Norwegian University of Life Sciences
Faculty of Biosciences

Philosophiae Doctor (PhD)
Thesis 2018:37

Identification of sperm parameters and gene variants influencing boar fertility

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Nina Hårdnes Tremoen

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Abstract

Boar fertility has a major impact on overall pig reproductive efficiency, and good semen quality is essential for successful fertilization and proper embryo development. Thus finding semen characteristics that can predict fertility traits like pregnancy rate and total number of piglets born (TNB) is of great importance. However, the results of pregnancy rate and TNB are not available until the boars have been used in production for a period, and determination of phenotypes with effects on fertility at an earlier stage in the boars' lives would be beneficial for estimating breeding values. To find methods to evaluate the boar's sperm quality in relation to TNB and thus the boar's fertility is of great importance also for the semen produced for sale. Identifying gene variants affecting these traits is of equal importance.

We found significant breed differences in the motility characters comparing ejaculates from Norwegian Landrace (NL) and Norwegian Duroc (ND). The percentage of hyperactivated sperm cells increased significantly upon storage in NL. In ND a larger portion of sperm cells with a hyperactive swimming pattern were detected at day 0, and the size of this population decreased upon storage. A significant decrease in the ATP level ($p<0.0001$) was also found in both breeds during storage. The motility characters linearity and wobble showed an effect on TNB in NL, at the day of collection and after storage, respectively. For ND, the percentage of motile cells, curvilinear velocity and amplitude of lateral lateral head displacement at the day of collection and linearity after storage showed an effect on TNB.

A significant negative effect on TNB was found for boars with contrasting DNA fragmentation index (DFI). This might explain some of the variation in TNB caused by the sperm quality of the individual boars, although the effect was moderate.

Transcriptome profiling by RNA sequencing (RNAseq) of testis tissue from NL and ND boars showed that 308 and 374 genes displayed significant different expression between high and low DFI boars, respectively. Of these genes, 71 were differentially expressed in both breeds. Gene ontology analysis revealed that significant terms in common for the two breeds included extracellular matrix, extracellular region and calcium ion binding capacity.

Two SNPs in *BMPR1* and one SNP in *COX-2* in NL were found significantly associated with an estimated breeding value for TNB. In ND, two SNPs in PLC_z, one SNP in *VWF*

and one SNP in *ZP3* were found significantly associated to TNB. These SNPs explained between 0.27% and 1.18% of the genetic variance, which is quite low and not interesting for direct selection in breeding programs. However, the associated variants can be of interest in SNP-panels used for genomic selection.

Based on the associations found between motility parameters and TNB, and between DNA fragmentation and TNB, this thesis shows that male fertility is an important part of the total fertility in pigs. In addition, this thesis have contributed on the knowledge on the genetics of male fertility and DNA fragmentation in sperm cells in pigs.

Sammendrag

Det er mange faktorer som påvirker fertilitet, og for hannfertilitet er evnen til å produsere og ejakulere normale fertile sædceller den viktigste faktoren. Fertilitet hos råne har en stor innvirkning på den totale fruktbarheten hos svin, og god sædkvalitet er essensielt for vellykket fertilisering og normal embryoutvikling. Tradisjonelt har utvelgelsen av råner fokusert på økonomisk gunstige egenskaper som for eksempel drektighetsprosent hos purka og totalt antall fødte grisunger (TNB). Resultatene for fertilitetsrate og TNB vil imidlertid ikke være tilgjengelig før rånene har stått i seminproduksjon en periode. Derfor ville det vært svært gunstig i forhold til avlsverdiberegninger å kunne fastslå fenotyper som påvirker fertilitet tidligere i rånens reproduktive liv. For seminproduktet til salg er det også viktig å finne metoder som kan evaluere rånens sædkvalitet i forhold til TNB og dermed rånens fertilitet.

Vi fant signifikante raseforskjeller i motilitetsparametere når ejakulater fra de to griserasene norsk landsvin (NL) og norsk duroc (ND) ble sammenlignet. Prosentandelen av hyperaktive celler økte etter lagring hos NL. Hos ND, derimot, hadde en høyere prosentandel av spermiene et hyperaktivt svømmemønster på uttaksdagen, og størrelsen på denne populasjonen ble mindre etter lagring. Det var en signifikant reduksjon i den totale prosentandelen motile spermier hos både NL ($p=0.01$) og ND ($p<0.0001$) etter lagring. Det var også en signifikant reduksjon i mengden ATP i ejakulatene hos begge raser ($p<0.0001$) etter lagring. For NL var det motilitetsparameterne lineariten på uttaksdagen og "wobble" etter lagring, som hadde effekt på TNB. For ND hadde prosentandelen motile spermier, "curvilinear velocity" og den laterale hodebevegelsen en effekt på TNB ved uttaksdagen, og lineariteten en effekt på TNB etter lagring.

En signifikant negativ effekt på TNB ble funnet for både NL og ND råner med en median DNA fragmenteringsindeks (DFI) på 1.37% (NL) og 1.61% (ND). Til tross for at effekten ikke var så stor, kan dette forklare noe av variasjonen i TNB forårsaket av sædkvaliteten hos de individuelle rånene.

Transkriptom-profilering ved RNA-sekvensering (RNAseq) i testikkelvev fra NL og ND råner resulterte i henholdsvis 308 og 374 gener som var differensielt uttrykt mellom lav og høy DFI. Av disse genene var 71 differensielt utrykt i begge raser. Ved hjelp av genontologianalyser ble det funnet at signifikante begreper for de to rasene inkluderte ekstracellulær matriks, ekstracellulær region og kalsiumionbinding.

To enkeltnukleotidpolymorfier (SNPer) i *BMPR1* og en SNP i *COX-2* i NL var signifikant assosiert med en estimert avlsverdi for TNB. I ND ble det funnet to SNPer i *PLCz*, en SNP i *VWF* og en SNP i *ZP3* som var signifikant assosiert med TNB. Disse SNPene forklarte mellom 0.27% og 1.18% av den genetiske variasjonen, noe som er lavt og ikke interessant for direkte seleksjon i avlsprogrammet. Likevel kan de assosierte variantene være av interesse for SNP-paneler som brukes i genomisk seleksjon.

Assosiasjonene mellom motilitetsparameterne og TNB, og mellom DNA fragmentering og TNB som ble funnet i denne avhandlingen, viser at fertilitet hos hanndyr har en viktig rolle i den totale fertiliteten hos gris. I tillegg har denne avhandlingen bidratt til økt kunnskap innen genetikken bak hanndyr fertilitet og DNA fragmentering i sædceller hos råne.

List of abbreviations

ACTB	β-actin	GWAS	Genome wide association study
AI	Artificial insemination	HCO_3^-	Bicarbonate
ALH	The amplitude of the lateral head displacement	HDS	High DNA stainable
AO	Acridine Orange	LIN	Linearity
AR	Androgen receptor	NL	Norwegian Landrace
BCF	Beat cross frequency	ND	Norwegian Duroc
BMP1	Bone morphogenetic protein 1	PLCz	Phospholipase C zeta
BMP15	Bone morphogenetic protein 15	QTL	Quantitative trait loci¨
BMPR1B	Bone morphogenetic protein receptor 1B	RNAseq	RNA sequencing
Ca^{2+}	Calcium	SCSA	Sperm chromatin structure assay
CASA	Computer assisted semen analysis	SNP	Single nucleotide polymorphism
CD9	Cluster-of-differentiation antigen 9	SRD5A	Steroid 5α-reductase
COX-2	Cyclooxygenase isoenzyme type 2	STR	Straightness
DFI	DNA fragmentation index	TNB	Total number of piglets born
EBV	Estimated breeding values	VAP	Average path velocity
ESR1	Estrogen receptor 1	VCL	Curvilinear velocity
ESR2	Estrogen receptor 2	VSL	Straight line velocity
FR	Farrowing rate	WOB	Wobble
GDF9	Growth differentiation factor 9	ZP	Zona pellucida
GEBV	Genomic estimated breeding values	ZP3	ZP glycoprotein-3
GLM	General linear model		

List of papers

I Tremoen, N. H., Gaustad, A. H., Andersen-Ranberg, I., van Son, M., Zeremichael, T. T., Frydenlund, K., Grindflek, E., Våge, D. I., Myromslien, F. D. (2018). The relationship between sperm motility characteristics and ATP levels, and its effects on field fertility in two different pig breeds. *Manuscript submitted to Animal Reproduction Science*

II Myromslien, F. D., Tremoen, N. H., Andersen-Ranberg, I., Fransplass, R., Stenseth, E. B., Zeremichael, T. T., van Son, M., Grindflek, E., Gaustad, A. H. (2018). Sperm DNA integrity in Landrace and Duroc boar semen and its relationship to litter size. *Manuscript submitted to Reproduction in Domestic Animals*

III van Son, M., Tremoen, N. H., Gaustad, A. H., Myromslien, F. D., Våge, D. I., Stenseth, E. B., Zeremichael, T. T., Grindflek, E. (2017). RNA sequencing reveals candidate genes and polymorphisms related to sperm DNA integrity in testis tissue from boars. BMC Veterinary Research 13:362

IV Tremoen, N. H., van Son, M., Andersen-Ranberg, I., Grindflek, E., Myromslien, F. D., Gaustad, A. H., Våge, D. I. (2018). Association between SNPs within candidate genes and fertility in Landrace and Duroc pigs. *Manuscript submitted to Animal Genetics*

1. Introduction

Fertility is defined as the capacity of producing offspring. There are many components affecting fertility. For males, the main factors affecting fertility are the abilities to produce and ejaculate normal fertile sperm. In females there are other factors affecting fertility, e.g. a reproduction system that maintain sperm transport and keep the right conditions for final maturation of sperm cells and fertilisation of the egg, and also an uterine environment facilitating embryo and fetal development, and giving birth to the offspring (Foote 2003). Modern pig breeding schemes aimed at improving fertility are usually focused towards the female fertility. The reason is probably that both the fertilization and the embryogenesis occur in the female reproduction tract. However, successful fertilization and proper embryo development is also heavily dependent on male fertility. Male fertility may be affected by the external parts of the reproduction system. For example, the testicular size has been found to be correlated with sperm production. In addition, the reproduction efficiency in cattle, sheep and pigs have been reported to be significantly related to testicular size. The sperm quality traits are frequently used to quality control the semen doses, and the sperm cells are typically evaluated based on their motility, morphology and defects in sperm organelles and DNA (Foote 2003). Although sperm quality traits are not commonly used for selection in pig breeding, male fertility has a major impact on overall reproductive efficiency and proper evaluation of semen quality is essential.

Due to large geographical distances in Norway, more than 70% of artificial insemination (AI) is performed with liquid preserved semen stored for more than 24 hours. Boar semen also needs to be kept unfrozen since cryopreserved porcine sperm are sensitive to cellular stress (Waterhouse et al. 2006). Sperm storage capacity in liquid state is therefore important for semen quality. In addition, the boars' ability to produce spermatozoa and ejaculates of high quality are critical since only a limited number of doses can be obtained from one ejaculate (Bonet et al. 2013). In order to obtain sufficient sperm quality in boars used for AI, threshold values for sperm concentration, motility and morphology have been established. This has resulted in a selection of boars with relatively high fertility. However, individual differences are still observed and boars with lower fertility may be disguised behind the high sperm numbers in the doses. To be able to utilize the boars with the highest genetic potential to the fullest, it would be ideal to reduce the sperm numbers in the doses. Thus, knowing the fertility potential of the semen before it is inseminated would be economically and practically beneficial (Tardif et al. 1999).

The aims of this thesis was to investigate new methods for assessments of semen quality in boars that are associated to field fertility. We also investigated a limited number of potential candidate genes that could be related to male fertility in pigs and the underlying genetics for one of the semen quality parameters.

1.1 Reproduction physiology in pigs

1.1.1 Boar reproductive system

The boar reproductive system consists of two testicles, two epididymis, two deferent ducts, the urethra and its accessory sex glands (two seminal vesicles, the prostate and two bulbourethral glands or Cowper's glands), and the penis (Senger 2012) (Figure 1).

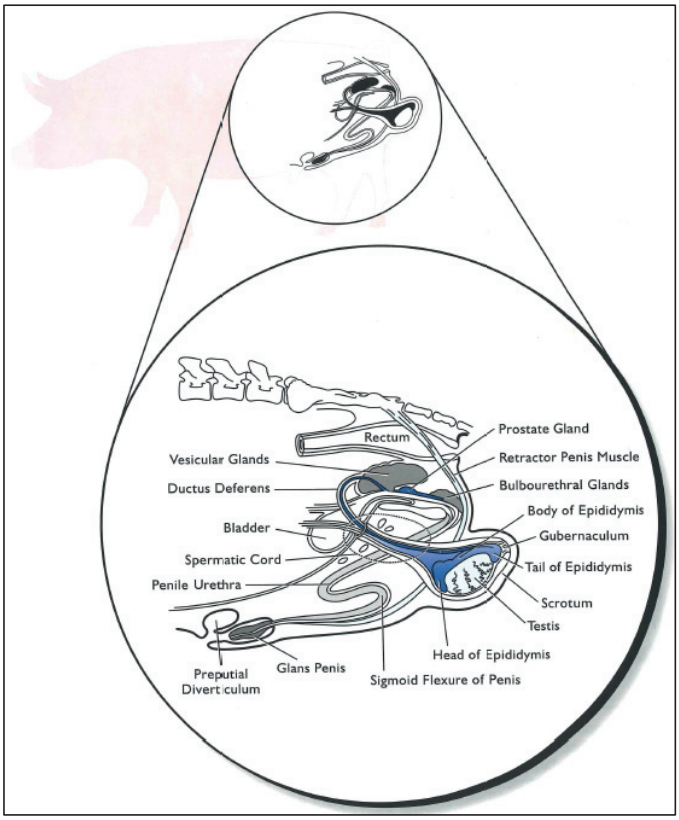


Figure 1. *Illustration of the male reproductive tract in pigs. The boar reproductive system consists of two testes, two epididymides, two deferent ducts, the urethra and its accessory sex glands (two seminal vesicles, the prostate and two bulbourethral glands or Cowper's glands), and the penis (Senger 2012).*

The main function of the boar reproductive system is the production and the ejaculation of semen (Knobil and Neill 2006). The testes are the male gonads and their main function is sperm production, but they are also endocrine glands that secrete hormones (Garcia-Gil et al. 2002). The testes consists of the testicular capsule, the parenchyma, the mediastinum and the rete tubules. The testicular capsule is covering the testicles, the mediastinum is the central connective tissue core of the testis and the rete tubules are tiny channels that transport spermatozoa out of the testes. The parenchyma consists of seminiferous tubules, interstitial Leydig cells that produce testosterone, capillaries, lymphatic vessels and connective tissue. The seminiferous tubules are composed by a layer of seminiferous epithelium which is resting on a basement membrane. Sertoli cells are anchored to the basement membrane and is thought to have a supportive function for the germ cells inside the seminiferous tubules. In addition, the Sertoli cells have the capability of producing various substances, such as androgen binding protein, sulphated glycoproteins, transferrin and inhibin. The Sertoli cells form junctional complexed that prevents large molecules reaching the germ cells. These junctions and the peritubular cells surrounding the seminiferous tubules form the blood-testis barrier which prevents autoimmune reactions destroying the developing germ cells (Senger 2012). To summarize, the male reproduction system has three distinct functions; the endocrine function performed by the testicular Leydig cells and Sertoli cells which regulates the sperm production, sperm production in the seminiferous tubules of the testis and sperm maturation in the epididymis (Hafez and Hafez 2000).

1.1.2 Spermatogenesis and the maturation of the sperm cells

The spermatogenesis is the process where spermatozoa are produced, and is essential for the transfer of genetic material from one generation to the next (Manku and Culty 2015). The spermatogenesis is a complex process consisting of a mitotic phase and a meiotic phase, giving rise to haploid spermatids. The spermatids undergo a differentiation process to become a mature sperm cell (Garcia-Gil et al. 2002).

The spermatogenesis occurs in the testis (Figure 2). The seminiferous tubules of the testis is lined by seminiferous epithelium, and consist mainly of two cell-types; Sertoli cells and germ cells. The Sertoli cells have, as mentioned, nourishing function for the germ cells which undergo cellular divisions while they are progressing towards the lumen of the tubule

(Figure 2). The stem-cell, the spermatogonia, may either divide to form new spermatogonia or differentiate to form spermatocytes (Hafez and Hafez 2006). For males that have reached puberty, there is a continuous production of spermatogonia throughout a lifetime (De Jonge and Barratt 2006).

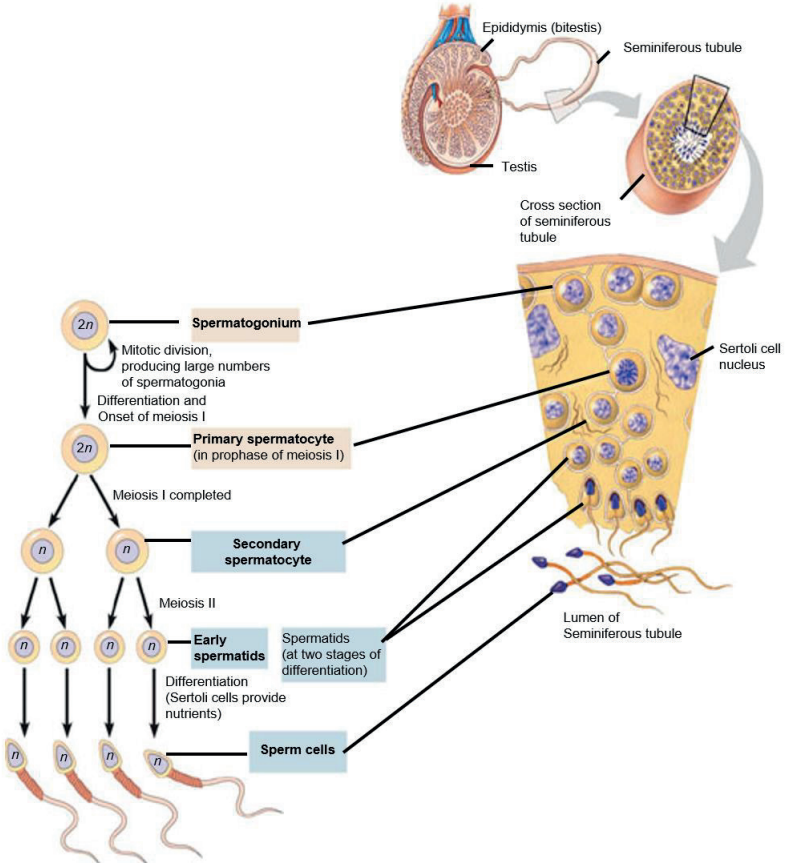


Figure 2. *Illustration of the production of spermatozoa by spermatogenesis in the seminiferous tubules of the testis. In this process, diploid spermatogonia undergo cell proliferation, meiosis and differentiation and form the haploid spermatozoa. The spermatozoa are released into the lumen of the seminiferous tubules, and are transferred to the epididymis for further maturation and storage (Campbell 2008).*

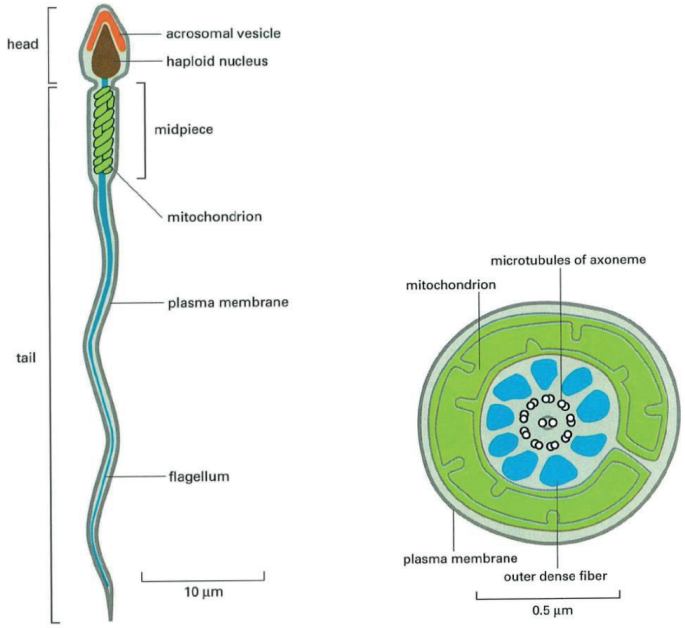
The spermatocytes undergo meiosis, reducing the DNA content forming a haploid cell. This is known as the spermatocytogenesis, and the end product is the haploid spermatid. The spermatid undergoes structural and developmental changes to form a spermatozoon, and this process is known as the spermiogenesis. The spermiogenesis includes condensation of

chromatin, formation of the sperm tail (the flagellum) and development of the acrosome. The chromatin undergoes progressive condensation where transitional proteins are replaced by protamines. The spermiation is the final process of the spermatogenesis where the spermatozoa are released into the lumen of the seminiferous tubules. In boars, this cycle takes about nine days. The testicular spermatozoa are transported from the testis to the epididymis for the final maturation process. The final maturation includes development of potential motility and an additional compaction of the chromatin in the nucleus. The spermatozoa in boars are transported through the epididymis in 12 days, and the major site of storage is the caudal portion of the epididymis (Hafez and Hafez 2006).

