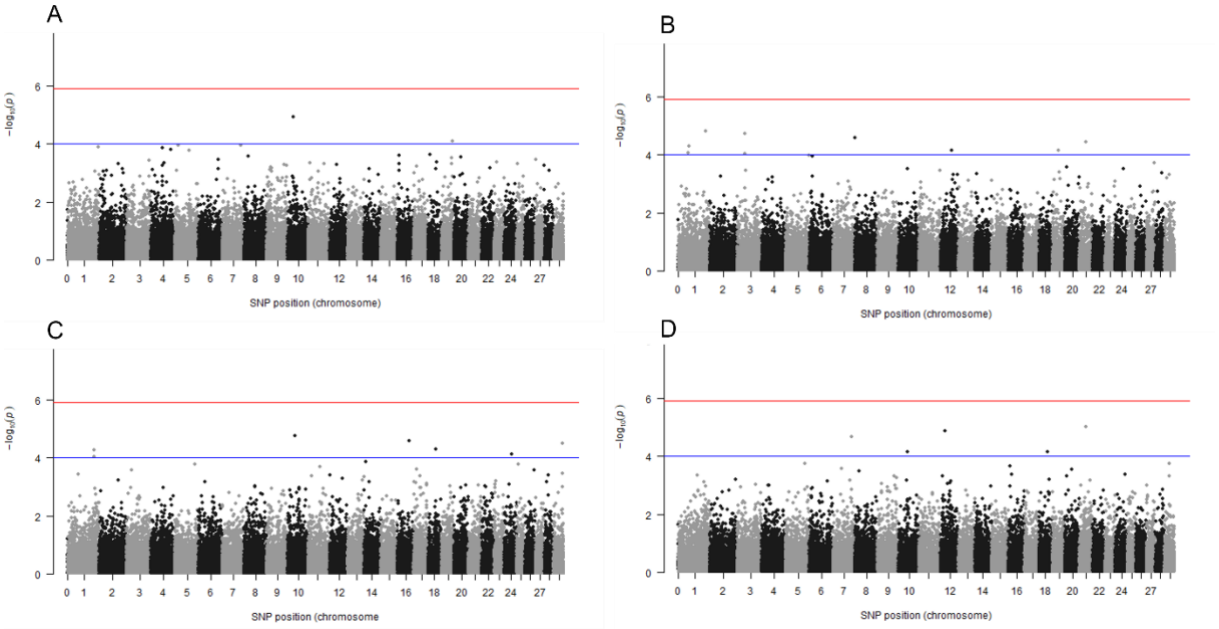

Figure 19: GWAS for dominance effect for Sperm Quality traits: Volume of Sperm(A), Concentration of Sperm(B), Percentage of motile Spermatozoa(C) and Number of Sperms (D) and the significant threshold 4.0 (blue line)

The dominant effect analysis (Figure 19) showed a total of 23 significant signals for the four different sperm quality traits. In addition, two SNP, nine SNP, seven SNP and five SNP showed traits for the volume of sperm, the concentration of sperm, the percentage of motile spermatozoa and the number of sperm, respectively. When analysing the volume of sperm (Figure 20A), we found that BTA10 had two genes of interest that were neighboring the significant SNP, namely AVEN (apoptosis and caspase activation inhibitor) and FMN1 (formin 1). The genes are related to a decrease in spermatogenesis (Laurentino *et al.*, 2011) and cells and muscle movement (Buzanskas *et al.*, 2017), respectively. The only significant signal that raised interest was on BTA1, which harbored the TOPBP1(DNA topoisomerase II binding protein 1) gene. TOPBP1 deletion causes testis reduction, as well as reduced sperm counts and compromisation of fertility is observed (Jeon *et al.*, 2019). This gene was associated with the concentration of sperm. Gene NEK1 (NIMA related kinase 1) around the significant signal on BTA8, which was also associated with the concentration of sperm, would be of interest. NEK1, in homozygous form in animals, was seen to affect testis size and morphology and causes complete infertility in males (Holloway *et al.*, 2011).