1.1.3 The spermatozoa

The mature boar spermatozoa are approximately 43-45 μm in length and are composed of two main components; the head and the tail (Figure 3a). These two are joined by the connecting piece (or neck). The head is oval-shaped and bilaterally flattened (Bonet et al. 2013), and contains the nucleus and acrosome. Moderate amounts of cytoskeletal components and a small amount of cytoplasm are surrounding the two components. The cytoplasm consists of a thin layer, and is believed to be important in the early events of capacitation and acrosome reaction. It may also be important in the sperm-egg membrane fusion (Knobil and Neill 2015).

The nucleus contains highly condensed chromatin. The chromatin consists of DNA packed around histones and protamines. The protamines are able to pack the chromatin more tightly, compared to the histones. Studies have reported that there is a connection between the amount of protamines in the chromatin – the more protamins present – the more tightly packed DNA – the lesser chance for the DNA to be damaged (Rathke et al. 2014). Even if most of the histones are replaced with protamines, some of the histones remain non-randomly distributed and associated with specific genes (Knobil and Neill 2015).



a. *b.*

Figure 3. *Schematic overview of a sperm cell. The sperm cell consists of two main parts; the head and the tail (a). The head contains the haploid nucleus, the acrosome and a small amount of cytoplasm. The tail is a strong flagellum and contains mitochondria strategically placed where they can power the flagellum. The core of the flagellum is composed of an axosome, which consists of two central singlet microtubules, surrounded by nine microtubule doublets, and further surrounded by nine outer dense fibres (b) (Alberts 2002).*

There are few or none repair mechanisms for DNA damage in the sperm cells. Thus, DNA damages are not repaired as they would be in somatic cells. The sperm cells with DNA damage may be able of fertilizing the oocyte. However, embryos with paternal DNA damage will die in utero, so-called embryonic loss (Fatehi et al. 2006, Wdowiak et al. 2015). The non-randomly distributed histones are associated with the activity of specific genes and the transmission of certain genes to the newly fertilised oocyte (Knobil and Neill 2015).

The acrosome is a unique sperm organelle which originates from the Golgi complex. It is a membrane-enclosed vesicle and contains enzymes necessary for the sperm to be able to penetrate the zona pellucida. The acrosome is a cap over the nucleus and is responsible for

the shape characteristics of the sperm head in different species. The outer acrosomal membrane lies close to the inner surface of the plasma membrane of the anterior sperm head. These two membranes fuse during the acrosome reaction and vesiculate. The acrosome contains multiple enzymes that serve a critical role in the fertilization process and is discharged by exocytosis in response to Ca^{2+} signals. After the release and activation of enzymes, the spermatozoon is able to penetrate the ZP (Knobil and Neill 2015).

The cytoskeleton has a structural role in defining the shape of the sperm head, and has a functional role in helping the sperm penetration into the egg and regulation of other functional molecules required for the acrosome reaction (Knobil and Neill 2015). Actin is one of the cytoskeletal domains that is proposed to be involved in the organization of the acrosome (Abou-Haila and Tulsiani 2000).

The sperm tail is connected to the head at the basal plate between the connecting piece and the nucleus, and provides motility for the spermatozoa. The tail has a cylindrical and filamentous shape, and may be divided into four segments; the connecting piece, the midpiece (or the mitochondrial region), the principal piece and the terminal piece. The main structures within the flagellum are axoneme, the mitochondrial sheath, the outer dense fibers and the fibrous sheath (Figure 3b) (Knobil and Neill 2015). The axoneme consists of nine microtubule doublets arranged around a central pair of microtubules. The nine outer doublets are paralleled by nine outer dense fibers that provide a flexible and firm support during flagellar movement (De Jonge and Barratt 2006). The midpiece of the flagellum contains 75-100 sperm mitochondria forming a helix shaped mitochondrial sheath. The mitochondria generate energy for the flagellar movement of the sperm cell. The principal piece of the flagellum is covered and protected by the fibrous sheath which both protects the axoneme and contains protein kinases necessary for the final sperm maturation steps prior to fertilization (De Jonge and Barratt 2006).

The flagellar movement of the sperm cell is an ATP dependent process. Calcium ions (Ca^{2+}) have been reported to be an important factor for the motility of the sperm cell (Li et al. 2016). There are two main metabolic pathways providing the energy for supporting the functions, such as the motility, of a spermatozoon: glycolysis in the head and the principal part, and oxidative phosphorylation in the mitochondria. There has been discussion about which of these methods of ATP production is primarily utilised by the spermatozoa. Human spermatozoa are suggested to rely mainly on glycolysis as their source of ATP (du Plessis

et al. 2015, Hereng et al. 2011). Most likely, the spermatozoa are dependent on both metabolic pathways, either in a combination or restricted to one at a time.

1.1.4 Sow reproductive system

The female reproductive tract includes the ovaries, oviducts, uterus, vagina and the external genitalia (Figure 4). The vagina extends from the vulva to the cervix, and the primary functions are to be a copulatory organ, to serve as a birth canal during parturition and as the site for expulsion of urine. The cervix is located between the vagina and the uterus, forming a cervical canal. It provides lubrication, a flushing system and a barrier during pregnancy.

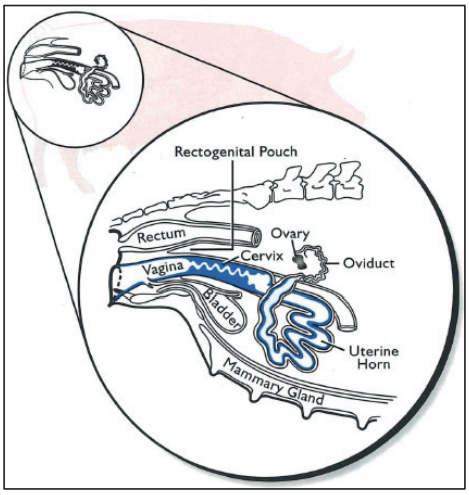


Figure 4. *Illustration of the female reproductive tract in pig. The sow reproductive tract includes the ovaries, oviducts, uterus, vagina and the external genitalia (Senger 2012).*

The uterus of the sow consists of two highly developed uterine horns (cornua) and a small uterine body. The primary functions of the uterus are sperm transport, luteolysis (degradation of corpus luteum) and control of cyclicity, creating optimal environment for pre-attachment embryo, maternal contribution to the placenta and expulsion of the fetus and fetal placenta (Senger 2012).

The oviduct consists of the infundibulum, the ampulla and the isthmus. The mucosa of the oviduct produce secretes important for providing the optimum environment for the unfertilized oocyte. The secretes also influence the function of the spermatozoa in the oviduct until the oocyte arrives. The main function of the oviduct is transportation of the sperm cells and the oocyte to the ampullary-isthmic-junction, which is the site of the fertilization (Senger 2012).

The ovaries are located in the upper oviduct segment, and the primary functions of the ovaries are to produce female gametes and the hormones estrogen and progesterone. The different stages of follicular development and maturity is represented by the various types of ovarian follicles. There are four types of follicles in the ovaries: the primordial follicles, the primary follicles, the secondary follicles and the antral follicle. Females are born with a lifetimes' supply of primordial and primary follicles, and these are not capable of division into other primary follicles. Instead, they develop into more advanced secondary follicles (Senger 2012). The maturation of the oocyte occurs continuously throughout the females' reproductive lifetimes (Hafez and Hafez 2006). The mammalian oocyte is the most advanced follicle, is highly differentiated, and will not survive more than 24-48 hours without fertilization. The relationship between the oocyte and the somatic follicular cells is essential for the maturation of the oocyte, and the follicular cells surrounding the oocyte is forming the corona radiata (Figure 5) (Hunter 2000).

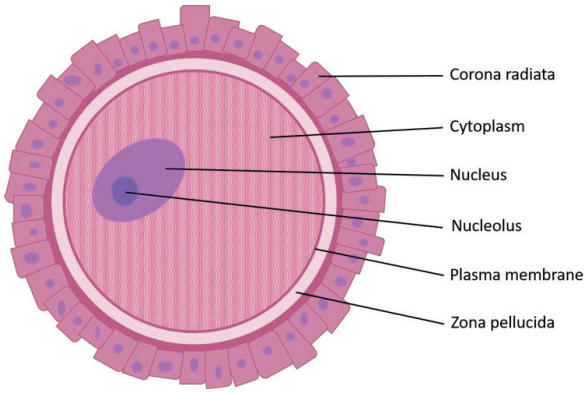


Figure 5. *Illustration of a mammalian oocyte. The haploid nucleus containing a nucleolus is located in a large volume of cytoplasm. The plasma membrane is coated by the zona pellucida. The outside of the oocyte is covered by follicular cells forming the corona radiata.*

The oocyte (Figure 5) is characterized as being surrounded by the zona pellucida (ZP) (Senger 2012). The ZP is a specialized extracellular matrix constructed from glycoproteins, ZP-glycoproteins, synthesized, processed and secreted from the oocyte. This structure has been recognized as a protective barrier to the oocyte (Knobil and Neill 2015). The ZP provides receptors for attachment and binding of sperm cells, and serve as site of secondary block to polyspermy after penetration of the first spermatozoa (Sinowatz et al. 2001).

1.2 Sperm transport in the female reproductive organ and fertilization

The spermatozoa are exposed to various environments along the female genital tract prior to encountering the oocyte. After entering the female genital tract, the spermatozoa spends most time within the oviduct, which provides a suitable environment for sperm transport, storage and the final maturation step (Rodriguez-Martinez 2007). The spermatozoa have limited ability of repairing damages, and the correct environment for protecting the cells are essential for survival (Rodriguez-Martinez et al. 2005).

The semen deposition is dependent on the use of AI or natural mating. In natural pig mating, the semen is deposited in the narrow cervical canal (Rodriguez-Martinez 2007). However, a majority of the piglets born in Norway originates from AI. Using AI, the semen is deposited intra-cervically (Figure 4). In boars, the semen volume varies from 200-300 mL and contains between 10 x 10^9 and 100 x 10^9 sperm cells in natural mating (Bonet et al. 2013). The breeding company Norsvin typically produces semen doses containing 89 mL of semen diluted in a long-term extender to a final concentration of 25 x 10^6 sperm cells. The relative large volume flushes the semen directly into the lumen of the uterine body, and further into the uterine horns to prevent that the sperm cells are retained in the cervical folds (Langendijk et al. 2005). The sperm cells need to be transported to the end of the uterine horn, and this process is suggested to be very fast – within minutes after the insemination, fertile sperm cells may be found in the oviducts (Baker and Degen 1972). Uterine contractions seems to contribute to the transfer of semen into the oviducts (Langendijk et al. 2005). Even though billions of spermatozoa are inseminated, the portion of spermatozoa reaching the oviduct is smaller, only a few thousand. There are two main phenomena that may explain this; the backflow and a possible selection of spermatozoa before entering the oviduct (Matthijs et al. 2003, Taylor et al. 2008). The transport of the

male gametes through uterus appears to be a passive process driven by a gravitational force and uterine contractions rather than by the motility of the spermatozoa (Langendijk et al. 2002, 2005). The seminal plasma has an effect on the uterine activity (Langendijk et al. 2005). In addition, relationships between seminal plasma proteins and semen traits have been reported, such as association between lactadherin and sperm motility (Gonzalez-Cadavid et al. 2014).

1.2.1 The sperm reservoir and capacitation process

By providing an appropriate microenvironment for gamete support and transport, the oviduct has a significant role in the fertilization process. The spermatozoa that reaches the oviduct, form a functional sperm reservoir. As the ovulation approaches, the spermatozoa in the sperm reservoir will be continuously distributed and undergo capacitation and attain the hyperactivated motility (Rodriguez-Martinez 2007).

Capacitation is the final maturation step of the spermatozoa (Figure 6), which takes place in the female genital tract, and is necessary for the ability of interaction with the oocyte.

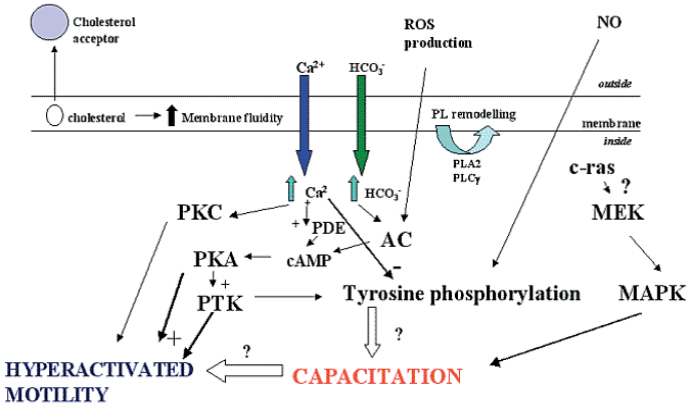


Figure 6. *Illustration of the capacitation process. Loss of cholesterol from the membrane changes the membrane fluidity, and allows influx of Ca^{2+} and HCO_3^-. This starts a cascade of intracellular signalling events, including activation of adenylyl cyclase activity and the production of cyclic adenosine monophosphate (cAMP), stimulation of protein kinase A (PKA), and tyrosine phosphorylation of sperm protein molecules (Baldi et al. 2000).*

The capacitation is induced by changes in membrane permeability in response to Ca^{2+} and bicarbonate (HCO_3^-) in the seminal fluids. Loss of cholesterol from the membrane causes an increased membrane fluidity. These events leads to increased tyrosine phosphorylation and an increased activity of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway. The consequence is capacitation of the spermatozoa which in turn lead to a hyperactivated motility, the ability of acrosome reaction and interaction with the oocyte (Baldi et al. 2000, Knobil and Neill 2015). The possibility of decapacitation have been suggested (Senger 2012).

Hyperactivated spermatozoa are characterized by a vigorous and non-linear movement, caused by an increased amplitude of flagellar beats (Schmidt and Kamp 2004). This swimming pattern is reported to be important for fertilization of the oocyte, probably due to an improved penetration of zona pellucida by this movement (Stauss et al. 1995, Yanagimachi 1969). Hyperactivated motility is a highly ATP-consuming process, which, if initiated too early, pose a risk for depleting the energy store of the sperm cells before they reach the oocyte for fertilization (Mortimer et al. 1997, Suarez and Ho 2003). Contrary, if the hyperactivated motility is induced too late, the spermatozoa will not be able to penetrate the oocyte. Thus, the timing for hyperactivation is of great importance. In AI, this means that the storage ability of each ejaculate is of great importance, as the hyperactivation is not preferred to happen already before the insemination.

1.2.2 Fertilization

The fertilization process (Figure 7) occurs in the ampullary-isthmic-junction of the oviduct. The spermatozoa are capacitated in the oviduct and the motility pattern changes from progressive motile to a hyperactivated swimming pattern. Specific zona-binding proteins on the plasma membrane of the spermatozoa binds to ZP molecules on the surface of the ovulated oocyte. The binding of one of the ZP molecules, ZP3, is believed to initiate the acrosomal reaction. The acrosomal reaction enables the spermatozoon to penetrate the ZP by fusion of the outer acrosomal membrane to the outer plasma membrane of the spermatocyte and dispersion of the acrosomal content following a vesiculation. The acrosome contains enzymes that allow the spermatozoa to penetrate the ZP. The flagellum generates a mechanical force which enables the maintenance of the contact between the sperm head and the ZP. After membrane fusion between the oocyte and the spermatozoa,

a zona block is formed to prevent polyspermy. The final step of the fertilization process is the fusion of the male and female pronuclei, referred as syngamy, and the zygote enters the first stages of the embryogenesis (Senger 2012).

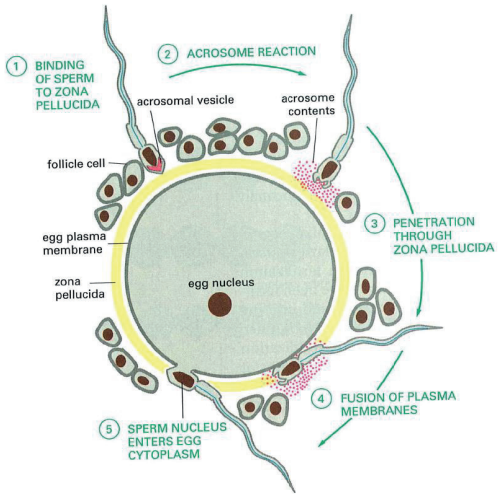


Figure 7. *Illustration of the fertilization process. A capacitated sperm cell binds to the zona pellucida of the oocyte (1) and the acrosome reaction is initiated (2). The acrosome-reacted sperm cell penetrates the zona pellucida (3), the plasma membranes of the sperm cell and the oocyte fuses (4) and the sperm nucleus enters the egg cytoplasm (5) (Alberts 2002).*

1.3 Evaluation of fertility

Selection of boars has traditionally focused on economically important traits that are easy to record in field, like e.g. pregnancy rate and the total number of piglets born (TNB), treated as a female fertility in the breeding scheme. The trait TNB is influenced by several factors, including ovulation rate, uterine capacity and embryonic survival. The boar as sire of the dam (female fertility) is also affecting of all these traits through his genetics. Considering the boar as sire of the litter (male fertility), the sperm quality will affect the fertilisation rate, while the genetic contribution of the boar might affect the viability of the embryo (van der Lende et al. 1999). A positive correlation between farrowing rate and TNB have been reported, implying a direct boar effect on the TNB (Swierstra and Dyck 1976). As the TNB is a fertility parameter measured in the field, it is dependent on registration by the pig farmers. On the contrary, TNB is a direct assessment of fertility.

The genetic progress difficult to achieve as the TNB is characterized by low heritability and influenced by many loci (Trenhaile et al. 2016). Due to the economic importance of this trait, it has been included in the breeding program since the 1990s in Norway.

1.4 Evaluation of semen quality

To optimize the use of AI in pig production, the assessment of fertilizing capacity of boar ejaculate is of great importance (Foote 2003). The sperm cells' ability to undergo capacitation at the correct point of time, hyperactivation, acrosome reaction, binding to the ZP and penetrate the oocyte are essential for the fertilization process (Foxcroft et al. 2008). Factors affecting these abilities will influence the quality of the semen. Some of these factors will be described in the next section.

The morphology of the spermatozoa appears to be related to fertility (Gadea 2005). Several studies have found a relationship between motility parameters of the spermatozoa and fertilizing capacity of boar ejaculates (eg.Broekhuijse et al. 2012, Vyt et al. 2008). The spermatozoa is dependent of ATP for various reasons, and motility is one of the most important ATP dependent actions (Jones and Bubb 2000). It has been proposed that loss of motility during storage might be a consequence of a decreased ATP production (Gogol et al. 2009).

Variations in sperm chromatin integrity is reported to be correlated to fertility in different species, including pig (Ballachey et al. 1987, Boe-Hansen et al. 2008, Broekhuijse et al. 2012, Didion et al. 2009, Evenson and Wixon 2006, Evenson et al. 1980).

Seminal plasma facilitates the transport of spermatozoa into the female genital tract, acts as a buffer solution and is a nutrient source for the spermatozoa. The seminal plasma proteins play therefore an important role in the fertilization process by preventing early capacitation, maintenance of sperm viability, sperm-ZP interaction and oocyte-sperm binding as well as enhancing uterine contractions (Foxcroft et al. 2008).

Predicting the storage capacity of diluted boar semen is important due to the fact that semen is fresh goods and the fertility of diluted semen have been reported to decline within the first 72 hours *in vitro* (Waberski et al. 2011). The storage capacity of a semen sample during liquid storage depends on the extender, storage time, storage temperature, dilution and semen quality (Haugan et al. 2007, Martin-Hidalgo et al. 2011, Waberski et al. 1994,

Waterhouse et al. 2004). Differences between pig breeds regarding to storage capacity have been indicated by variation in sperm motility (Martin-Hidalgo et al. 2013).

Other factors influencing the semen quality includes temperature, the year-season effect and age of the boar (Smital 2009).