ZFHX3 (zinc finger homeobox 3) and SIN3A (SIN3 transcription regulator family member A) were related to the percentage of motile spermatozoa on BTA18 and BTA21, respectively. TRPC4 (transient receptor potential cation channel subfamily C member 4) and EHD4 (EH domain containing 4) on BTA12 and BTA10 were connected to the number of sperm. SINE3A and EHD4 are both essential for male fertility and spermatogenesis (George *et al.*, 2010; Pellegrino, Castrillon and David, 2012; Miyamoto, 2015), TRPC4 was linked to the motility of sperm (Castellano *et al.*, 2003), and ZFHX3 was found to be a gene related to growth and reproduction in goat (Snyman *et al.*, 2020). Overall, out of the 9 genes that were significant for the traits, 3 were affecting semen production. The 7 other SNPs were crucially related to spermatogenesis and semen traits, including motility. The dominant effect analysis showed that the genes were related to their traits compared to the additive effect. The AVEN and FMN1 gene could cause a decrease of sperm over the years if expressed as they influenced spermatogenesis. NEK1

as it could reduce sperm count and therefore concentration per ejaculation if expressed. The same was observed for the number of sperm where genes next to the SNP signal were related to sperm production, growth, and reproduction. FMN1 is important as its relationship to cells and muscle movements could impact on spermatozoid cells and elongation of penis and if expressed could have an impact on sperm production.

4.2.3. GWAS – The Effect of RoH on Semen Quality

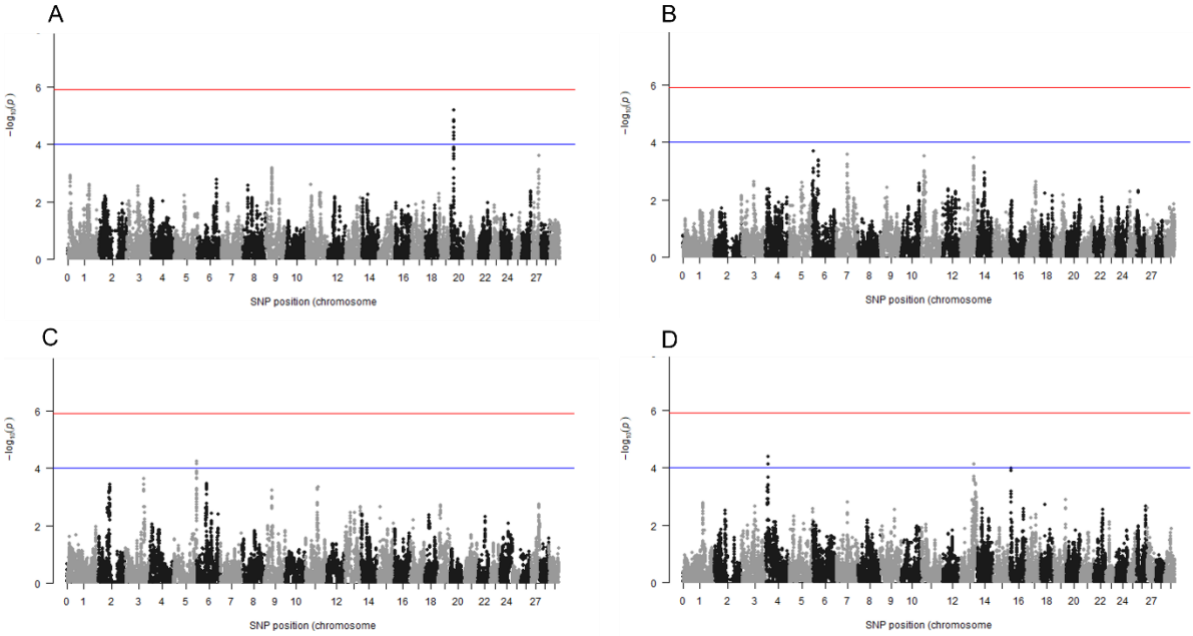

Figure 20 GWAS for the effect of ROH (≥2 Mb) for Sperm Quality traits: Volume of Sperm(A), Concentration of Sperm(B), Percentage of mobile Spermatozoa(C) and Number of Sperms (D), and the significant threshold 4.0 (blue line)

Investigating the effect of ROH (Figure 20) has shown peaks on BTA20 (13.7 Mb to 15.5 Mb) for the volume of sperm and two significant SNP on BTA6 for percentage of motile spermatozoa and two SNPs on BTA4 and one SNP for BTA13 for the number of sperms. The genes in the regions of 13.7 Mb and 15.5Mb on BTA20 are the following: NLN (neurolysin), SGTB (small glutamine rich tetratricopeptide repeat co-chaperone beta), TRAPPC13(trafficking protein particle complex subunit 13), PPWD1(peptidylprolyl isomerase domain and WD repeat containing 1), CENPK (centromere protein K), ADAMTS6(ADAM metallopeptidase with thrombospondin type 1 motif 6), CWC27(spliceosome associated cyclophilin), RGS7BP(regulator of G protein signaling 7 binding protein) and RNF180(ring finger protein 180). One gene in this region ADAMTS6 (14.0 Mb to 14.3Mb) is a protein gene of interest as the ADAMT has been correlated to infertility as dysregulation in the ADAMTS family protease has negatively impacted fertility (Russell, Brown and Dunning, 2015). Furthermore, ADAMTS1 and ADAMTS5 were confirmed to be related to sperm production (Aydos *et al.*, 2016). Another important SNP signal significant for the number of sperm per ejaculations is on BTA13 with gene SRC, which in humans plays an essential role in spermatogenesis; it is vital in increasing sperm count (Lawson, Goupil and Leclerc, 2008).

The effect of ROH and sperm traits quality was not informative for the concentration of sperm and percentage of motile spermatozoa, but the two genes that were reported are extremely interesting. The ADAMTS6 could be potentially related to infertility due to its family group, and therefore it would be helpful to do further analysis on this gene and its effect on the volume of sperm. SRC (SRC proto-oncogene, non-receptor tyrosine kinase) is another fascinating gene as it is directly connected to the trait it is associated with, i.e., the sperm count.

4.3. Success of Insemination

The effect of bulls, effect of cows age and the interaction effect of bulls age were significant for the NRR at 25 days, 56 days, and 90 days ($P < 0.001$). The non-return rate based on the age of bulls increases in all three groups: NNR at 25 days, 56 and 90 days (Figure 21). The surprising results come from the mean that increases with age which was not expected. The younger bull of 12 to 17 months old seemed to be less successful in all three NNR group, nevertheless a higher success for NRR is observed at 25 days (0.75) than 96 days (0.52). Which are interesting results, showing that older mature bulls of 8 years old were being more successful in inseminating cows than 1 year old bulls (Table 4). A big drop is observed around 36 months old to 60 months old due to a resting period for the animals, we can see that through the number of observations dropping from 23,495 to 2,544 observations from 24 months to 48 months.

Table 3 Descriptive table of mean non-return rate of cows at 25, 56 and 90 days with age of bulls at insemination day (n=number of observations)

Age (months)	*12*	*24*	*36*	*48*	*60*	*72*	*84*	*96*
NRR-25	0.76±0.43 n=451	0.80±0.39 n=23495	0.81±0.39 n=5780	0.80±0.40 n=2544	0.83±0.37 n=7819	0.82±0.39 n=26394	0.81±0.39 n=16267	0.81±0.39 n=8678
NRR-56	0.61±0.49 n=446	0.66±0.47 n=23362	0.66±0.47 n=5740	0.65±0.48 n=2529	0.68±0.46 n=7780	0.68±0.47 n=26257	0.67±0.47 n=16189	0.68±0.47 n=8628
NRR-90	0.52±0.50 n=444	0.58±0.49 n=23178	0.57±0.49 n=5696	0.57±0.49 n=2505	0.60±0.49 n=7707	0.59±0.49 n=26034	0.59±0.49 n=16060	0.59±0.49 n=8556

The descriptive analysis of sperm quality in chapter 4.2, we observe a similar pattern with low production from 12 months to 16 months. We also observed low sperm count of less than 37 million per spermatozoa per ejaculation at 12 months old. In this situation sperm is considered to not have reached their maturity (Killian and Amann, 1972) and therefore explaining the lowest non-return rate recorded for bulls of 12 months old in all NRR group. Given the opportunity of dosing on the insemination station, there is a possibility of increasing the success of insemination given that the doses contain semen with sperm of minimum 30% motility (Kastelic, 2013).