1.4.1 Evaluation of sperm motility parameters

Motility is the most widely used indicator of sperm quality, and has traditionally been manually and subjectively assessed using phase contrast microscopy. An objective computer assisted sperm analysis (CASA) system is currently available for evaluation of sperm motility characteristics. CASA was proposed to obtain objective semen measurements more than 30 years ago (Dott and Foster 1979), but it has not been taken into routinely use for farm animals until the last decade. The advantage of an objective analysis of sperm motility and classification of spermatozoa subpopulations has led to an increased use of CASA in mammals (Mortimer et al. 1997, Verstegen et al. 2002). The most commonly reported CASA parameters include curvilinear velocity (VCL), the average path velocity (VAP), the straight line velocity (VSL), the amplitude of the lateral head displacement (ALH), the beat cross frequency (BCF), straightness (STR), linearity (LIN) and wobble (WOB) (Mortimer 2000) (Figure 8). In kinematic analysis of the movement of the spermatozoa, the movement of the sperm head is assessed even though the flagellar movement is the determining factor of the head movement (Mortimer et al. 1997). Based on these parameters, individual motile sperm cells may be divided into subpopulations, and hyperactivated spermatozoa is an important subpopulation (Yanagimachi 1969). The hyperactive swimming pattern varies from species to species, and for boar spermatozoa the thresholds have been suggested to be VCL>97µm/s, ALH>3.5µm, LIN<32% and WOB<71% (Schmidt and Kamp 2004).

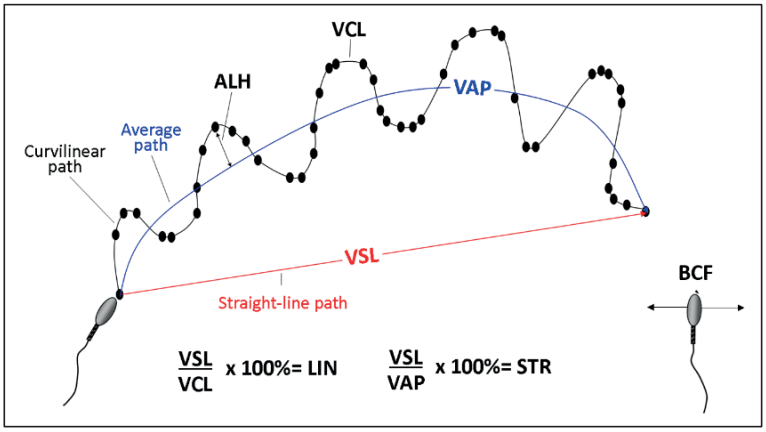


Figure 8. *Schematic illustration of the motility characteristics important for measurement of motility using computer assisted semen analysis (CASA). The most commonly reported CASA parameters include curvilinear velocity (VCL), the average path velocity (VAP), the straight line velocity (VSL), the amplitude of the lateral head displacement (ALH), the beat cross frequency (BCF), straightness (STR), linearity (LIN) and wobble (WOB).*

1.4.2 Evaluation of ATP levels in semen

The ATP needed to obtain a hyperactivated swimming pattern is most likely dependent on glycolysis in the principal piece of the spermatozoan flagellum and respiration in the mid-piece (Schmidt and Kamp 2004, Westhoff and Kamp 1997). To determine the ATP in semen samples, luminescence assays may be used. In short, the ATP is extracted from the spermatozoa by lysing the cells. Luciferin is added to the lysate and is converted to oxyluciferin by an ATP-dependent luciferase (Figure 9). This reaction produces a light signal proportional to the number of living cells, which is detected by a luminometer. The bioluminescence value in semen samples, measured in relative luminescence units (RLU), is converted to the corresponding ATP value in nM using the standard curve values.

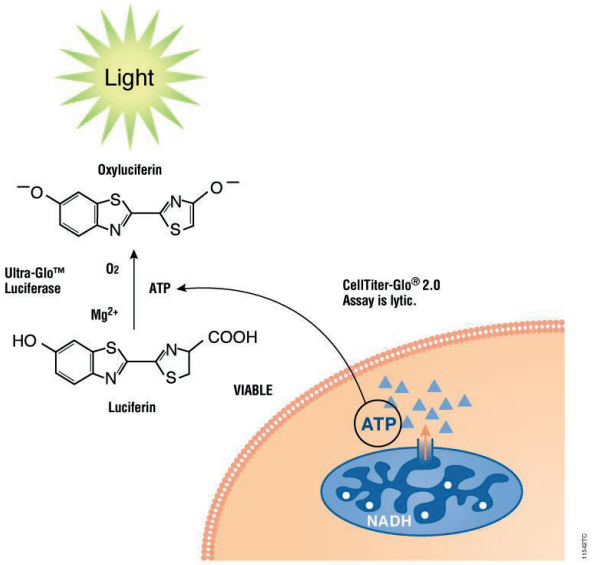


Figure 9. *Illustration of the ATP assay used for determining ATP content in semen. Conversion of luciferin by a recombinant luciferase produces oxyluciferin and light. The light may be measured by a luminometer and is proportional to the number of living cells. Obtained from manufacturer (Promega 2018).*

1.4.3 Flow cytometric evaluation of sperm DNA integrity

Flow cytometry is a technology that allows investigation of single cells by forcing the cells into a stream of fluid that passes through a flow cell with an analysis point. A thousand cells within a minute may be analysed. Cell suspension may be incubated with fluorochromes or fluorochrome conjugated antibodies against cellular components of interest. Light is scattered as the cells move through a laser beam. Electrons in the fluorochromes are excited and fluorescent light is emitted. The amount of fluorescent emission is proportional to the amount of bound fluorochromes. The signals are collected, amplified by a detector, transformed and transferred to a computer (Figure 10). The relative fluorescence is plotted against the number of events in a scatter plot.

The study of thermal denaturation of DNA using acridine orange (AO) was reported as early as the 1970s (Darżynkiewicz et al. 1974). The sperm chromatin structure assay (SCSA) is a flow cytometry-based method using DNA staining properties of acridine orange (AO), which fluoresces green and red, respectively, in native dsDNA and denatured

ssDNA (Evenson and Jost 1994). The SCSA has many advantages compared to other methods of assessing DNA integrity. There is only one protocol available for the method, which means that all laboratories reporting results from this method have done exactly the same giving that the recommendations have been followed. Other methods, e.g. TUNEL, COMET or HALO have variations in their protocols, making the reproducibility difficult to maintain. Further, the SCSA has low standard deviation, and thus the repeatability and precision is very good. Another important advantage with the SCSA, is the maintenance of the normal nucleus morphology (Evenson 2016). In short, acid added to the samples denatures the DNA at sites were the DNA is already damaged. The SCSA measures the relationship between the single-stranded, denatured DNA and the native double-stranded DNA for each sperm cell. The ratio between the red and total (red+green) fluorescence is called the DNA fragmentation index (DFI) and will give a quantitative number of the chromatin integrity of a sperm sample (Evenson and Wixon 2006).

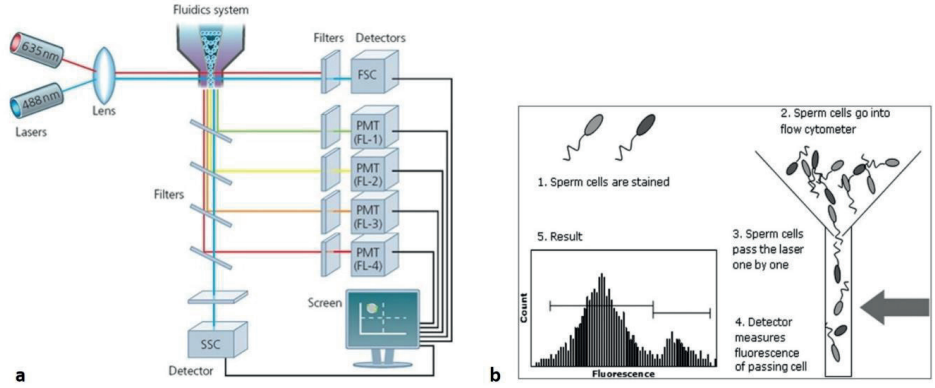


Figure 10. *Schematic overview over the flow cytometrical principle. The flow cytometer is built up by three components: the fluidics, the optics (laser, lens and filters) and the electronics (a) (Rahman 2006). The cells move through the flow cytometer, and pass the laser one by one (b) (Broekhuijse et al. 2012).*

1.5 Fertility genomics

The most economically important traits in pigs are quantitative, and are influenced by multiple genes or QTLs, but are also influenced by the environment (Ernst and Steibel 2013). This makes the identification of genetic variants underlying these traits difficult. This is also the case for spermatogenesis, which is a highly specialized cellular process

occurring in the testis, with a number of genes involved (Chalmel and Rolland 2015, Lin et al. 2006). The testis has been identified as the organ that expresses the greatest number of tissue-specific genes and proteins, and contains the highest number of alternative splicing (Chalmel and Rolland 2015). In addition, numerous genes are involved in fertility through several different pathways, such as steroidogenesis, spermatogenesis, fertilization and embryo development (e.g. Hunter et al. 2004, Ito and Kashiwazaki 2012, Mutembei et al. 2005, Robic et al. 2014, Signorelli et al. 2012, Sutovsky 2015).

1.5.1 Candidate genes

There are numerous genes involved in male fertility and female fertility. In the present study, a number of candidate genes was further investigated to find associations to litter size. The candidate genes were chosen based on a literature search, and the genes selected for male fertility are as follows:

- Phospholipase C zeta (*PLCz*): Contributes to Ca^{2+} oscillation, which is important for successful fertilization in pigs, and *PLCz* is also indicated to be involved in prostaglandin synthesis. Prostaglandins have been suggested to play an important role in the spermatogenesis (Kaewmala et al. 2012).
- Cyclooxygenase isoenzyme type 2 (*COX-2*): Involved in prostaglandin synthesis, and thus the spermatogenesis. A polymorphism within this gene has been reported to be significantly associated with litter size through the prostaglandin production in pigs (Kaewmala et al. 2012, Sironen et al. 2010).
- The ZP glycoprotein-3 (*ZP3*): Suggested to initiate sperm binding to the ZP of the oocyte and induce the acrosome reaction. The *ZP3*-induced acrosome reaction has been thought to be dependent on extracellular Ca^{2+} (Chiu et al. 2008).
- Cluster-of-differentiation antigen 9 (*CD9*): Crucial for the fusion of the sperm cell and the oocyte, and also has a role during sperm development (Inoue et al. 2011, Kaewmala et al. 2011).
- CatSper family proteins: Sperm-specific ion channels in flagellar membranes and are suggested to play an important role in the Ca^{2+} oscillation necessary for the hyperactivated motility (Carlson et al. 2003).
- Steroid 5α-reductase (*SRD5A*): A key enzyme in spermatogenesis by the involvement in converting testosterone into a more potent androgen,

dihydrotestosterone (*DHT*), in male reproductive organs. There are two types of this gene, and genetic variants in the *SRD5A2* are suggested to be associated to semen quality (Zhao et al. 2012).

- The androgen receptor (*AR*): A steroid receptor essential for male sexual differentiation and maturation, and spermatogenesis among others (Dirac and Bernards 2010).
- β-actin (*ACTB*): Located in the acrosomal and postacrosomal region of ejaculated spermatozoa and has a possible function during the acrosome reaction (Lin et al. 2006). Actin polymerization and acrosome reaction have been reported to be important for the fertilization process (Castellani-Ceresa et al. 1993).
- Protamines: Important for sperm chromatin condensation (Domenjoud et al. 1991). Protamin 1 (*PRM1*) is omnipresent in mammals and replaces the histones in the chromatin packing to make it more compact and less exposed for DNA damage (Dogan et al. 2015).
- Estrogen receptor 1 (*ESR1)*: Estrogen is considered to be a female hormone. However, estrogen is present with high concentrations in semen (Ganjam and Amann 1976). A previous study have demonstrated that the male fertility is impaired in mice when the *ESR1* is lacking, suggesting that *ESR1* has a possible role in spermatogenesis and sperm maturation (Eddy et al. 1996). Associations between *ESR1* polymorphisms and boar sperm quality and fertility traits have been reported previously (Gunawan et al. 2011).
- Estrogen receptor 2 (*ESR2*): Suggested to act as a negative regulatory partner for *ESR1* (Weihua et al. 2000). Reduced levels of *ESR2* lead to reduction of sperm motility and fertilizing abilities (Couse and Korach 1999), while overexpression of *ESR2* leads to germ cell cycle arrest or apoptosis and infertility (Selva et al. 2004). An earlier study indicated the important role of *ESR2* in the spermatogenesis in boars (Gunawan et al. 2012).

Also when it comes to female reproduction, several genes are known to play an important role both in pigs and in other mammals. In this thesis, sows were not included, but genes linked to female reproduction were included to investigate the boars as maternal grandsires. The most well-known are probably the genes related to ovulation rate and oocyte quality, like bone morphogenetic protein 15 (*BMP15)* and bone morphogenetic protein receptor 1B (*BMPR1B)* and growth differentiation factor 9 (*GDF9)* (de Castro et al. 2016, Juengel et

al. 2004, Paradis et al. 2009, Persani et al. 2014, Våge et al. 2013). Several genes have proved to be involved in both male and female reproduction, such as *ESR1* and *ESR2* (Gunawan et al. 2012, Gunawan et al. 2011).

1.5.2 Identifying gene variants affecting fertility

Two principal approaches can be used to identify genetic variants affecting quantitative traits, either a genome wide association study (GWAS), or a more restricted analysis of association to selected candidate genes. The GWAS method has its obvious strength in covering the whole genome without any prior assumptions about involved genes. On the other hand, sequencing candidate genes may reveal causative SNPs or variation located very close to causative SNPs, which might show stronger associations than SNPs on a commercial chip, which are selected just to be evenly spaced across the whole genome. Another way to investigate underlying genetics is transcriptome sequencing. The differential gene expression identified by transcriptome sequencing may point towards molecular mechanisms involved in a chosen trait. The gene expression is analysed by directly sequencing the cDNA synthesized from all the RNA extracted from a sample. The expression level is quantified by counting the number of reads for each gene, and this measure is normalised by accounting for gene size. Transcriptome sequencing is more sensitive than microarrays and the result is not affected by array-design. In addition, all mRNAs in the sample are detected regardless of prior knowledge of the expressed genes. Transcriptome sequencing gives the opportunity of identifying genetic networks and biological processes involved in the studied trait, as well as detecting SNPs by aligning the sequences to reference-sequences (Mane et al. 2009). Both the transcriptome sequencing approach and the candidate gene approach were used for evaluation of underlying genetics for male fertility in this thesis.

1.6 Pig breeding in Norway

Norsvin SA is a farmer-owned cooperative started in 1958 as the Norwegian Pig Breeders' Association. Norsvin is the sole Norwegian swine breeding company, focusing on genetic improvement of important production traits. The international part of the company merged with the Dutch pig breeding company Topigs in 2014, and this company, Topigs Norsvin, is the second largest provider of pig genetics in the world. Boar fertility is of great

importance for overall pig reproduction efficiency and the economy for the pig producers. An increase of 0.1 piglet per litter per year is estimated to give an increased income for Norwegian pig production of 10 million NOK per year. Subfertility is currently hard to predict, and finding sperm quality parameters correlating to TNB or fertility rate would be of high value. Also identification of genetic markers explaining boar fertility could be highly valuable in order to improve the male fertility and to identify the more efficient boar semen producers prior to AI boar selection. Semen with high fertility will enable a reduction in the number of sperm cells per dose, and thus reduce the number of boars needed for semen production. This would to reduce the production cost considerably, as well as enabling more effective use of the best breeding boars at the AI station. Today, national breeding programs exist for two breeds in Norway, the Norwegian Landrace and the Norwegian Duroc.

Ingris is a national database system and is an important registration and management tool for pig farmers. The registration of data from the herd is done directly into the database. The collected data for the basis for breeding of pigs, statistics, research and prognosis for slaughter amongst other. Approximately 70% of the pig producers in Norway are members of Ingris (Norsvin 2017).

1.6.1 Norwegian Landrace

The Norwegian Landrace (NL) (Figure 11) has been used since the 1950s, and this breed has continuously been improved, through genetic selection. The breeding goal has changed from focusing on growth efficiency in the 1960s, 70s and 80s, to nowadays include maternal productivity, health and meat quality. Litter size was included in the 1990s and days from weaning to breeding as a reproduction trait was included in the breeding goal in 2004. Per 2016 the breeding goal includes production traits, slaughter quality, meat quality, reproduction, maternal ability, robustness and health. The NL is used as the maternal line in the Norsvin breeding program (Norsvin 2017). The average TNB was 13.7 piglets for NL in 2016.

Figure 11. *A Norwegian Landrace boar, used in Norsvin's breeding program in Norway (Norsvin 2017).*

1.6.2 Norwegian Duroc

The Norwegian Duroc (ND) (Figure 12) has not been subject to organized genetic selection for as long time as NL. The systematic breeding started in the 1990s. The breeding goals in the 1990s included growth, feed efficiency, meat percentage, slaughter percentage and carcass quality. Litter size was included for a few years, but per 2016 the breeding goal includes production traits, slaughter quality, meat quality and health. The ND is used as the paternal line in the Norsvin breeding program (Norsvin 2017). The average TNB for ND was 9 piglets in 2016.

Figure 12. *A Norwegian Duroc boar, used in Norsvin's breeding program in Norway (Norsvin 2017).*

2. Objectives

The main goal of this PhD project is to find suitable boar fertility parameters that can be used in practical breeding systems to produce boars with good fertility. To reach this goal we have compared and evaluated current and experimental sperm quality analysis parameters, related them to field fertility records and finally investigated possible genetic factors underlying these traits.

The overall objective of this project was to find correlations between boar semen quality parameters obtained by laboratory analysis, and field fertility. The lab assays we used included evaluation of motility characteristics using computer assisted semen analysis (CASA), measuring ATP levels in semen and analysis of sperm DNA integrity (DNA fragmentation index) using a flow cytometer. The genetic analyses included RNA sequencing of contrasting groups and testing candidate genes for association with male and female fertility EBVs.

There are reports showing differences between the Landrace and Duroc in their ability to reproduce and in semen quality. There are also results indicating breed differences in storage of semen (from day of collection to day 4 after collection). Possible breed differences were therefore tested by using the same assays/parameters as described above.

3. Results: summary of individual papers

Paper I:

The relationship between sperm motility characteristics and ATP levels, and its effect on fertility in two different pig breeds

Boar fertility has a major impact on overall pig reproductive efficiency. However, finding accurate and objective *in vitro* sperm parameters for predicting *in vivo* fertility is challenging. Motility is the most widely used indicator of sperm quality and in this paper motility characteristics in two pig breeds, Norwegian Landrace (NL) and Norwegian Duroc (ND), were assessed using CASA. ATP levels in semen from the same samples were measured parallel to the CASA. To assess the storage capacity of each ejaculate, measurements were performed both at the day of collection (Day 0) and after of liquid storage at 18°C (Day 4). To find possible associations between the CASA parameters, ATP and fertility, the TNBs for each ejaculate were obtained. Comparing ejaculates from the two breeds showed significant differences in the motility characters. The motility pattern in NL developed towards more hyperactivation during storage, while in ND a larger portion of sperm cells with a hyperactive swimming pattern were detected at Day 0. The size of this population decreased upon storage. The total percentage of motile sperm cells significantly decreased in both NL ($p=0.01$) and ND ($p<0.0001$). A significant decrease in the ATP level ($p<0.0001$) was also found in both breeds during storage. In correlation with TNB, linearity at the day of collection and the wobble after storage showed an effect in NL, while the percentage of motile cells, curvilinear velocity and lateral head displacement at the day of collection and linearity after storage showed an effect in ND.

Paper II:

Sperm DNA integrity in Landrace and Duroc semen and its relationship to litter size

Improved quality assessment methods are needed for routine semen quality evaluation at AI stations, as standard semen parameters are poor in predicting the fertility of a semen sample. In sperm cells, there are few or none reparation mechanisms for DNA damage. However, sperm cells with DNA damage are still able to fertilize the oocyte, and even though oocytes and early embryos can repair some types of DNA damage, this might result in embryonic loss and a decreased litter size. The sperm chromatin structure assay (SCSA) is a flow cytometry method assessing DNA fragmentation by evaluation of the susceptibility of DNA denaturation *in situ* under acidic conditions. The aim of this study was to use the SCSA to assess the DNA fragmentation in semen samples from boars in relation to the total number of piglets born (TNB). Comparing the DNA integrity in the two breeds Norwegian Landrace (NL) and Norwegian Duroc (ND), there were differences in the DNA fragmentation index (DFI). The medians for NL and ND were 1.37% and 1.61%, respectively. For both breeds, DFI had a significant negative effect on TNB, indicating that lower DFI values results in higher TNB. No threshold values were suggested for either breed due to the knowledge on other factors affecting the TNB. However, boars with the 5% lowest TNB had a mean DFI of 2.77% and 2.08% in NL and ND, respectively, compared to 1.32% and 1.18% for the boars with the 5% highest TNB. This underlines the importance of DNA integrity for male fertility in pigs.

Paper III:

RNA sequencing reveals candidate genes and polymorphisms related to sperm DNA integrity in testis tissue from boars

Proper chromatin packaging of sperm DNA is known to be important for boar fertility outcome, as this protects the DNA against fragmentation. The aim of this study was to investigate the molecular mechanisms underlying the differences in sperm DNA fragmentation using transcriptome sequencing. Testis tissue from Norwegian Landrace and Duroc boars, with stable high or low sperm DFI, were analysed. The mean (± SD) of the DFI values for the low and the high groups were 1.04% (± 0.44%, n = 5) and 4.79% (± 1.12%, n = 4) in Landrace and 1.09% (± 0.03%, n = 5) and 4.79% (± 0.62%, n = 6) in Duroc, respectively. Altogether, 308 and 374 genes were found to display significant differences in expression level between high and low DFI in Landrace and Duroc boars, respectively. Among these genes, 71 were differentially expressed in both breeds. Gene ontology analysis revealed that significant terms in common for the two breeds included extracellular matrix, extracellular region and calcium ion binding. Moreover, different metabolic processes were enriched in Landrace and Duroc, whereas immune response terms were common in Landrace only. Variant detection identified putative polymorphisms in some of the differentially expressed genes, and validations showed that predicted high impact variants in five genes were particularly interesting for sperm DNA fragmentation in boars. We have identified differentially expressed genes between groups of boars with high and low sperm DFI, and functional annotation of these genes point towards important biochemical pathways. Moreover, variant detection identified putative polymorphisms in the differentially expressed genes.

Paper IV:

Association between SNPs within candidate genes and fertility in Landrace and Duroc pigs

Finding effective predictors of relative boar fertility is essential for increasing the efficiency of AI systems in pig breeding. The main objective of this study was to find associations between SNPs within candidate genes and fertility in two Norwegian pig breeds; Landrace and Duroc. The candidate genes were selected based on either previous reports of association with reproduction traits or involvement in pathways related to reproduction. To detect genetic variants, boars with contrasting breeding values for male fertility were compared. In addition, the breeding value for fertility routinely used in the Norsvin breeding scheme was used to make corresponding contrast groups for female fertility. Animals with contrasting breeding values for fertility were re-sequenced to detect genetic variants. Out of the 13 re-sequenced genes, 57 SNPs were found in eight different genes. Due to difficulties in primer design, only primers for 52 SNPs were designed. In addition, one extra SNP was added based on a previous result from the RNA sequencing. Out of the 53 SNPs, 14 did not work in the assay or were monomorphic, and were excluded. A total of 619 Landrace boars and 513 Duroc boars were genotyped for the detected candidate gene SNPs. After filtration on MAF > 0.001, HWE > 0.0001 and call rate > 0.97, there were 25 and 21 SNPs left for association analysis in Landrace and Duroc, respectively. Two SNPs in *BMPR1* and one SNP in *COX-2* in Landrace were found significantly associated with litter size. In Duroc, two SNPs in PLC_z, one SNP in *VWF* and one SNP in *ZP3* were found significantly associated to litter size. These SNPs explained between 0.27% and 1.18% of the genetic variance. These effects are too low for being used directly for selection purposes, but the associated variants can be of interest in SNP-panels used for genomic selection.

4. Discussion

Increased knowledge of sperm quality parameters affecting field fertility, and elucidation of possible linkage to genetic variants is essential to increase the understanding of the relationship between sperm phenotypes and male genotypes related to fertility. This knowledge can be used to establish new genetic markers for boar fertility to improve the selection of the best boars in the breeding program.

4.1 Semen quality parameters and boar fertility

To be able to evaluate the male fertility, parameters linked to the sperms' ability of successful fertilisation of the oocyte and subsequent development of healthy embryos should be assessed (Roca et al. 2015). In the present thesis, evaluating motility parameters represent the capability of fertilisation and the evaluation of DNA integrity represent the potential impairment of early embryonic development.

4.1.1 Sperm motility parameters

Using CASA systems has great advantages compared to the traditional, subjective evaluations of sperm motility and morphology. Even though the laboratory technicians are well-trained and experienced, the subjective evaluation of motility will vary between assessors. The CASA system gives the opportunity of a more standardised and objective measure on the sperm motility patterns, is less time consuming, requires less resources, and is easy to implement in the production line. In addition, the CASA gives the opportunity of evaluating all the parameters that define motility, either for each sperm cell or as a mean for each ejaculate. This will be advantageous, both for the production of good breeding animals, but also for the farmers that need to keep up an efficient production.

In paper I, possible associations between motility parameters and fertility (TNB) were investigated. The most frequently used parameter for evaluation of semen quality, total motility, did not show an effect on TNB in NL and in ND, total motility influenced on TNB only in samples measured at the day of semen collection (Day 0). This is in accordance with a previous study reporting that the sperm motility has limited effects on TNB in pigs (Broekhuijse et al. 2012). One of the objectives in the present study (Paper I) was to find other CASA parameters that could be of more importance for the validation of the ejaculates. We hypothesised that hyperactive motility would be of great interest and could

possibly have an effect on TNB, as this swimming pattern is essential for the fertilisation. The spermatozoon needs to obtain this swimming pattern when it is in contact with the oocyte and preferable not in the tube prior to insemination. Our study showed that the NL boars have a lower percentage of hyperactive sperm cells at Day 0 compared to ND, and it increases during storage. The ND boars, however, maintain the higher percentage of hyperactivity. The sperm cells from ND boars have a more circular, less linear swimming pattern to begin with that changes little during storage, while the sperm cells from NL have a more linear swimming pattern at Day 0 which changes to a more circular swimming pattern upon 96 hours storage (Day 4). However, results from this study showed that the percentage of hyperactivated sperm cells in the ejaculates did not have an effect on the TNB in any of the breeds, neither at the day of collection or after storage, and our hypothesis was rejected. The definition of hyperactive motility is based on threshold values of CASA parameters (Schmidt and Kamp 2004), and in our study, several of these parameters showed significant associations to TNB. This indicates that the involvement of a hyperactive motility pattern might be important for fertility in boars in terms of TNB after all. The high number of sperms cells in the dose ($28x10^6$ cells/mL) may explain the lack of association between hyperactivity and TNB, as the high cell number may compensate increased hyperactivity or decreased motility in a dose. Decreasing the number of cells per dose used for AI, would be a huge advantage, as it would enable more efficient utilisation of the best boars used for sperm production as well as being of economic value as the litter size is most likely to be increased. However, this will increase the importance of the evaluation of the individual parameters for motility, in addition to overall motility, in prediction of fertility.

In Paper I, a screening was done to obtain an adequate number of ejaculates for the evaluation of the relationship between CASA parameters and TNB. Semen doses were obtained from the AI centre twice a week, and were analysed both at the day of collection and upon 96 hours storage. The doses were randomly chosen according to three criteria; the doses were accepted for costumer use, the doses were purebred NL or ND and that both breeds must be represented in each sampling. This data collection was introduced before the AI station started using CASA as routine. Up to five ejaculates were collected from some of the animals, while other animals were only represented by one ejaculate. This was part of the randomisation process, but it might have been better to ensure that several ejaculates from all the boars in the study were available. The aim of the current study was

to investigate if the results from the CASA method could predict how the ejaculates performed in field in terms of TNB. However, ensuring several ejaculates from all boars would make it possible to both look at the boars' reproductive abilities as well as the individual ejaculates' reproductive potential. In this thesis we have aimed to investigate methods for identifying the most fertile boars within a group of boars with acceptable fertility. Therefore, some of the rejected ejaculates should possibly have been included in the study, ensuring more variation in the samples used for evaluating the effect of motility. However, we investigated the ejaculates that were approved for AI, to be able to find methods for ranking the ejaculates that already were considered to have good fertility. For fertility evaluation of rejected ejaculates, an agreement with pig producers including an economic compensation of possible production loss would be needed. However, this was not in agreement with the budget in the current project.

The overall motility in an ejaculate is the most commonly used parameter for evaluation of male fertility. However, several studies report that sperm cells within each ejaculate can be divided into subpopulations based on their swimming patters (Holt 2009). There are several reports on motility subpopulations in boars (Henning et al. 2014, Quintero-Moreno et al. 2004, Ramio et al. 2008), and cluster analysis may be useful to detect differences in semen quality that are not revealed by just considering mean values of motility in the sample (Ibanescu et al. 2017). Subpopulation analysis was not included in our study, but should be assessed in future studies on motility parameters in NL and ND.

There are many different CASA systems on the market, both for research and production purposes. One challenge is that the different systems might give slightly different results. The percentage of sperm motility is probably the most complicated parameter to evaluate in a CASA system, and it is likely affected by the CASA settings (Boryshpolets et al. 2013). Comparing the motility patterns from one instrument to another is therefore not possible, as the manufacturers might have applied different settings. Although we used another CASA system in our study (Paper I) than the one used at Norsvin's AI station, the overall results in motility characteristics, storage capacity of ejaculates and breed differences may be used as a guideline for the AI station.

Another challenge measuring the motility patterns with CASA is the general settings used for different species. For example, the sperm motility characterizations are not identical for boars and for bulls (Shojaei et al. 2012). However, the possible differences between breeds

in the same species have not been properly investigated. In this thesis, big variations in the motility characteristics were observed between the two breeds NL and ND. Previous studies have implied a difference in the plasma membrane structure between pig breeds (Waterhouse et al. 2006). It would be interesting to investigate if the differences in motility patterns in the two breeds are linked to these structural differences in the plasma membrane. In addition, further studies should be performed to assess whether this might result in different threshold values for assessing the motility patterns, especially hyperactive motility.

4.1.2 Sperm DNA integrity

The production and maturation steps of the spermatozoa, which occur in the testicles and the epididymis, are of great interest in the assessment of sperm quality. However, these processes are not practical to monitor *in vivo*, but they are to some extent reflected in the ejaculated sperm cells (Foote 2003). Our results showed that increased DNA fragmentation have a significant negative effect on TNB in both NL and ND. This implies that the production and maturation steps of the spermatozoon have a role in male fertility, and are related to the overall fertility in pigs.

The percentage of DNA fragmentation, the DFI, was measured by the SCSA-method. This is a measure of the ratio between cells with highly packed DNA resistant to DNA damage (stained with green fluorescence) and cells with more loosely packed DNA, hence more sustainable to DNA damage (stained with red fluorescence). However, there is a population of cells that was not considered in this thesis: the high DNA stainable (HDS) sperm population. This is a population of cells with an abnormal high DNA staining (green fluorescence), caused by the lack of full protamination. This population has an increased amount of retained histones and indicates sperm chromatin (protein) defects (Evenson 2016). The HDS population in an ejaculate will affect the overall ratio between red and green fluorescence and thereby result in a normal DFI in samples with a high DFI. Thus, there is a hypothetical chance that high DFI might be masked, resulting in a false low DFI. The founder of the SCSA recommends that, in addition to DFI, this population should be determined as well as the standard deviation of DFI (Evenson and Jost 2000). This was not done in the studies presented in this thesis (Paper II). However, in-house data (data not shown) shows that there are few cells with HDS in boars and it would have a relatively low impact on the result for the AI station in Norway. In addition, a software is used for calculating the ratio between red and total fluorescence in the protocol described in earlier

studies (Evenson and Jost 2000). In our study, the ratio between red and total fluorescence was calculated directly by the flow cytometer, using specific "gating" for red and green fluorescence visualised by a cytogram. However, in-house data showed that there was no significant difference between the two methods, with the exception of a few samples with high DFI, which tended to be a bit lower using the software. These are relative values, not absolute, so the main goal is to be consistent in the choice of method to be able to compare and monitor boars' DFI levels over time.

An advantage with the evaluation of DNA fragmentation in our study (Paper II) is the high number of samples included in the dataset. This increases the reliability of the results in the study. In addition, the two breeds have been evaluated separately, eliminating the breed effect in the relationship to TNB.

A threshold value for when the SCSA DFI has a damaging impact on fertility has been suggested in several studies. This threshold value varies across species, e.g. pigs: 6%, bulls 10–20%, horses: ~28%, humans: 25–30% (Evenson 2016). Another study have reported threshold values as low as 2.1 or 3.0% in liquid stored boar semen (Boe-Hansen et al. 2008). The results from our study (Paper III) also indicated a low threshold value for liquid stored boar semen. However, the effect of DFI on TNB is relatively low, indicating that the impact on TNB is limited and that in addition to sperm DNA fragmentation, several other parameters influenced on the litter size. Although no threshold value was proposed, boars giving ejaculates with high DFI value should be monitored.

4.2 Gene variants and boar fertility

To be able to increase the understanding of the underlying mechanisms behind the sperm quality traits, the identification of genetic factors and relationship between genotypes and fertility-phenotypes is of great interest.

4.2.1 Assessment of underlying genetics

A low number of sows inseminated from one ejaculate makes it difficult to evaluate boar effects independently from the effects of the sow (Flowers 2008).

The RNA sequencing performed in Paper III, revealed several differentially expressed genes involved in calcium ion binding. To our knowledge, this has never been reported earlier. Ca^{2+} is critical in different cellular signalling processes, and must be regulated both

intra- and extracellularly. For spermatozoa, Ca^{2+} has a role in maturation, motility and acrosome reaction and is a fundamental regulatory factor for sperm hyperactivation (Darszon et al. 2011). In addition, Ca^{2+} affects protein phosphorylation and sperm motility through changes in cyclic adenosine monophosphate and ATP concentrations (Li et al. 2016). The Ca^{2+} is also important for the mammalian embryo development through elevation in the oocyte's intracellular free Ca^{2+}, triggered by fertilizing sperm cells (Machaty 2016). One of the genes that were found up-regulated in ND boars with a high sperm DFI was *PLCZ1*. *PLCz* has been suggested to be the strongest candidate for a sperm factor that initiates the oocyte activation and early embryonic development (Yoneda et al. 2006). It is suggested that sperm binding to *ZP3* induces a Ca^{2+} influx which in turn leads to activation of *PLCz* and further to acrosome reaction (Fukami et al. 2003). In addition, *PLCz* have been reported to be important in spermatogenesis (Kaewmala et al. 2012). In the candidate-gene study (Paper IV), SNPs in both *ZP3* and *PLCz* were found significantly associated to EBV for TNB. For *PLCz*, SNPs associated to both male and female fertility were found, supporting earlier findings of involvement in both male and female mechanisms. The results from Paper II and Paper IV, which indicate the involvement of Ca^{2+}, supports earlier reports of the importance of the Ca^{2+} in male reproduction. Further studies are needed to clarify the role of testicular calcium signalling for sperm DFI levels. Analysing intracellular calcium through calcium markers samples with high and low DFI using a flow cytometer, as described in an earlier study (Yeste et al. 2015), would enable us to elucidate the relationship between calcium and DFI.

The results from the RNA sequencing in Paper III indicate that genes involved in different stages of spermatogenesis affect the DNA fragmentation in sperm cells. The gene ontology terms "extracellular matrix" and "extracellular region" were significant for both breeds. The "extracellular matrix" plays an important role in regulation of the spermatogenesis as the Sertoli and germ cells are structurally and hormonally supported by the extracellular matrix during their development in the seminiferous tubes (Siu and Cheng 2008). There are cell junctions situated in the "extracellular region", that are important for completing the spermatogenesis. The germ cells must migrate across the seminiferous epithelium which is a process controlled by restructuring events in the cell junctions in the "extracellular region", called the ectoplasmic specialization (Lee and Cheng 2004, Siu and Cheng 2008). In this stage, the chromatin condensation occurs (O'Donnell 2014), and the results from Paper III suggests that genes involved in processes at the extracellular matrix and jell

junction affect the DNA fragmentation in sperm cells. In the current study, genes encoding extracellular matrix compounds such as collagens, laminins, fibulins and cytokines were differentially expressed. Moreover, peroxiredoxins and actins of the ectoplasmic specialization were up- and down-regulated. Genes involved in regulation of these compounds, like proteases, protease inhibitors and cathepsins, were also differentially expressed. The results confirm previous findings, as well as reporting a number of new genes, highlighting the importance of testicular steroidogenesis in the outcome of sperm DFI. In addition, variant calling identified five high impact SNPs within five differential expressed genes that are interesting for DNA fragmentation in boars. These SNPs have been validated, and include a stop lost variant (*GIMAP6*), a start lost variant (*ENSSSCG00000000712*), a stop gained variant (*ENSSSCG00000028326*) and two frameshift variants (*RAMP2* and *ENSSSCG00000009348)*. Their association to DNA fragmentation should be further examined in a larger animal material.

In Paper IV, SNPs within candidate genes were found associated to EBVs for TNB. Two EBVs for litter size were estimated, one with the boar as a father to the sow mothering the litters and one with the boar as father to the litters. This gave us the opportunity of investigating SNPs within candidate genes both associated to male and female fertility. Interestingly, we obtained three SNPs significantly associated to both male and female fertility, one SNP in *COX-2*, and two SNPs in *BMPR1*. This implies an importance of these genes for both male and female fertility traits. An earlier study has reported that BMPs have an impact on female fertility (Shimasaki et al. 1999). In addition, the *BMP* gene family has previously been reported to play a role in male reproductive biology (Itman and Loveland 2008) and may process both pre-collagens and laminins in the extracellular matrix (Trackman 2005). This gene family has also been suggested to play a role in follicular development and follicle/oocyte maturation (Paradis et al. 2009). Thus, this gene is suggested to be important for both male and female fertility, as our study implies. Also, another gene family member *BMP1* was found differentially expressed in both NL and ND boars with high/low levels of DFI (Paper III).

The candidate gene approach (Paper IV) may be considered as “old-fashioned”, when SNP - panels for whole genome studies are available (GWAS). However, SNPs within candidate genes may show stronger trait associations than SNPs just selected according to even chromosomal distribution (in SNP panels). In the current study, SNPs in a limited number of candidate genes were detected and investigated, none of them were shown to have a

major effect on TNB, even though they were significant. To increase the understanding of the underlying genetics of male fertility, more genes should be investigated in the future in the search of causative SNPs, combined with genome wide association studies.

Litter size is a low heritability trait, indicating limited genetic influence of this trait. Selection is therefore less effective on this trait compared to other traits with higher heritability. This is probably the main reason why few significant causative SNPs were found in candidate genes related to litter size in our study (Paper IV).

An increased knowledge on the underlying mechanisms behind DFI in sperm cells from NL and ND was obtained from the RNA sequencing. Also, significant SNPs in candidate genes associated to both male and female fertility and TNB, supports that TNB in pigs as a fertility parameter, is composed and influenced by several factors, both in relation to male and female fertility. RNA sequencing has revealed some interesting genes that are differentially expressed between groups of individuals contrasted with respect to DNA fragmentation. To confirm that these differences also are realised on the protein level, proteome profiling will be performed for similar contrasting groups in near future. In addition, the variant detection showed that high impact SNPs in five genes are of interest for DFI. Thus, these genes might be candidates for the detection of molecular markers for sperm DFI for use in selection towards improved sperm quality. In addition, RNA sequencing will be performed to elucidate differentially expressed genes between groups of individuals contrasted with respect to hyperactive motility.

4.3 Breed differences

Differences between the breeds concerning sperm quality parameters have not been sufficiently studied. However, some studies estimated a coefficient of variation for semen volume to be 30-40 % between several purebred pig breeds (Smital et al. 2004). Differences in androstenone concentrations between NL and ND have been reported (Grindflek et al. 2011), suggesting that there are differences related to steroidogenesis pathways between the breeds. The results in Paper I showed different sperm motility patterns between the two breeds, NL and ND. Both breeds showed a significant decrease in the percentage of motile sperms cells during storage. The percentage of progressively motile cells significantly decreased in ND, while the decrease in NL was not significant. The most visible difference observed between the two breeds was the percentage of

hyperactive sperm cells, as already discussed, as well as the level of ATP measured in semen. The ATP levels were considerably higher in the ND boars, compared to the lower level of ATP measured in NL. This either indicates that the ND has a capacity of a more circular, hyperactive swimming pattern due to higher levels of ATP, or that the cells have the ability of obtaining higher levels of ATP due to the vigorous swimming pattern. In both breeds, the ATP levels in semen decreases, as expected, during storage.

The RNA sequencing (Paper III) also revealed differences between the two breeds investigated. Although a portion of the differentially expressed genes was common to the breeds, a large portion of the differentially expressed genes were found to be breed specific. The breed specific differentially expressed genes might reflect breed specific mechanisms in chromatin condensation and DFI levels with regards to these two breeds. Gene ontology classification of the differentially expressed genes found "cholesterol metabolic process" and "oxidation-reduction process" specific to ND and "collagen catabolic process", "hydrolase activity" and "proteolysis" specific to NL.