We can also observe that in the three groups the mature bulls are doing as good as the 24 months old bulls. The factor that is considered as impactful on age is the scrotum circumference, which grows until around three years old, and as the testis grow, literature explained that the sperm morphology and production gets better, providing better quality semen (Barthle and Reiling, 1999). Based on Norman et al. (2015), McWhorter et al. (2020) and Shorten et al. (2015), there was a positive effect on insemination rate when bulls ranging from 1.5 to 7 years old were used and this can be observed in Figure 22 if we do not consider the resting period (40-48 months old). The results provide grounds that older bulls are still beneficial to AI Stations and question if it is economically beneficial for the stations to use younger bulls of 12 months old.

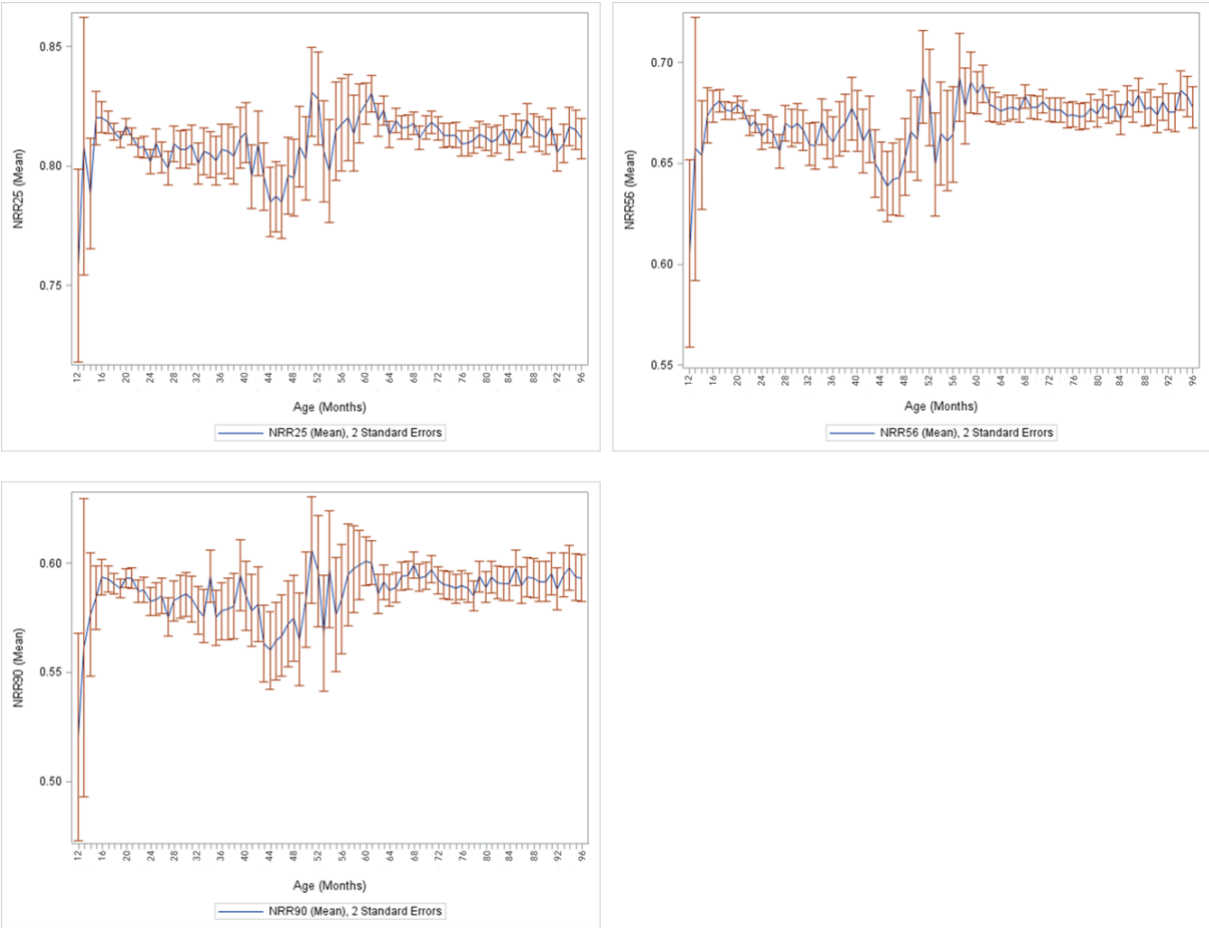

Figure 21 Graph of mean non-return rate of cows at 25,56 and 90 days with age of bulls in months on insemination day