The breed differences have been reported previously, in terms of differences in sperm physiology and semen plasma composition. In addition, differences in the composition of fatty acids in the sperm cell membrane, capacitation and ability for cryopreservation have been reported (Waterhouse et al. 2004, Waterhouse et al. 2006). However, the differences might be dependent on the individual male, and not the breed. Furthermore, significant differences were indicated in sperm shape and dimensions among pig breeds, including Landrace and Duroc (Saravia et al. 2007). Differences in semen volume, number of total sperm and number of viable sperm between breeds have also been reported (Smital et al. 2004). An earlier study reports that Duroc boars have a higher sperm cell concentration as well as a lower semen volume compared to Landrace (Smital 2009). Thus, the spermatozoa of Duroc boars have reduced volume of seminal plasma per cell compared to Landrace. Plasma proteins in the seminal plasma have several roles and effects (Flowers et al. 2016). One of the plasma proteins found in seminal plasma, albumin, plays a role on the protection of sperm against oxidative stress caused by lipid peroxidation. In addition, spermadhesins, also found in seminal plasma, bind to the acrosome and act to preserve membrane integrity, motility and mitochondrial activity (Gonzalez-Cadavid et al. 2014). These effects will possible be lower in Duroc semen as the ratio between seminal plasma and spermatozoa is lower, compared to Landrace semen.

A breed difference was also seen in the DFI measured in liquid stored semen (Paper II). The DFI levels were higher in ND compared to NL. This is to be expected as the NL has been used in breeding for a longer amount of time and selected for TNB.

4.4 Storage capacity

The storage capacity of the sperm cells is of great importance as the semen doses rarely are used within the first 24 hours. Due to large geographical distances in Norway, more than 70% of AI is performed with semen stored for more than 24 hours. In paper I, there were significant differences in the motility parameters of sperm cells and in the ATP levels in semen during storage. Although the mean percentage of motility in NL and ND did not show a significant decrease after storage, a significant decrease after storage was seen at the ejaculate level. This implies that there are individual differences in the storage capacity in each ejaculate. The percentage of progressive motility and ATP level also significantly decreased at the ejaculate level in both breeds. Altogether, this implies that the semen doses should be used as soon as possible, and within the recommended time suggested by the breeding company. Interestingly, the hyperactive motility increased in the NL samples after storage. The sperms cells' ability of obtaining this swimming pattern is a prerequisite for fertilizing the oocyte. However, this should not occur in the tube during storage.

In Paper II, the storage capacity was tested with emphasis on DFI. The result from this showed a significantly increased DFI during storage in both NL and ND. For this study, the stored samples were used to investigate the "worst case scenario", as the doses rarely are used at the day of collection. Increased levels of DFI are reported to influence the TNB in pigs (Boe-Hansen et al. 2008, Didion et al. 2009). Considering both the increased hyperactive motility pattern in NL during storage and the increased percentage DFI during storage in both breeds, it is of great importance for the farmers to use the doses within the recommended time for AI to avoid a decrease in TNB.

5. Concluding remarks

The association found between motility parameters and TNB, and between DNA fragmentation and TNB imply that male fertility is an important part of the total fertility in pigs. Thus, the sperms' ability to successfully fertilize the oocyte and the subsequent development of healthy embryos have an overall effect on TNB. Therefore, some of these parameters are suggested to be included in the evaluation of the fertility potential and approval of ejaculates used for AI.

Gene expression profiling with RNAseq in testis tissue revealed differences between boars with high and low DNA fragmentation, and putative polymorphisms were identified in some of the differentially expressed genes. Several selected candidate genes were found to influence the litter size in both NL and ND, although the effects were modest.

Sperm quality parameters related to fertility have been found during this work. These might be used in practical breeding systems to select boars with good fertility. The results from this thesis have also contributed to knowledge on the genetics underlying male fertility and DNA fragmentation in pig sperm cells. Finally, this thesis have revealed differences between NL and ND in terms of sperm quality influencing fertility and storage capacity.

7. References

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Paper I

The relationship between sperm motility characteristics and ATP levels, and its effect on fertility in two different pig breeds

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Abstract

Boar fertility has a major impact on overall pig reproductive efficiency. However, finding accurate and objective *in vitro* sperm parameters for predicting *in vivo* fertility from a single ejaculate is challenging. Motility is the most widely used indicator of sperm quality, and a computer assisted sperm analysis (CASA) system is now available for objective assessment of sperm motility characteristics. In this study sperm motility characteristics using CASA and measured ATP levels and their effect on total number of piglets born (TNB) was investigated in Norwegian Landrace (NL) and Norwegian Duroc (ND) boar semen. Subsequently, a possible effect of these parameters on the total number of piglets born (TNB) were evaluated. In addition, breed differences in semen storage abilities were investigated. The CASA results showed differences between NL and ND sperm motility characters. The percentage of motile sperm cells significantly decreased in both NL ($p=0.01$) and ND ($p<0.0001$) during storage. A large proportion of sperm cells with a hyperactive motility pattern were detected in ND semen on the day of collection, with no significant changes upon storage. On the contrary, the sperm motility pattern in NL developed towards more hyperactivation during semen storage. A significant decrease in ATP level during storage ($p<0.0001$) was found in both breeds. The linearity at the day of collection and the wobble after storage influenced TNB in NL, while the percentage of motile cells, curvilinear velocity and lateral head amplitude at the day of collection and linearity after storage influenced TNB in ND.

Keywords:

Boar spermatozoa; Motility characteristics; CASA; ATP; Fertility

1. Introduction

Boar fertility has a major impact on overall pig reproduction efficiency. Selection of boars with high fertility is economically essential both for farmers and breeding companies. At the production level of AI centres, however, finding accurate and objective parameters for predicting field fertility results from a single ejaculate is challenging. Motility and morphology are the most widely used indicators of sperm quality and is the daily tool for decision of approval or rejection of ejaculates. Motility has traditionally been manually and subjectively assessed using phase contrast microscopy, but currently an objective computer assisted sperm analysis (CASA) is available for evaluation of sperm motility characteristics (Amann and Waberski, 2014). The advantage of an objective analysis of sperm motility has led to an increased use of CASA in mammals (Mortimer et al., 1997; Verstegen et al., 2002). However, to utilize the potential of CASA analysis at AI centres, there is a need for more studies of specific sperm motion characteristics within ejaculates and how they potentially correlate to male fertility.

The CASA default reports usually include mean values for hundreds of single sperm cell tracks presented as curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), amplitude of the lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and wobble (WOB) (Mortimer, 2000). In addition to these parameters, evaluation of hyperactive motility is of interest. Hyperactivated spermatozoa are characterized by a vigorous and non-linear movement, caused by an increased amplitude of flagellar beats (Schmidt and Kamp, 2004). This swimming pattern varies from species to species, and for boar spermatozoa the thresholds have been related to VCL, ALH, LIN and WOB (Schmidt and Kamp, 2004). Sperm hyperactivity is reported to be important for fertilization of the oocyte, but it is a highly ATP-consuming process. If initiated too early, hyperactivity pose a risk of depleting the energy store of the sperm cells before they

reach the oocyte for fertilization (Mortimer et al., 1997; Suarez and Ho, 2003). Therefore, sperm motility parameters and ATP content in semen were analysed in this study for further evaluation of the relationship between hyperactive motility and ATP levels.

The fertility of liquid preserved spermatozoa declines gradually during storage, and differences in sperm storage capacity between individual boars has been reported (Waberski et al., 2011). In Norway, the liquid diluted semen used in pig production is recommended to be used within 96 hours after collection (Norsvin, 2017). However, several farmers ordering semen for insemination are not able to use the doses until two or more days after collection, due to factors like large distances and long shipment time. Therefore, the sperm storage capacity is also essential in pig production.

The aim of this study was to investigate specific sperm motility characteristics and ATP levels in ejaculates from Norwegian Landrace (NL) and Norwegian Duroc (ND) boars, and to evaluate the possible effect of these parameters on field fertility measured as total number of piglets born (TNB). In addition, breed differences in semen storage abilities were investigated.

2. Material and methods

2.1. Animals, collection and processing of semen

This study was based on semen collected at the AI station run by Norsvin at Hamar, Norway, between February 21st, 2014 and March 20th, 2015. Ejaculates from 103 purebred NL boars (n=239) and 88 purebred ND boars (n=179) were included in the study. The boars were housed in individual 6 m^2 pens, fed a standard commercial diet and had access to straw and sawdust as rooting materials. All animals were cared for according to laws, internationally recognized guidelines and regulations for keeping pigs in Norway (The

Animal Protection Act of December 20th, 1974, the Animal Welfare Act of June 19th, 2009 and the Regulations for keeping of pigs in Norway of February 18th, 2003). All boars were routinely used for AI. The age of the boars at semen collection for sampling ranged from 241 to 1041 days (median age = 338 days).

The sperm-rich fraction of the ejaculates was collected using the gloved hand technique. At the AI station, motility and morphology were subjectively evaluated using phase contrast microscopy (Leica DM 4000B, Leica Microsystems, Wetzlar, Germany) at 37°C, and ejaculates with <70% motile and/or >20% morphologically abnormal spermatozoa were discarded. The total concentration of sperm cells was assessed by NucleoCounter® SP-100TM (Chemotec, Denmark). Ejaculates approved by the quality check were diluted to achieve a concentration of $25x10^6$ cells/mL in Androstar® Plus extender (Minitube, 84184 Tiefenbach, Germany), transferred to airtight tubes containing doses of 89 mL, and stored at 18°C until shipment. Only semen accepted for AI was used in this study. All doses were marked with donor ID, breed, and the latest day of recommended use, which is the 4th day after collection. Customers had their doses delivered either by overnight mail, including domestic and international air transport, courier cars or buses, or they picked them up themselves at a drop point near the AI centre. During the 15 minutes long transportation from the AI station to the laboratory, the samples were packed in a styrofoam box to ensure a stable temperature. At the laboratory, semen was transferred to 15 mL falcon tubes and the samples were taken for CASA and ATP analysis at the day of collection (Day 0) and after storage at 18°C for 96 hours (Day 4). In the current study, each boar has given from one to five ejaculates (Table 1) and different ejaculates from the same boars have been treated as separate samples.

2.2. Assessment of sperm motility

Sperm motility analysis was performed using Sperm Vision CASA system (SpermVision, Minitube GmbH,, Tiefenbach,Germany), with Leja-4 standardized counting chambers (Leja products, Nieuw-Vennep, the Netherlands) and analysed using a phase contrast microscope (Axio Lab.A1, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with Basler avA1000-120km 1024 x 1024 pixels digital camera (Basler Vision Technologies, Ahrensburg, Germany). The Sperm Vision and the Leja-4 slides were pre-warmed at 38°C. Boar semen, diluted in Androstar® Plus extender, as described above, was incubated at 38°C for 10 min prior to CASA analysis. The capillary flow chambers of the Leja counting slides were filled with 3 µL pre-warmed semen. Samples were single analysed (one chamber filled, one sample analyzed) at the day of collection and after 4 days of storage at 18°C, each in two parallels. Analysis was performed on eight microscope fields with a total of at least 500 cells analysed per sample. Mean of the eight fields was used for statistical analysis. The motility parameters measured were total motility, progressive motility, hyperactive motility, average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and average orientation change of the head (AOC). The manufacturer's microscope settings for boar semen were used with sperm cell detection based on head area (35 μm^2-100 μm^2), 60 Hz frame rate and 30 frames captured per object. Sperm cells were defined as motile if AOC>7º (manufacturer's default setting for boar semen). In addition, motile cells with VSL<10 µm/s were defined as locally motile and cells with VSL>10 µm/s as progressive motile. The criteria for hyperactive motility for each single sperm cell track were VCL>97 µm/s, ALH>3.5 µm, LIN<32% and WOB<71% (Schmidt and Kamp, 2004).

2.3. Assessment of ATP levels in semen

The ATP content in semen was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Technical Bulletin, Promega, 2012). Repeated evaluation tests were performed to determine the optimal sperm cell number for the analysis. The standard curve was generated from ATP disodium salt hydrate (Sigma, A7699-1G) by dilution in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4, 8.1 mM Na_2HPO_4 x $2H_2O$, pH 7.4.). Boar semen, diluted in Androstar® Plus extender, as described above, was diluted 1:10 in PBS, and 50 µL was transferred to a white 96-well microtiter plate (NUNC™, Denmark). Subsequently 50 µL CellTiter-Glo® Reagent was added to each well and the mixture was gently shaken for 2 min in a rotary shaker (IKA® MS 3 digital, USA) to induce cell lysis. After further 15 min incubation at room temperature, bioluminescence measurement was performed using FLUOstar OPTIMA multiwell plate reader (BMG LABTECH GmbH, Offenburg, Germany) with MARS data analysis software (Version 1.10, BMG LABTECH, Germany). Samples were analysed at the day of collection and after 4 days of storage at 18°C, each in three parallels. The bioluminescence value for each sample, measured in relative luminescence units (RLU), was converted to the corresponding ATP value in nM using the standard curve values. The average of the three parallels was used for statistical analysis.

2.4. Fertility records

Insemination dates of gilts and sows with doses from any given ejaculates were collected through the national litter recording system "ingris" (https://ingris.animalia.no/IngrisWeb) and semen storage time was determined based on semen collection date of each individual boar. Only herds that were situated geographically close enough for courier car or self-service would be able to use doses at the day of semen collection. Insemination records indicating

that doses had been used more than 4 days post collection were omitted from the data set. The litter records were included in the dataset provided they matched the semen collection dates at which the CASA and ATP analyses were performed. In addition, only litter results from purebred litters were included in the dataset. The total number piglets born (TNB) for each litter was calculated as the sum of liveborn and stillborn piglets and mummified foetuses. Litter records with zero TNB or with >29 TNB were deleted from the dataset collected from ingris. Among the females that farrowed between 109 and 125 days after the latest insemination date, only 6.3% and 6.0% of the preceding inseminations in NL and ND respectively, were performed at the day of semen collection. In contrast, 24.9% and 28.3% in NL and ND respectively, were performed at the recommended last day of usage. The semen samples collected and analysed in this study resulted in 677 NL and 166 ND purebred litters.

2.5. Statistical analyses

The analyses of TNB were divided into Day 0 and Day 4 within breed due to estimate effects affecting TNB at the day of collection (Day 0) and after storage at 18°C for 96 hours (Day 4). The difference between the two breeds were large (13.8 and 8.9 for NL and ND, respectively) and therefore the two breeds were separated into different analyses.

The statistical analyses were performed using the software package Statistical Analysis Software (SAS) version 9.4 for Microsoft Windows (SAS Institute Inc., Cary, NC, USA). The CASA and ATP data were normally distributed (tested by Shapiro-Wilk test), and the results were analysed using paired t-test for testing the storage (Day 0 and Day 4) and unpaired t-test for testing the breeds (NL vs ND). Correlations (Pearson) were tested using PROC CORR. The results were considered statistically significant when $p<0.05$. Boxplots were made using RStudio version 3.4.0 (RStudio, Inc., 250 Northern Avenue Suite 420, Boston, Massachusetts

02210, US). The possible effects on TNB were analysed by the General Linear Model (GLM) procedure. First, the type of insemination (single/double), age of the boar, parity (divided into three classes; 1, 2 and >2), herd number, the storage time of the semen when inseminated (0-4 days), season (divided into four classes based on the four seasons in Norway (winter (December, January, February), spring (March, April, May), summer (June, July, August) and autumn (September, October, November)), batch number (a measure for the arrival of the boars to the AI station, meaning that every group wise intake of boars is given a specific number) and interval class (the interval in days since previous collection from each boar (<4, 4, >4)) were tested in the model. Further, models were constructed by including the semen quality parameters, measured at the Day 0 and Day 4, with a significant effect on TNB upon a correlation analysis (Table 4). Following this, a backwards selection approach was used where all the variables of interest were fitted into a model. The variable with the highest p-value was excluded, if the variable had no significant effect. This re-fitting of the model was continued until all the variables were statistically significant ($p<0.1$). The final models were as followed:

Model 1) NL, Day 0: TNB = herd + LIN

Model 2) NL, Day 4: TNB = herd + WOB

Model 3) ND, Day 0: TNB = parity + batch number + % motile cells + VCL + ALH

Model 4) ND, Day 4: TNB = parity + batch number + LIN

3. Results

3.1. Assessment of sperm motility and ATP levels in semen

A significant effect of storage (Day 0 and Day 4) and breed (NL and ND) were found for both ATP-levels and CASA-parameters. The mean values (± SD) of the kinematic sperm motility parameters (VAP, VCL, VSL, STR, LIN, WOB, ALH and BCF) obtained from CASA in the two breeds are shown in Table 2 and Table 3.

For NL boars (Table 2), there were significant differences between Day 0 and Day 4 in all CASA parameters, except percentage of progressive cells ($p=0.63$). The p-values for the significant differences were <0.001, except from the difference in percentage motile cells, in which it was <0.01. The change in ATP levels in semen was also found significantly different between Day 0 and Day 4 ($p<0.0001$) (Figure 1). The ND boars showed a slightly different pattern in differences between Day 0 and Day 4. Percentage motile cells, percentage progressive cells, VAP, VCL, VSL, ALH and ATP level were significantly different from Day 0 to Day 4 ($p<0.001$), while the rest of the parameters were not significantly different (Table 3 and Figure 1).

All the CASA parameters and ATP measurements were significantly different between the two breeds ($p<0.0001$) with two exceptions: VSL ($p=0.09$) and WOB ($p=0.09$) at Day 4. Due to the statistically significant difference between the breeds, all the further calculations and interpretations have been performed for each breed separately. Also within breeds there were individual differences in the ejaculates as shown in Figure 1.

To estimate the correlations, the motility parameters analysed by CASA were aligned with the levels of ATP in the semen samples to evaluate any correlations. Both motility and progressive motility showed a significantly positive small correlation to the levels of ATP at Day 0 and Day 4 in both breeds (range correlation coefficient: 0.09 - 0.20). Thus, the higher

the levels of ATP measured in the semen, the higher the percentage of motile cells and progressive cells, as shown in Figure 2. In addition, VSL at Day 0 and WOB and BCF at Day 4 showed a positive small correlation to the levels of ATP in NL (range correlation coefficient: 0.12-0.17, $p<0.05$). For ND, the parameters VSL, STR, LIN, WOB and BCF at Day 0 and VSL, STR, LIN and WOB at Day 4 were found to have a positive small correlation to the levels of ATP (range correlation coefficient: 0.15-0.26, $p<0.05$) (data not shown).

3.2. Field fertility, CASA parameters and ATP levels

The total number of piglets born per litter (TNB) in the farms, using commercial semen doses at 0 to 4 days post collection of ejaculates sampled for the CASA and ATP measurements, constitute the field fertility in this analysis. The mean of TNB in NL was significantly higher compared to the mean of TNB in ND ($p<0.0001$). The average TNB per litter was 13.8 (SD=3.49) in NL and 9.0 (SD=3.01) in ND. The minimum and maximum TNB were 2 and 24, and 1 and 16 for NL and ND, respectively.

The Pearson correlation and p-values between the CASA parameters and ATP levels measured and TNB were calculated (Table 4). Significant correlations were found for several of the parameters, however the correlations were found to be week (range correlation coefficient: -0.065 to -0.17 and 0.077 to 0.20, for NL and ND, respectively).

For further evaluating the effects of the semen quality parameters on TNB, separate GLM for each breed were constructed. The correlation analysis indicated that the ATP level in NL at Day 0 was weakly correlated to TNB, and therefore it was tested in the GLM. However, the effect of ATP was not found significant in the model and was therefore excluded.

The CASA parameter LIN had an effect on TNB in NL semen used at day 0 and were included in the model 1. In model 2, the CASA parameter WOB was included, in model 3 the CASA parameter motile sperm cells (%), VCL and ALH was included and in model 4 the CASA parameter LIN was included.

Both, the NL model at Day 0 (model 1) and at Day 4 (model 2) explained each 19% of the variability in TNB. While for ND, the Day 0 model (model 3) and Day 4 model (model 4) explained 26% and 23% of the variability in TNB, respectively. The p-values are listed in Tables 5-8.

4. Discussion

The aim of the present study was to investigate sperm motility parameters and ATP levels in boar semen from two breeds, NL and ND, and to evaluate their possible effect on field fertility in terms of TNB. Using CASA for measurement of sperm parameters is a huge advantage compared to the traditional subjective methods of microscopically assessment of sperm motility. These subjective methods have been successful in terms of separating out the samples with poor motility. The CASA instrument, on the other hand, gives an objective and more repeatable count of the number of motile sperm cells in a sample, as well as measuring several other parameters. This will not only separate out the poor samples, but can also be a useful tool in predicting the very best boars to be used in AI based on fertility parameters. In this study, there were statistically significant differences both in the sperm motility parameters measured by CASA and ATP levels in semen between the two breeds. There were also differences between the breeds in which prediction parameters that influenced on TNB. In addition, significant differences were detected between Day 0 and Day 4 within the breeds.

The largest difference between NL and ND was the percentage of hyperactive sperm cells. On average, ND had a higher percentage of hyperactive sperm cells at Day 0, but the

difference between Day 0 and Day 4 was not statistically significant. In contrast, the percentage of hyperactive sperm cells in NL was much lower, compared to ND, and increased significantly after storage (6.3% increase). The breed differences have been reported previously, in terms of differences in sperm physiology and semen plasma composition. For example, differences in the composition of fatty acids in the sperm cell membrane, capacitation and ability for cryopreservation have been shown (Waterhouse et al., 2004; Waterhouse et al., 2006). Furthermore, differences in sperm shape and dimensions among pig breeds, including Landrace and Duroc, have been reported (Saravia et al., 2007) and it has been shown that small differences in head size and morphology can result in large differences in sperm hydrodynamics and thereby motility parameters (Dresdner and Katz, 1981). Breed differences in semen volume, number of total sperm and number of viable sperm has also been detected (Smital et al., 2004). It has been reported that Duroc boars have a higher sperm cell concentration as well as a lower semen volume compared to Landrace (Smital, 2009). Thus, the spermatozoa of Duroc boars have reduced volume of seminal plasma per cell compared to Landrace. Plasma proteins in the seminal plasma have several roles and effects (Flowers et al., 2016). For example, one of the groups of plasma proteins found in seminal plasma, spermadhesins, bind to the acrosome and act to preserve membrane integrity, motility and mitochondrial activity (Gonzalez-Cadavid et al., 2014). These effects will possibly be lower in Duroc semen as the ratio between seminal plasma and spermatozoa is lower, compared to Landrace semen.

The ATP levels in the semen correlated to several of the motility parameters in sperm cells, including motility and progressive motility at Day 0 and Day 4 in both breeds. The correlation showed that the higher the level of ATP in the semen, the higher percentage of motile cells and progressive cells. The connection between ATP and motility in boar sperm cells has previously been explained by the amount of calcium in the sperm cells, which affects

the motility through regulation of the ATP concentration in the cell (Li et al., 2016). Previous studies have reported that loss of motility may be due to loss of ATP production in the cells and a decrease in ATP has been observed upon storage in various media (Jones and Bubb, 2000; Fraser et al., 2001; Gogol et al., 2009). This supports the results in the current study indicating that the decrease in the number of motile cells could be related to the significant decrease in ATP levels upon storage.

It has been reported that hyperactive motility requires ATP to obtain the vigorous swimming pattern (Suarez and Ho, 2003; Li et al., 2016). Therefore, in the current study it was of interest to evaluate a possible correlation between the percentage of hyperactivated sperm cells and the level of ATP in semen. However, only a non-significant positive trend was observed. A reason for not detecting a correlation might be that the cells are continuously synthesising and using ATP (Medrano et al., 2006). A previous study reported that even though the percentage of motile sperm cells decreased upon short-time storage, the sperm cells may still be able to maintain the potential to obtain a hyperactive motility (Henning et al., 2014).. In addition, in the current study the ATP measurements were performed in semen including both intracellular and extracellular ATP. The lack of relationship between the ATP content and the percentage of hyperactive cells observed in this study could therefore be concealed by a high level of extracellular ATP. However, previous studies have reported that the ATP content in seminal plasma is negligible (Long and Guthrie, 2006).

Significant differences in most of the motility parameters and the semen ATP levels were observed between Day 0 and Day 4. The difference between Day 0 and Day 4 is important since semen rarely is used at the day of production. Semen doses are transported over large distances and will in most cases not be available until the day after collection. The general recommendation is to use the doses within 96 hours, which was the reason for using this test-point (Day 4) for semen quality analysis in the current study. However, some farmers use AI

doses older than the recommended 96 hours. Individual variation in boar semen storage capacity has been shown both by an *in vitro* study evaluating long-term semen-extenders (Waterhouse et al., 2004) and by an *in vivo* study using a short-term semen-extender (Haugan et al., 2005). Therefore, we tested the effect of age of semen on TNB, but no significant effect was obtained. In addition, the magnitude of CASA parameter-changes between Day 0 and Day 4 had no effect on TNB.

The percentage of motile cells has previously been reported to have an effect on TNB (Vyt et al., 2008; Broekhuijse et al., 2012). In this study, a small positive effect of the percentage motile cells on TNB was found. However, this effect was only significant in ND at Day 0. In addition, the CASA parameters VCL and ALH were found to influence on TNB in ND at Day 0 with a negative and positive effect, respectively. In NL at Day 0, the CASA parameter LIN had a significant positive effect on TNB. In addition, this parameter was found to have a significant positive effect in ND at Day 4. For NL at Day 4 none of the above mentioned CASA parameters influenced TNB, however the results showed that WOB had a significant positive effect on TNB. This indicates that there are differences in the motility patterns in NL and ND that affect TNB. In addition, it indicates that the specific sperm motion parameters analysed by CASA are related to TNB.

Altogether, our results show that spermatozoa in ejaculates from NL boars adopt a less straight forward pattern upon 96 hours storage expressed by increased VCL and ALH values, and decreased LIN and WOB values. This has an unfavourable effect on TNB and therefore we suggest that, the AI station should consider the LIN and WOB values in ejaculates from NL boars, in addition to general motility evaluation, for decision of approval or rejection of ejaculates. For ND, lower LIN values and higher VCL and ALH values compared to NL indicates that the ND spermatozoa are in transition to “hyperactive-like” motility already at the day of semen collection. In addition, our results indicate that a more straightforward

motility pattern has a positive effect on TNB. Thus, in addition to motility in general, the AI station should consider the values for LIN, VCL and ALH ejaculates from ND boars, for decision of approval or rejection of ejaculates. Altogether, the parameters VCL, ALH, LIN and WOB showed a significant effect on TNB in one or more of the models and all these parameters are among the threshold values defining the swimming pattern of hyperactive boar spermatozoa (Schmidt and Kamp, 2004). This possible link between hyperactive motility and TNB in pigs has to our knowledge, not been reported earlier.

In conclusion, the current study showed that there are differences between the NL and ND breeds in terms of the CASA parameters and their effect on TNB. Although motility is the most widely used sperm quality parameter at AI stations, results in the present study indicate that, surprisingly, only in the ND ejaculates motility at Day 0 were associated to TNB. However, several of the CASA parameters with threshold values defining hyperactive motility were found to be associated with TNB in both breeds. Therefore, it is suggested that these parameters should be taken into consideration when evaluating the fertility potential and approval of the ejaculates used for AI.

Conflict of interest

The authors have no conflicts of interest to declare.

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Table 1

Number of ejaculates per boar in the breeds Norwegian Landrace (NL) and Norwegian Duroc (ND).

No. of ejaculates per boar	1	2	3	4	5
NL (103 boars)	27	33	31	7	5
ND (88 boars)	33	27	22	4	2

Table 2

Means (± SD), minimum and maximum values for the kinematic sperm motility parameters in ejaculates from 103 Norwegian Landrace boars (n=239) at the day of collection (Day 0) and after 96 hours of storage (Day 4).

	Day 0			Day 4		
Variable	**Mean ± SD**	**Min**	**Max**	**Mean ± SD**	**Min**	**Max**
VAP (µm/s)	50.32 ± 6.11	35.09	71.83	54.53 ± 7.01	39.48	74.25
VCL (µm/s)	99.38 ± 15.00	66.83	154.58	114.14 ± 16.86	77.69	171.42
VSL (µm/s)	41.33 ± 4.52	29.57	57.14	42.57 ± 4.60	32.65	57.02
STR (%)	82.06 ± 3.24	73.00	89.00	78.09 ± 3.92	64.00	86.00
LIN (%)	41.73 ± 3.72	32.00	51.00	37.39 ± 3.10	28.00	46.00
WOB (%)	50.64 ± 2.72	44.00	58.00	47.66 ± 1.89	43.00	54.00
ALH (µm)	2.67 ± 0.48	1.80	4.43	3.24 ± 0.58	2.04	5.32
BCF (Hz)	32.07 ± 2.54	26.68	40.88	30.21 ± 2.82	23.13	37.69

Table 3

Means (± SD), minimum and maximum values for the kinematic sperm motility parameters in ejaculates from 88 Norwegian Duroc boars (n=179) measured at the day of collection (Day 0) and after 96 hours of storage (Day 4).

	Day 0			Day 4		
Variable	**Mean ± SD**	**Min**	**Max**	**Mean ± SD**	**Min**	**Max**
VAP (µm/s)	54.37 ± 6.32	38.14	67.17	57.14 ± 5.73	40.21	72.68
VCL (µm/s)	114.48 ± 15.50	76.57	146.10	120.29 ± 13.98	81.57	156.65
VSL (µm/s)	39.80 ± 3.94	29.72	49.34	41.85 ± 3.70	30.25	51.52
STR (%)	73.20 ± 3.68	64.00	84.00	73.21 ± 4.72	62.00	84.00
LIN %)	34.78 ± 2.95	25.00	44.00	34.80 ± 3.49	28.00	44.00
WOB (%)	47.40 ± 1.86	4.00	53.00	47.34 ± 1.95	41.00	52.00
ALH (µm)	3.80 ± 0.54	2.55	5.04	4.02 ± 0.60	2.34	5.51
BCF (Hz)	26.47 ± 1.88	22.74	31.55	26.28 ± 2.65	19.92	32.72

Table 4

The Pearson correlation coefficient (corr) and p-values for the correlations between the total number of piglets born (TNB), ATP and CASA parameters for Norwegian Landrace and Norwegian Duroc at the day of collection (Day 0) and after 96 hours of liquid storage (Day 4).

	NORWEGIAN LANDRACE				NORWEGIAN DUROC			
	Day 0		Day 4		Day 0		Day 4	
Variable	**Corr**	**p-value**	**Corr**	**p-value**	**Corr**	**p-value**	**Corr**	**p-value**
ATP (nM)	-0.075	0.050[a]	-0.020	0.61	0.12	0.11	0.068	0.39
Motile (%)	0.049	0.20	0.082	0.034[a]	0.20	0.0089[a,b]	0.18	0.017[a]
Progressive (%)	0.078	0.043[a]	0.091	0.017[a]	0.16	0.044[a]	0.17	0.029[a]
Hyperactivated (%)	0.060	0.12	0.082	0.033[a]	-0.096	0.22	-0.13	0.11
VAP (µm/s)	0.033	0.39	0.050	0.19	-0.15	0.048[a]	-0.099	0.20
VCL (µm/s)	-0.030	0.44	0.019	0.62	-0.18	0.022[a,b]	-0.16	0.046[a]
VSL (µm/s)	-0.071	0.064[a]	-0.011	0.77	-0.11	0.16	0.010	0.90
STR (%)	0.013	0.73	0.044	0.25	0.16	0.036[a]	0.17	0.031[a]
LIN (%)	0.11	0.0049[a,b]	0.044	0.25	0.17	0.32[a]	0.19	0.012[a,b]
WOB (%)	0.13	0.001a	0.077	0.045[a,b]	0.15	0.060[a]	0.20	0.011[a]
ALH (µm)	0.12	0.0014[a]	0.11	0.0052[a]	-0.15	0.047[a,b]	-0.17	0.027[a]
BCF (Hz)	-0.065	0.092[a]	-0.023	0.55	0.070	0.34	0.16	0.044[a]

Significance of correlation ($p<0.1$) is indicated by [a] and parameters with a significant effect ($p<0.1$) in general linear models are indicated by [b].

Table 5

The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (Pr>F) for the parameters with significant effect in Norwegian Landrace at Day 0 on the total number of piglets born.

Source	**DF**	**SS**	**Mean Square**	**F Value**	**Pr > F**
Herd	61	1392.58	22.83	2.09	<.0001
LIN	1	117.22	117.22	10.73	0.0011

Note: R-square for the model is 0.19.

Table 6

The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (Pr>F) for the parameters with significant effect in Norwegian Landrace at Day 4 on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	Pr > F
Herd	61	1392.58	22.83	2.09	<.0001
WOB	1	69.48	69.48	6.32	0.012

Note: R-square for the model is 0.18.

Table 7

The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (Pr>F) for the parameters with significant effect in Norwegian Duroc at Day 0 on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	Pr > F
Parity	2	81.76	40.88	5.28	0.0061
Batch number	14	192.83	13.77	1.78	0.047
Motile sperm cells (%)	1	41.60	41.60	5.37	0.022
VCL	1	63.45	63.45	8.19	0.0048
ALH	1	34.45	34.45	4.45	0.037

Note: R-square for the model is 0.26.

Table 8

The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (Pr>F) for the parameters with significant effect in Norwegian Duroc at Day 4 on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	Pr > F
Parity	2	82.23	41.11	5.19	0.0066
Batch number	14	226.35	16.17	2.04	0.018
LIN	1	53.22	53.22	6.72	0.011

Note: R-square for the model is 0.23.

Figure legends:

Fig. 1. The percentage of motile spermatozoa, progressive spermatozoa, hyperactive spermatozoa and levels of ATP measured in boars from two breeds, Norwegian Landrace (NL) and Norwegian Duroc (ND), at the day of collection (Day 0) and after 96 hours of storage (Day 4). Significance levels are indicated: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Fig. 2. The relationship between measured ATP levels in semen and motility parameters measured by computer assisted semen analysis (CASA) in boar from two breeds, Norwegian Landrace (NL) and Norwegian Duroc (ND) at the day of collection (Day 0) (A, C and E) and after 96 hours of storage (Day 4) (B, D, and F).

Figure 1

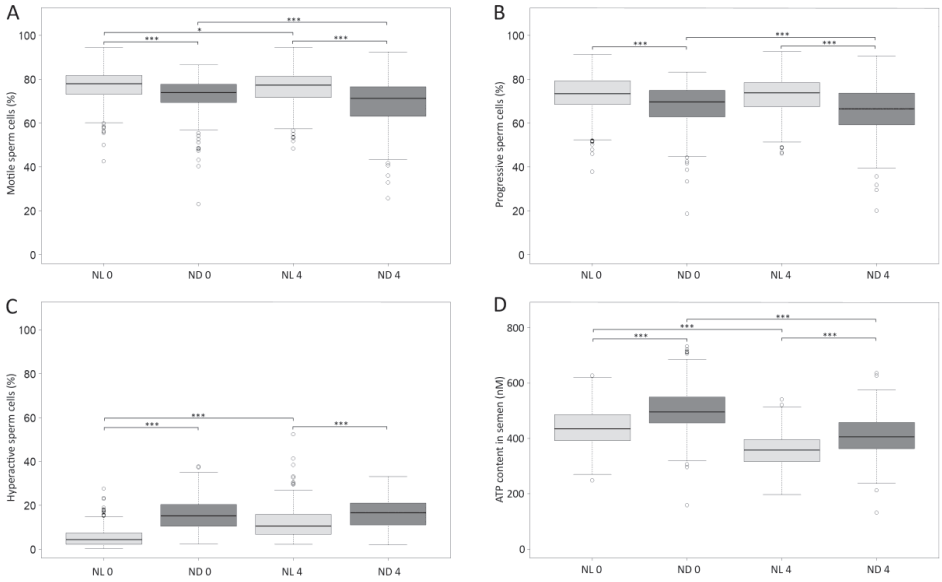
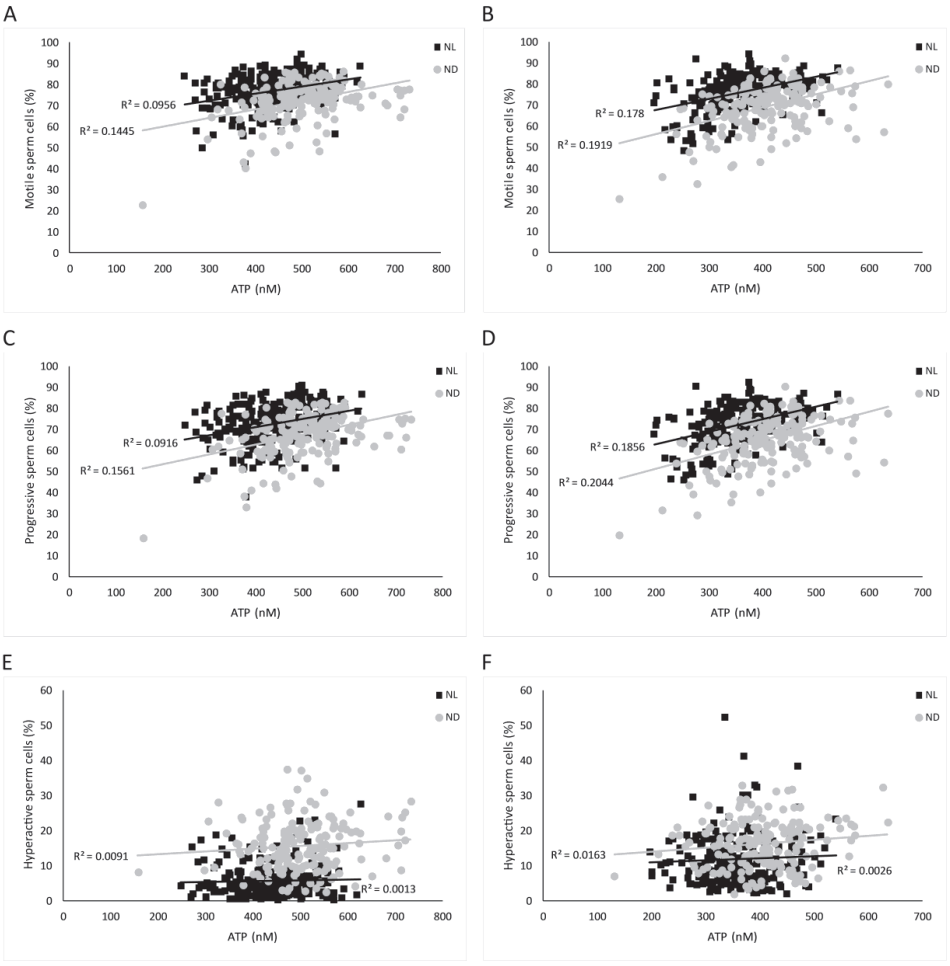

A
Motile sperm cells (%)
100
80
60
40
20
0
***
*
***
***
NL 0
ND 0
NL 4
ND 4
B
Progressive sperm cells (%)
100
80
60
40
20
0
***
***
***
NL 0
ND 0
NL 4
ND 4
C
Hyperactive sperm cells (%)
100
80
60
40
20
0
***
***
***
NL 0
ND 0
NL 4
ND 4
D
ATP content in semen (nM)
800
600
400
200
0
***
***
***
***
NL 0
ND 0
NL 4
ND 4

Figure 2


A
Motile sperm cells (%)
ATP (nM)
NL
ND
R² = 0.0956
R² = 0.1445
B
Motile sperm cells (%)
ATP (nM)
NL
ND
R² = 0.178
R² = 0.1919
C
Progressive sperm cells (%)
ATP (nM)
NL
ND
R² = 0.0916
R² = 0.1561
D
Progressive sperm cells (%)
ATP (nM)
NL
ND
R² = 0.1856
R² = 0.2044
E
Hyperactive sperm cells (%)
ATP (nM)
NL
ND
R² = 0.0091
R² = 0.0013
F
Hyperactive sperm cells (%)
ATP (nM)
NL
ND
R² = 0.0163
R² = 0.0026

Paper II

Sperm DNA integrity in Landrace and Duroc boar semen and its relationship to litter size

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Contents

The routine procedures for evaluation of sperm quality at AI stations show a limited relationship to field fertility. The sperm chromatin structure assay (SCSA) is a quality evaluation method for assessment of sperm DNA fragmentation, reported to be negatively related to field fertility in several mammal species. This method calculates a DNA fragmentation index (DFI) whose high values indicate abnormal chromatin structure. The aim of this study was to assess sperm DNA fragmentation in stored liquid extended semen from two different pig breeds, Norwegian Landrace (NL) and Norwegian Duroc (ND) and to evaluate the influence on field fertility measured as total number of piglets born (TNB). There was a significantly higher median DFI in ejaculates from ND boars compared to NL boars. For either breed herd, semen storage time, semen collection month as well as DFI showed significant effects on TNB. DFI was negatively correlated to TNB in both breeds. The boars with the 5% lowest TNB had a mean DFI of 2.77 and 2.08 in NL and ND, respectively, compared to 1.32 and 1.18 for the boars with the 5% highest TNB. This indicates the importance of DNA integrity in boar sperm cells for fertility in pigs.

Keywords: Boar, Semen analysis, DNA integrity, Sperm chromatin structure assay, Fertility

1. INTRODUCTION

Examination of sperm concentration, motility and morphology are routine procedures for semen quality evaluation at AI stations (Foxcroft, Dyck, Ruiz-Sanchez, Novak, & Dixon, 2008). However, *in vivo* effect of these standard semen parameters can be masked by high sperm number in the semen dose, which can explain why they only to a limited extent are directly related to field fertility. Therefore, improved quality assessment methods have been developed such as the sperm chromatin structure assay (SCSA) for assessment of sperm DNA fragmentation (Evenson & Jost, 1994, 2000).