Further investigation should be done on measuring the scrotum circumference with age and analysing sperm morphology, as it was investigated in Australia that sperm from semen of a bull group of more than 30 months old had 34% more chance of passing the sperm morphology test than bulls younger than 30 months old (Felton-Taylor et al., 2020).These results should be taken with a pinch of salt, as it is only a descriptive analysis and the following factors were not considered: the sperm quality post-freezing, parity, and age of the cows. These could be included in a linear model to have a better overview of success on insemination. Other factors that influenced success of insemination, like genetic ability and environmental impact involving feed management, diseases, and cattle and mating management (Hamilton, 2015) should also be included to complete the further analysis to assess the non-return rate and age of bulls at insemination day.

5. Conclusion

In the study, we analysed two critical factors of Fleckvieh production in Austria AI stations: semen trait quality and success of insemination of cows with semen obtained from AI stations. The research was done with data from the three AI stations over the last 12 years. The limitation in this study was the inability to merge the additive, dominant and ROH effects in one genome analysis, this would have possibly provided a more powerful result. The software used didn't provide this option even with the various coding trials to find a solution and merge the three effects. Furthermore, the lack of literature on the effect of dominance and run of homozygosity regarding sperm quality was slowing down our analysis. Another limitation was the limited time frame this topic is such a vast topic that is influenced by so many factors, and we couldn't include all the factors specially when analysing the success of insemination. The next step for further investigation and to relatively decrease this limitation, would be to assess frozen semen for the success of insemination, a separate analysis of heifers age and cows' age at first service and to include the genotypic information of the females used in the non-return rate.

One of the first findings was that sperm production was indeed affected by season and year. We also observe that the younger bulls have shown lower sperm volume and concentration and hence a lower sperm count. Bulls under 13 months of age were seen to have sperm counts of less than 50 million sperm per ejaculation which shows that the bulls reached puberty only as of 14-17 months old. The older the bulls, the higher the semen production, except for the percentage of motile spermatozoa, which seem to be stabilised after 13 months. We also demonstrated that the reduction in sperm volume with years could be due to the increase in temperature in the past 10 years in Austria. Another significant result was that bulls older than 30 months old were able to produce better semen quality than younger ones.

The GWAS analysis on additive effect, dominant effect, and effect of ROH has provided us with interesting genes that have an impact on spermatogenesis and bull fertility, even if the signals were not that significant across the genome using the Bonferonni Threshold. It was observed that out of all the significant 18 SNP signals, 18 genes of interest were found to have an impact on semen quality and 2 genes to growth and development. An interesting outcome was related to the success of insemination, where younger Fleckvieh bulls' rates of success of insemination were lower than the older ones pointing that the bulls age influences both semen quality and the success of insemination.

The study could be used by AI stations to avoid passing down the genes that would decrease semen quality. In addition to that, they could further investigate if the inclusion of pre-puberty bulls in the insemination process is essential with lower quantitative traits. But it is important to consider the opportunity of dosing paillettes for insemination according to the number of sperm that can be done in the different stations. Furthermore, including young bull in breeding programme, reduce generation interval and consequently increasing the genetic gain. Therefore, including young bulls with low quantitative traits is important for AI station. In general, with the production of Fleckvieh being stable in Austria and the breeding values getting better with years, we can assume that the AI stations in Austria are already accomplishing excellent and efficient work with their herds. This study could be useful as it englobes the phenotypic, genomic and the environmental impact on semen production. This would be helpful in genomic selection as it could improve heritability estimation and prediction accuracy. The integration of these results to other breeding tools could accelerate breeding process.

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