During spermatogenesis, the sperm chromatin is condensed by protamines to a highly compact structure (Rathke, Baarends, Awe, & Renkawitz-Pohl, 2014). This protects the sperm genetic material against damage during transport through the male and female reproductive tracts. Defects in sperm chromatin packaging are associated with DNA damage which can i.e. be caused by germ cell apoptosis in the testis, incomplete epididymal sperm maturation or oxidative stress (Aitken, Bronson, Smith, & De Iuliis, 2013). Sperm DNA integrity is protected by DNA repair mechanisms during spermatogenesis (Gonzalez-Marin, Gosalvez, & Roy, 2012). However, upon spermatogenesis, sperm cells lack mechanisms to repair DNA damage and thus the compact chromatin structure is essential for its protection.

DNA damaged spermatozoa can fertilize oocytes, and upon fertilization, oocytes and early embryos can repair some types of DNA breakage (Gonzalez-Marin et al., 2012). However, the extent of this repair is associated with the level and type of DNA damage and the repair capacity of the oocyte (Wdowiak, Bakalczuk, & Bakalczuk, 2015). Both in human (Wdowiak et al., 2015) and bovine (Fatehi et al., 2006), low DNA integrity has been reported to negatively affect embryo development and it is related to early embryonic mortality.

The SCSA is a flow cytometry method assessing sperm DNA fragmentation (Evenson & Jost, 1994, 2000). The assay evaluates the susceptibility of sperm DNA to denaturation *in*

situ under acidic conditions. It utilizes the metachromatic properties of acridine orange (AO), which fluoresces green when bound to double stranded DNA (dsDNA), and red when bound to single stranded DNA (ssDNA). Flow cytometer results are used to calculate a DNA fragmentation index (DFI) for each spermatozoon. High DFI values are indicative of abnormal chromatin structure (Evenson & Jost, 2000). Accumulating evidence suggests that increased levels of DFI are negatively related to field fertility (Evenson, 2016; Love & Kenney, 1998; Waterhouse et al., 2006). For example in swine, sperm DNA fragmentation is reported to influence the total number of piglets born (Boe-Hansen, Christensen, Vibjerg, Nielsen, & Hedeboe, 2008; Broekhuijse, Sostaric, Feitsma, & Gadella, 2012; Didion, Kasperson, Wixon, & Evenson, 2009). Therefore, the level of DNA integrity may be a promising parameter for selecting and ranking boars within AI stations with regards to potential litter size.

The aim of the present study was to assess sperm DNA fragmentation in stored liquid extended semen from two different pig breeds, Norwegian Landrace (NL) and Norwegian Duroc (ND) and to evaluate the influence on field fertility measured as total number of piglets born.

2. MATERIALS AND METHODS

2.1. Animals, semen collection and preparation

Semen was collected from 451 purebred Norwegian Landrace (NL) and 475 Norwegian Duroc (ND) boars routinely used for artificial inseminations (AI), located at the AI station run by Norsvin at Hamar, Norway. The age of the boars at the day of sampling ranged from 221-1000 days (mean = 312.3 days, SD = 86.7) for NL and from 228-829 days (mean = 297.3 days, SD = 62.5) for ND. All animals were cared for according to internationally recognized guidelines and regulations for keeping pigs in Norway (The Animal Welfare Act of June 19th,

2009 and the Regulations for keeping of pigs in Norway of February 18th, 2003). During the collection period, a total of 1345 samples (NL: n=695; ND: n=650) were analyzed upon storage. In addition, 75 of these samples (NL: n=49; ND: n=26) were analyzed at the day of collection.

The sperm-rich fraction of the ejaculates was collected with the "gloved hand" technique, during a period from March 2010 to June 2017. Upon collected, the samples were diluted in the commercial extender Tri-X-cell® (IMV technologies, L'Aigle, France) to a concentration of $28x10^6$ spermatozoa/mL, according to the normal routines of the AI center. However, from July 2013 the extender used was Androstar Plus® (Minitüb GmBH, Tiefenbach, Germany).

Upon semen collection, motility and morphology were evaluated at the AI station. Ejaculates with <70% motile and/or >20% morphologically abnormal spermatozoa were discarded and only semen accepted for AI was included in this study. Tubes with diluted semen (89 mL) were stored at 18°C until shipment. Regular single-sire semen doses were shipped to commercial swine producers for use within the next four days after collection date. Samples for analysis of DNA fragmentation were transported from the AI station to the laboratory. At the laboratory, semen aliquots were snap-frozen and stored at -80°C until analyzed for DNA fragmentation. Aliquots from semen samples collected during the period from March 2010 to February 2011 were snap-frozen at the day of collection (Day 0) and after storage at 18°C for 96 hours (Day 4). These samples were included in an initial study to evaluate the effect of liquid semen storage on sperm DNA fragmentation. Later, the samples were only snap-frozen at -80°C upon storage for 48, 72 or 96 hours depending on the weekday the collection was performed.

2.2. Sperm chromatin structure assay

The SCSA protocol was performed according to the procedure described by (Evenson & Jost, 2001) and later modified by Boe-Hansen (Boe-Hansen, Ersboll, Greve, & Christensen, 2005). For sample analysis, a Cell Lab QuantaTM SC MPL flow cytometer equipped with an argon laser with excitation at 488 nm and 22 mW power (Beckman Coulter, Fullerton, CA, USA) was used. Snap-frozen semen samples were thawed in water bath at 37°C and diluted to $2x10^6$ sperm cells/mL in TNE buffer (10 mM Tris-HCL, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final volume of 200 µL. Thereafter, 400 µL acid detergent solution (0.38 M NaCl, 80 mM HCL, 0.1 % (w/v) Triton X-100, pH 1.2) was added to the sample. A stopwatch was started and upon exactly 30 seconds incubation, 1.2 mL AO staining solution (6 µg/mL AO (A3568, Life Technologies, OR, USA) in a buffer containing 37 mM citric acid, 0.126 M Na_2HPO4, 1.1 µM EDTA, 0.15 M NaCl, pH 6) was added. Further, the sample was placed in the flow cytometer and run in setup mode for 3 minutes. Then the data acquisition started and 5000 events were collected for each sample. Signals were first separated by 550 nm dichroic long pass mirror. Subsequently, fluorescence was collected through a 525 nm band pass filter (green) and a 670 nm long pass filter (red). Prior to sample analysis, the flow cytometry instrument was AO saturated by running AO equilibration solution (1.2 mL AO staining solution and 400 µL acid detergent solution) through the system for 5 minutes. At the start and after analyzing every fifth sample, mean green and red fluorescence signals were set to 425±5 and 125±5, respectively, using reference boar semen of known DFI to control laser stability. The percentage of red (ssDNA) and green (dsDNA) fluorescence was determined using Cell Lab QuantaTM SC MPL Analysis software (Beckman Coulter, Software Version 1.0 A). Based on the fluorescence ratio red/(red + green), percentage DFI was calculated and mean of two parallels was used for further statistical analysis.

2.3. Fertility records

Insemination data from gilts and sows were retrieved from “Ingris”, the national litter recording system. Type of insemination as an effect in the models was classified as either single or double with the same sire. For double insemination dates, the latest insemination date was used as the valid date. Storage time of semen doses before use was determined based on corresponding semen collection and insemination dates of any given boar. Only herds situated geographically close enough for courier car or self-service would be able to use doses at the day of semen collection. Insemination records indicating that doses had been used later than the fifth day post collection were omitted from the data set. Only purebred litters were included in the dataset (gestation lengths between 109 and 125 days). The total number of piglets born (TNB) for each litter was calculated as the sum of liveborn and stillborn piglets and mummified foetuses. Litter records with zero TNB or with >29 TNB were deleted from the dataset and litter data with at least 15 and 10 from each NL and ND boar, respectively. The full litter data set included 20511 litters from 323 NL boars and 3927 litters from 219 ND boars with analyses of DFI. Among these, 75.3% and 74.4% of the NL and ND litters, respectively, were from semen stored from 48 to 96 hours prior to AI.

2.4. Statistical analyses

Statistical analyses were performed using the software package Statistical Analysis Software (SAS) version 9.4 for Microsoft Windows (SAS Institute Inc., Cary, NC, USA). A Shapiro-Wilk test showed that the DFI data were not normally distributed. Therefore, a log transformation of the DFI data was performed prior to further statistical analysis. The log-transformed DFI data (log DFI) were analyzed using paired t-test for testing the effect of storage (Day 0 and Day 4). Statistical analyses were performed separately for the two breeds.

Least Square Means (LS-means) for log DFI of each boar was calculated using a General Linear Model (GLM) procedure. First, possible correlations (Pearson test) between log DFI and age of the boar, sperm cell concentration, collection interval (the interval in days since previous collection from each boar) and storage time of the semen when analyzed (2, 3, 4 days) were tested using PROC CORR (significance: $p<0.05$). Further, a GLM analysis was performed by evaluating the effect of the boar and other specific boar parameters on DFI values. The interval parameter was divided into four classes; "A" (a boar's first semen collection hence no interval), and "B", "C" and "D" representing 1-3 days, 4-5 days and >5 days, respectively. A backwards selection approach was used where all the variables of interest were fitted into a model. The variable with the highest p-value was excluded, if the variable had no significant effect. This re-fitting of the model was continued until all the effects in the model were statistically significant ($p<0.05$). In the final models for log DFI, boar and interval class were integrated as effects in both NL and ND. In addition, the effect of boar age was integrated in the ND model. Based on the GLM results, LS-means for log DFI per boar were calculated and included in the model for TNB.

In addition to LS-mean log DFI per boar, the following variables were tested in the model for TNB: sow herd, sow parity (divided into three classes; 1, 2 and >2), type of insemination (single/double), storage time of the semen when inseminated (0, 1, 2, 3, or 4 days) and semen collection month (month/year) were evaluated by using the GLM procedure with the backwards selection approach. The following model 1) for NL and model 2) for ND were used:

Model 1): TNB = herd + parity + type of insemination + semen storage time + semen collection month + LS-means log DFI

Model 2): TNB = herd + semen storage time + semen collection month + LS-means log DFI

In order to investigate threshold values for DFI of the boars, GLM models for each breed were constructed as in model 1 and model 2, with the exception that the effect of log DFI per boar was changed into the effect of the boar. The LS-means for TNB per boar was then aligned with the LS-means for DFI per boar, backtransformed from log DFI. The percentiles of LS-means TNB were used as limits to classify boars as lowest 5%, medium 90% and highest 5% with regards to litter size. The mean values of LS-means for DFI as well as the mean values of LS-means for TNB per percentile group were calculated.

3. RESULTS

3.1. Semen storage and sperm DNA fragmentation

Initially, the effect of liquid semen storage on sperm DNA fragmentation was evaluated by analyzing 75 of the ejaculates at the day of collection and upon 96 hours storage. Descriptive data from the analysis are presented in Table 1. At the day of collection, the median of DFI was higher in ND compared to NL. However, in NL the median of DFI was higher upon storage at 18°C for 96 hours (Day 4). In addition, the range of DFI was highest in NL both at Day 0 and Day 4.

Results from a paired t-test with log-transformed DFI values showed a significant increase in log DFI from Day 0 to Day 4 in both breeds (NL p=0.002, ND p=0.04). Norsvin recommends to use the semen within 96 hours upon collection. Thus, in order to mimic the status at the day of use in the herds, DFI was screened in liquid semen samples stored at 18°C for 48, 72 or 96 hours depending on the weekday the collection was performed.

3.2. Sperm DNA fragmentation in liquid stored semen samples

DNA fragmentation was analyzed in all the stored samples (NL n=695, ND n=650) and the result showed that median DFI for NL was 1.37% (range 0.19-28.39%) and for ND 1.61%

(range 0.26-36.58%) (Figure 1). DFI values above 10% was observed for 1.7% and 0.5% of the NL and ND ejaculates, respectively. Results from a t-test with log-transformed DFI data showed a significant effect of breed ($p<0.0001$). Therefore, further statistical analyses were performed separately for the two breeds.

In the study, factors that contributed to the differences in measured log DFI were evaluated. First, the Pearson correlation coefficient and p-values for the correlations between the log DFI and parameters linked to the boar were calculated (Table 2). For NL, log DFI was significantly positively correlated to the storage of semen. However, for ND log DFI was significantly negatively correlated to age of the boar.

The effect of the parameters included in the correlation analysis on log DFI, were in addition to herd and interval class further evaluated by construction of separate GLM for each breed. In both models boar had a significant effect ($p<0.0001$). In addition, for NL boars, age had a significant negative effect on log DFI ($p=0.0031$). Thus, for NL boars included in this study log DFI decreases with increased boar age. In the models, interval class was found to have a significant effect on log DFI for both breeds ($p=0.0238$ and $p<0.0001$; NL and ND, respectively). In addition, for both breeds the interval class indicating boars' first semen collection represented significantly higher log DFI ($p=0.0043$ and $p=0.0017$, NL and ND, respectively) than the longest interval class, while for ND the "B" class (1-3 days) represented significantly lower log DFI ($p=0.0018$) than class "D" (> 5 days' interval).

3.3. The effect of DNA fragmentation on field fertility

Based on the results from the GLM analyses (NL: model 1 and ND: model 2), LS-means for log DFI of each boar were calculated and these values were used to evaluate the effect of DNA fragmentation on field fertility in form of TNB. The GLM results are shown in Table 3 and 4 and for both NL and ND litters, herd, semen storage time, semen collection month and

LS-means log DFI showed a significant effect on TNB. In addition, for NL sow parity and type of insemination (single/double) had a significant effect on TNB (Table 3). For both breeds, the effect of LS-means log DFI on TNB was found to be negative indicating that lower log DFI values give higher TNB. In the ND model (Table 4), LS-means log DFI had the highest impact (F value=44.86). However, in the NL model (Table 3) parity had the highest impact (F value=58.06) and the LS-means log DFI showed a lower impact (F value=13.13) than both parity and type of insemination (double/single).

For both breeds, the mean percentage DFI values within the 5% of boars with the lowest litter size were higher than the DFI values of boars within the other groups, and the DFI of the boars with the 5% highest litter size were the lowest, as represented in Table 5.

4. DISCUSSION

In swine, sperm DNA fragmentation has previously been reported to influence litter size. However, it is shown that the genetic line has an impact on the fertility outcome (Boe-Hansen et al., 2008; Broekhuijse et al., 2012). Therefore, in order to take in account the difference between genetic lines, the objective of the present study was to determine the level of DNA fragmentation in stored liquid extended semen from NL and ND boars and to evaluate the effect of DNA fragmentation on TNB.

In Norway, most of the semen is used for AI two or more days after collection and in the present study, around 75% of the litters were from semen stored from 48 to 96 hours prior to AI. Data from our initial work showed a small but significant increase in DFI upon 96 hours liquid storage. This is in agreement with several previous studies reporting that in liquid preserved boar semen, spermatozoa show increased DNA fragmentation upon storage (Bielas, Nizanski, Partyka, Rzasa, & Mordak, 2017; Boe-Hansen et al., 2008; Boe-Hansen et al., 2005; Broekhuijse et al., 2012). In contrast to this, De Ambrogi et al. (2006) reports that liquid

storage of boar semen for up to 96 hours does not cause loss of DNA integrity. However, only four ejaculates from four different boars were evaluated in this study. In addition, the boars represented three different breeds. Thus in the current study, screening of DFI from samples stored for 48 to 96 hours was performed in order to mimic the status at the day of use in the herds. In the studies by Bielas et al. (2017) and Broekhuijse et al. (2012) the greatest increase of DFI was observed between the day of collection and 24 hours storage. This supports our assumption that samples stored for 48 to 96 hours will adopt the "worst case" DFI value of the samples used for AI.

In the current study, the DNA fragmentation level in the analyzed samples was relatively low with DFI median values below 2% (Figure 1). However, individual variation was observed and a few samples showed DFI values above 10% and even up to values around 30%. The general low level of DNA fragmentation detected is in accordance to previous studies reporting mean DFI values from around 2 - 4% in liquid stored boar semen (Bielas et al., 2017; Boe-Hansen et al., 2008; Broekhuijse et al., 2012; Didion et al., 2009). However, these studies do also report on individual variation between boars and ejaculates regarding the DNA fragmentation level at the day of collection and upon storage.

The modeling of DFI showed that in addition to boar, the interval class influenced on the variation of DFI values in NL and ND semen. For both breeds, DFI was higher at the first semen collection compared to the longest interval class (> 5 days' interval). At the first semen collection, the age of the boars were around 7-10 months and this result could indicate immaturity of sperm structures in the young boars. This finding is supported by the observation that for NL boars, the level of DNA fragmentation decreases by increased boar age. These observations are in accordance to a recent study reporting a higher incidence of chromatin instability in semen from young boars (7-10 months) compared to mature boars (18-33 months) (Tsakmakidis, Khalifa, & Boscos, 2012).

Optimally, the relationship between DNA fragmentation and TNB should have been evaluated by linking litters from the exact same ejaculate as DFI samples. However, in pigs the number of inseminations performed for each ejaculate is low and therefore, in the present study all known litters from the boars with a DFI value for one or more ejaculates were included for calculation of TNB. The models of TNB showed that for both NL and ND, the DNA fragmentation had a significant effect on TNB. In the ND model, DNA fragmentation was found to have the highest impact on TNB of the effects in the model. For both breeds, it appears that litter size is significantly negatively affected by increased DFI. Thus, our findings confirm previous observations about the relationship between DNA fragmentation and field fertility (Boe-Hansen et al., 2008; Broekhuijse et al., 2012; Didion et al., 2009). Some studies have suggested threshold values for DFI in boar semen around 2-6% or between 2-18% (Boe-Hansen et al., 2008; Didion et al., 2009). The current study showed that the 5% boars with the lowest litter size had a mean DFI of 2.77% and 2.08% in NL and ND, respectively, compared to 1.32% and 1.18% for the 5% boars with the highest litter size. This gives a difference in litter size close to three piglets for NL litters and four piglets for ND litters indicating that TNB is markedly reduced by increased DFI values.

In conclusion, the present study shows that the sperm DNA fragmentation parameter measured in liquid stored semen provides important information regarding fertility of NL and ND boars. This parameter should therefore be taken into consideration for evaluation of NL and ND boars entering an AI center.

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CONFLICT OF INTEREST

The authors have no known conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

FDM, RF, MS, EG and AHG designed the study. RF, EBS and TTZ performed the SCSA analysis. NHT, IAR and AHG performed statistical analysis. FDM, NHT and AHG created the manuscript. All authors contributed to writing and approval of the finale manuscript.

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Table 1

Median, minimum and maximum (range) and storage difference (Day 4-Day 0) values for the sperm DNA fragmentation index (DFI) in ejaculates from Norwegian Landrace (n=49) and Norwegian Duroc (n=26) boars at the day of collection (Day 0) and after 96 hours storage at 18°C (Day 4).

	Norwegian Landrace (n=49)	**Norwegian Duroc (n=26)**
Median DFI Day 0 (%)	2.22	2.61
Range DFI Day 0 (%)	0.9 - 26.4	1.33 - 7.07
Median DFI Day 4 (%)	4.01	2.88
Range DFI Day 4 (%)	0.75 - 27.4	1.36 - 17.36
Difference median DFI Day 4 - Day 0 (%)	0.46	0.17
Range DFI Day 4 - Day 0 (%)	-2.56 - 9.38	-0.69 - 13.57

Table 2

The Pearson correlation coefficient (corr) and p-values for the correlations between the log-transformed sperm DNA fragmentation index (log DFI) and selected boar parameters in ejaculates from Norwegian Landrace (n=695) and Norwegian Duroc (n=650).

	Norwegian Landrace		**Norwegian Duroc**	
	Corr	**p-value**	**Corr**	**p-value**
Boar age	-0.019	0.62 [b]	-0.148	0.0001 [a]
Collection interval	0.013	0.76	0.054	0.22
Sperm cell concentration	0.053	0.27	0.045	0.33
Semen storage time	0.086	0.023 [a]	0.043	0.27

Significance of correlation ($p<0.05$) is indicated by [a] and parameters with a significant effect ($p<0.05$) in general linear models are indicated by [b].

Table 3

The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (Pr>F) for the parameters with significant effect in Norwegian Landrace on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	Pr > F
Herd	176	12739.07	72.38	6.92	<.0001
Parity	2	1214.00	607.00	58.06	<.0001
Type of insemination	1	347.60	347.60	33.25	<.0001
Semen storage time	4	112.19	28.05	2.68	0.0298
Semen collection month	91	1665.78	18.31	1.75	<.0001
LS-means log DFI	1	137.26	137.26	13.13	0.0003

Table 4

The degrees of freedom (DF), sum of squares (SS), mean squares, F values and p-values (Pr>F) for the parameters with significant effect in Norwegian Duroc on the total number of piglets born.

Source	DF	SS	Mean Square	F Value	Pr > F
Herd	13	1280.32	98.49	11.51	<.0001
Semen storage time	4	184.06	46.02	5.38	0.0003
Semen collection month	79	1028.87	13.02	1.52	0.0022
LS-means log DFI	1	384.03	384.03	44.86	<.0001

Table 5

The relationship between the estimated total number of piglets born (TNB) and the DNA fragmentation index (DFI) in Norwegian Landrace and in Norwegian Duroc.

Percenttiles LS-means TNB	Number of boars	Mean of LS-means DFI per boar (%)	Mean of LS-means TNB per boar
Norwegian Landrace			
<5%	17	2.77	11.96
5-95%	289	1.44	13.94
>95%	17	1.32	15.01
Norwegian Duroc			
<5%	11	2.08	6.57
5-95%	197	1.57	9.15
>95%	11	1.18	10.97

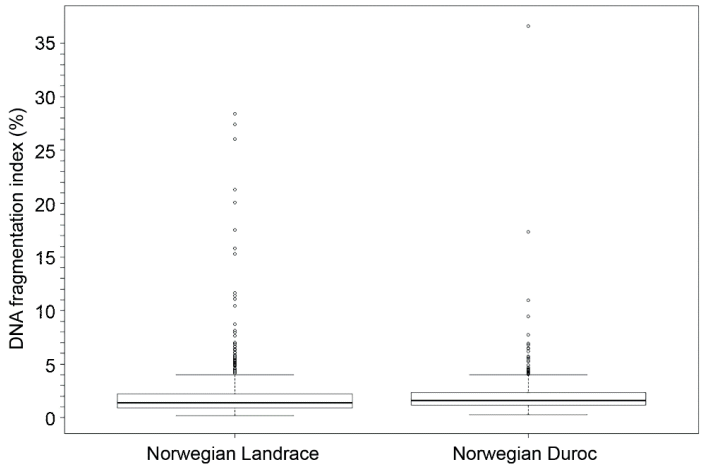

Figure 1. DNA fragmentation index (DFI) in semen samples from Norwegian Landrace (n=695) and Norwegian Duroc (n=650) upon storage at 18°C for 48-96 hours. The box plot presents the DFI median values, first and third quartile, range of the data and outliers.

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RESEARCH ARTICLE Open Access

RNA sequencing reveals candidate genes and polymorphisms related to sperm DNA integrity in testis tissue from boars

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Abstract

Background: Sperm DNA is protected against fragmentation by a high degree of chromatin packaging. It has been demonstrated that proper chromatin packaging is important for boar fertility outcome. However, little is known about the molecular mechanisms underlying differences in sperm DNA fragmentation. Knowledge of sequence variation influencing this sperm parameter could be beneficial in selecting the best artificial insemination (AI) boars for commercial production. The aim of this study was to identify genes differentially expressed in testis tissue of Norwegian Landrace and Duroc boars, with high and low sperm DNA fragmentation index (DFI), using transcriptome sequencing.

Results: Altogether, 308 and 374 genes were found to display significant differences in expression level between high and low DFI in Landrace and Duroc boars, respectively. Of these genes, 71 were differentially expressed in both breeds. Gene ontology analysis revealed that significant terms in common for the two breeds included extracellular matrix, extracellular region and calcium ion binding. Moreover, different metabolic processes were enriched in Landrace and Duroc, whereas immune response terms were common in Landrace only. Variant detection identified putative polymorphisms in some of the differentially expressed genes. Validation showed that predicted high impact variants in *RAMP2*, *GIMAP6* and three uncharacterized genes are particularly interesting for sperm DNA fragmentation in boars.

Conclusions: We identified differentially expressed genes between groups of boars with high and low sperm DFI, and functional annotation of these genes point towards important biochemical pathways. Moreover, variant detection identified putative polymorphisms in the differentially expressed genes. Our results provide valuable insights into the molecular network underlying DFI in pigs.

Keywords: Transcriptome profiling, Sperm DNA integrity, Differential expression

Background

Analysis of sperm parameters is important for predicting boar fertility and the outcome of artificial insemination (AI) in pig production. The classical way of evaluating sperm parameters is subjective scoring of viability, motility, concentration and morphology, to identify ejaculates with poor fertilization potential [1, 2]. However, this is insufficient for accurate prediction of the boar's reproductive capacity, since the sperm cells must have additional qualities to fertilize the oocytes and since it is a subjective score. Combining several assays is suggested to better predict the fertility of an ejaculate [3]. For example, combining sperm morphology parameters and evaluation of DNA chromatin integrity has been found to be related to field fertility, as measured by farrowing rate in pigs [4].

During the last phase of spermatogenesis, spermiogenesis, the DNA of sperm cells is tightly packed by protamine and results in a condensed chromatin structure [5]. This leaves the DNA protected against degradation during transport through the male and female reproductive tracts until fertilization. Altered sperm chromatin structure is

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associated with DNA fragmentation and the degree of sperm DNA fragmentation is shown to be correlated to fertility in different species [4, 6–13]. This parameter is a much more objective marker of sperm quality and function than standard subjective microscopic evaluations [14, 15]. The sperm chromatin structure assay (SCSA) is a flow cytometry-based method that measures abnormal chromatin structure, as an increased acid-induced degradation of sperm DNA in situ [11]. More specifically, the acid denatures DNA at the sites of DNA breaks, which again reflects chromatin integrity status. The SCSA thereafter measures the relationship between double-stranded (i.e. condensed chromatin) and single-stranded (i.e. denatured) DNA for each sperm cell. This relationship is quantified by the DNA Fragmentation Index (DFI) [12]. Previous studies in pigs showed that DFI was significantly associated with litter size [8]. Moreover, DFI is found to be an important parameter for predicting normal development of the embryo [11, 16] and is also associated with abortion in humans [17].

Although the amount of sperm DFI is shown to influence fertility outcome, little is known about the underlying molecular mechanisms. Differentially expressed proteins have been identified in human seminal plasma and spermatozoa [18, 19]. Studies in humans have also showed that a truncated form of KIT tyrosine kinase, expressed in testis, causes higher amounts of DNA damage in sperm cells [20]. Moreover, depletion of excision repair cross-complementing gene 1 (*ERCC1*) and tumor suppressor gene *p53* in mouse testis resulted in increased DNA breaks in sperm cells [21]. Recent studies indicate that the main reason of DFI in sperm is apoptosis, likely triggered by an impairment of chromatin maturation in the testis and by oxidative stress during the transit in the male genital tract [22].

The goal of this study was to use transcriptome sequencing to examine differential gene expression in testis tissue of boars with high and low sperm DFI. Testis tissue was chosen because chromatin condensation and DNA packaging in sperm cells occurs during testicular spermatogenesis [5, 23]. The biological functions of the differentially expressed genes were also investigated and a search for putative polymorphisms in the differentially expressed genes was performed. The results obtained in this study highly contribute to the knowledge of the molecular mechanisms underlying DNA fragmentation.

Methods

Animals and phenotypes

The sperm DFI was determined in a total of 241 Landrace and 302 Duroc AI boars in this study. All the boars were housed individually in pens sized approximately two by three meters and fed the same commercial diet. Nine Landrace and eleven Duroc boars were selected for transcriptome profiling based on their extreme high/low DFI values (Table 1). The boars' age at semen sample collection ranged from 221 to 1000 days (mean = 310 days, standard deviation (SD) = 84.5). The sperm-rich fraction of the ejaculates was collected with the "gloved hand technique" at the Norsvin AI center (Hamar, Norway), similar to other studies recently published [24, 25]. From each of the boars, samples from up to six different ejaculates were analyzed, and the mean of the measurements was used as the final score. The ejaculates were diluted to a concentration of 28×10^6 spermatozoa per ml, according to the normal routines of the AI center at each date. The ejaculates were shipped as regular semen doses to commercial swine producers for the use within the next four days. From each individually diluted ejaculate, a sample of approximately 12 mL was transferred to a plastic tube. The samples were stored at 18 °C for 48 to 96 h depending on day of the week, before they were frozen in −80 °C until used for the DFI analysis. Boars were culled according to normal culling procedures at the AI station. From these boars, the testicle tissue samples were collected at the slaughter line. A piece of sample was collected from the

Table 1 DFI measurements for the different boars used in this study

Group	Boar	n(ejaculates)	DFI mean	DFI SD
Landrace low	L1	2	0.62%	0.10%
	L2	1	0.73%	
	L3	1	0.82%	
	L4	3	1.50%	0.33%
	L5	4	1.52%	0.68%
Landrace high	L6	1	6.41%	
	L7	2	5.47%	1.69%
	L8	6	7.26%	5.11%
	L9	7	8.07%	3.85%
Duroc low	D1	1	1.05%	
	D2	2	1.07%	0.37%
	D3	2	1.08%	0.34%
	D4	1	1.11%	
	D5	3	1.13%	0.35%
Duroc high	D6	1	4.13%	
	D7	1	4.77%	
	D8	1	4.14%	
	D9	1	4.69%	
	D10	1	5.63%	
	D11	3	5.36%	3.64%

The number of ejaculates and mean DFI value with SD is presented for each boar

middle part of one of the testicles, approximately 3 × 1.5 cm in size, immediately frozen in liquid N_2, and thereafter stored at −80 °C until used for RNA extraction.

DFI measurements

The SCSA protocol was performed using Cell Lab Quanta™ SC MPL (Beckman Coulter, Fullerton, CA, USA), equipped with a 22 mW argon laser with excitation at 488 nm, according to the procedure described by Evenson and Jost [13] with modifications [26]. The method is based on DNA staining properties of acridine orange (AO) which fluoresces green and red when binding to native dsDNA and denatured ssDNA, respectively. Frozen samples were thawed at 37 °C and diluted to a concentration of 2×10^6 sperm cells/mL in TNE buffer (10 mM Tris-HCL, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final volume of 200 μL. Immediately afterwards, 400 μL of acid detergent solution (0.38 M NaCl, 80 mM HCL, 0.1% (*w*/*v*) Triton X-100, pH 1.2) was added. After exactly 30 s, 1.2 mL of AO staining solution (0.6 μg/mL AO (A3568, Life Technologies, OR, USA) in a buffer containing 37 mM citric acid, 0.126 M Na_2HPO_4, 1.1 μM EDTA, 0.15 M NaCl, pH 6) was added, and the sample was further incubated at room temperature in the flow cytometer. The sample was run in setup mode until 3 min after addition of the acid detergent solution, and then the acquisition of data was started. For each sample, 5000 events were collected with a flow rate of ~200 events/s. Prior to the analysis, the flow cytometer was AO saturated by running an AO equilibration solution (1.2 mL AO staining solution and 400 μL acid detergent solution) through the system for 5 min. The green fluorescence was collected by a 525 nm band pass filter, while the red fluorescence was collected by a 670 nm long pass filter. Prior to analysis and after every 10th sample, a reference sample was thawed, prepared and analyzed in the same way as the experimental samples to ensure the stability of the instrument and the laser throughout the experiment. The X-mean channel value was set to 125 ± 5 and Y-mean channel value was set to 425 ± 5. To identify the spermatozoa, a combination of electronic volume (EV)- and side scatter (SS)-signals were used, as described by Standerholen et al. [27]. The percentage of red and green fluorescence was determined using the Cell Lab Quanta™ SC MPL Analysis software package (Beckman Coulter, Software Version 1.0 A). Based on the ratio of red/(red + green), the DFI-value was calculated.

RNA extraction and sequencing

Total RNA for RNA sequencing was extracted from testicle tissue using the RNeasy Midi Kit from Qiagen according to the manufacturer's instruction (Qiagen, CA, USA). Concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA) and the RNA quality was examined by the 28S:18S rRNA ratio using the RNA 6000 Nano LabChip® Kit on 2100 Bioanalyzer (Agilent Technologies, CA, USA). All samples displayed a 260/280 ratio > 1.8 and RNA integrity numbers (RIN) >8.5. RNA sequencing was done using Illumina HiSeq 2000 by the Norwegian Sequencing Centre at Ullevål Hospital (http://www.sequencing.uio.no). and generated 50 basepair single end reads. TruSeq RNA v2 was used for non-stranded library preparation, V3 clustering and sequencing reagents were used according to manufacturer's instructions. Sample amount of 2 μg RNA was used as input, and 4 min fragmentation at 94 °C was employed. Image analysis and base calling were performed using Illumina's RTA software version 1.17.21.3. Reads were filtered to remove those with low base call quality using Illumina's default chastity criteria. The FASTQC software was used for quality control of raw sequence data (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). All reads had a per base sequence quality Phred score above 27 for all positions and were considered high quality. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) [28] and are accessible through GEO Series accession number GSE74934.

Differential expression

The high quality reads were mapped to the *Sus scrofa* genome build 10.2 using the software TopHat v.2.0.12 [29] and default parameters. The Picard AddOrReplaceReadGroups program (http://broadinstitute.github.io/picard/) was used to assign unique IDs to the files. Gene prediction coordinates (release 10.2.75) were obtained from the ENSEMBL web site (http://www.ensembl.org). Mapped reads were sorted and indexed using Samtools v.1.1 [30] and the software HTSeq [31] was used with the stranded = no option to calculate the number of reads mapped to each gene. The R software package "edgeR" v.3.2.4 from Bioconductor was used to analyze the data [32] [see Additional file 6 for code]. The breeds were analyzed separately and the boars were divided into "high" and "low" groups based on their DFI values. The package assumes that the data follow a negative binomial distribution and it uses raw counts without correcting for gene length as this bias is assumed to be the same in all samples. Filtering was done to keep genes that reached at least one count per million in at least half of the samples. A heatmap was made for the differentially expressed genes between the high (bad) and low (good) DFI groups using the heatmap function in R (default parameters).

Statistical analysis

Normalization was done using the trimmed mean of the *M* values method [33] as implemented in "edgeR". Moreover, tagwise dispersion was applied to estimate separate mean-variance relationships for the individual genes, and the generalized linear model likelihood test ratio method was employed to test for differential expression. The resulting *p*-values were adjusted for multiple testing by the Benjamini and Hochberg algorithm [34] and the level of significance for differentially expressed genes was set to an false discovery rate (FDR) of 0.05.

Gene ontology

Gene enrichment analyses make it easier to get an overview of functions that are overrepresented in gene expression datasets. Gene ontology (GO) tools can conveniently assign genes to different terms in the three categories "Molecular Function", "Cellular Component" and "Biological Process". In order to map all differentially expressed genes to corresponding GO terms, the R package "goseq" was applied [35]. The Wallenius approximation method was used to account for gene length bias before each GO term was tested for over-representation and under-representation of significant genes. The Benjamini and Hochberg algorithm [34] was used to correct for multiple testing and GO terms were considered significantly enriched at a 0.05 FDR cutoff.

Variant calling

Variant calling was done within breed using Samtools v.1.1 mpileup and bcftools call [30], and the Integrative Genomics Viewer (IGV) was used to visually inspect putative polymorphisms [36]. Using Samtools v.1.1 bcftools filter, variants (single nucleotide polymorphisms (SNPs)/insertions and deletions (indels)) were filtered to include only those having an alternate allele count of at least two, minor allele frequency above 0.01 and a read depth above 10. Moreover, only polymorphisms in differentially expressed genes were considered. The detected variants were annotated using SnpEff v.4.1 to classify variants (such as missense, nonsense, synonymous, stop gain/loss) and their impact (high, moderate, low, modifier) [37, 38]. Variants causing frameshift mutations or affecting start or stop codons are considered to have high impact, whereas variants e.g. in 3'UTR get the lowest impact (modifier). SnpSift was used to extract relevant information from list of variants files [39]. SNP validation was performed *in-silico* by matching putative polymorphism positions to known pig dbSNP entries [40]. SNPs not present in the database were considered novel. The putative variants identified in differentially expressed genes of this study have been deposited to the European Nucleotide Archive (EVA) under accession number PRJEB22189. For validation purposes, 15 of the high impact variants were genotyped using the KASP SNP genotyping system platform (KBiosciences, Herts, UK) using the 20 animals from the RNA-seq as well as 18 other pigs from Norsvin's boar testing station (nine from each breed), which are relatives to the RNA-seq boars. SNP validation was also performed in an independent next generation sequencing dataset of related boars [41]. The putative high impact variants were compared by sequence position, reference and alternate alleles to polymorphisms identified in this dataset. Corresponding variants were considered validated.

Results

Mapping

Gene expression in testis tissue from Landrace and Duroc boars, with high and low sperm DFI, was analyzed by transcriptome sequencing. The mean (± SD) of the DFI values for the low and high groups were 1.04% (± 0.44%; $n = 5$) and 6.80% (± 1.12%; $n = 4$) in Landrace and 1.09% (± 0.03%; n = 5) and 4.79% (± 0.62%; $n = 6$) in Duroc, respectively (Table 1). The sequence data was maximum 50 basepair reads and the total number of sequenced reads per animal ranged from 59.6 to 95.0 million of which on average 76.7% of the reads were uniquely mapped to the current porcine genome assembly (*Sus scrofa* build 10.2). Altogether, 22,059 genes in Landrace and 21,717 in Duroc had at least one count in at least one sample. After filtering, 14,609 (66.2%) and 14,713 (67.7%) genes were used for differential expression analysis in Landrace and Duroc, respectively.

Differential expression

A total of 308 genes in Landrace and 374 genes in Duroc were significantly differentially expressed in testis tissue from boars with high and low sperm DFI [see Additional file 1 and Additional file 2 for Landrace and Duroc, respectively]. Of these genes, 71 were common for the two breeds (Table 2). The most significant differentially expressed gene in Landrace and Duroc was actin *ACTA1* (FDR = 2.89e-09 and logarithmic fold change (logFC) = −1.78) and serum amyloid precursor *SAA4* (FDR = 1.90e-06 and logFC = −0.68), respectively. In Landrace, *ACTA1* was also the most down-regulated gene in the high DFI group, whereas neurexophilin *NXPH2* showed the highest up-regulation (FDR = 6.11e-04 and logFC = 3.44). In Duroc, L-dopachrome tautomerase *DCT* showed the most down-regulation (FDR = 2.65e-02 and logFC = −0.94), whereas metallopeptidase *ADAMTS4* was most significantly up-regulated (FDR = 1.88e-02 and logFC = 2.60). The majority of differentially expressed genes (94% and 78% in Landrace and Duroc, respectively) showed increased expression in the high DFI group compared to the low DFI group [see Additional file 5]. In

Table 2 Differentially expressed genes common for the two breeds Landrace and Duroc

Gene symbol	Gene name	FDR L	FDR D
ACER2	alkaline ceramidase 2	1.61E-02	3.74E-02
ACTN4	alpha-actinin	1.52E-02	1.34E-02
APP	amyloid beta A4 protein	2.73E-02	1.34E-02
ATG4A	autophagy related 4A, cysteine peptidase	4.02E-03	2.13E-02
BGN	Biglycan	2.64E-02	1.88E-02
BMP1	bone morphogenetic protein 1	4.60E-02	1.09E-02
SERPING1	Serpin family G member 1	2.68E-02	1.89E-02
GLMP	Glycosylated lysosomal membrane protein	3.66E-03	3.59E-02
C1R	complement component 1, r subcomponent	4.29E-02	2.13E-02
C4A	*Sus scrofa* complement C4 (C4), mRNA	2.86E-02	1.84E-03
CA4	carbonic anhydrase IV	4.06E-02	1.44E-02
CAT	Catalase	1.01E-02	1.24E-02
CDC42EP1	CDC42 effector protein (Rho GTPase binding) 1	4.93E-02	2.42E-02
CITED1	Sus scrofa Cbp/p300-interacting transactivator	2.31E-02	1.11E-02
ENSSSCG00000001711	Uncharacterized protein	3.89E-02	2.72E-02
COL3A1	collagen, type III, alpha 1	4.40E-02	3.59E-02
COPZ2	coatomer protein complex, subunit zeta 2	1.04E-02	4.75E-02
CPED1	cadherin-like and PC-esterase domain containing 1	4.51E-03	2.72E-02
CSF1R	colony stimulating factor 1 receptor	3.86E-02	2.92E-02
CTDSPL	CTD small phosphatase-like	1.52E-02	1.66E-02
CTSA	cathepsin A	2.43E-02	2.71E-02
CTSB	Sus scrofa cathepsin B (CTSB)	1.26E-02	9.65E-03
CTSH	Sus scrofa cathepsin H (CTSH), mRNA	2.07E-02	3.84E-02
CYP11A1	Sus scrofa cytochrome P450, family 11, subfamily A, polypeptide 1	1.58E-02	4.56E-02
ENSSSCG00000028912	Uncharacterized protein	3.02E-02	2.72E-02
CFD	Complement factor D	4.02E-02	1.98E-02
DNASE1L1	deoxyribonuclease I-like 1	4.51E-03	3.59E-02
ECHDC3	enoyl CoA hydratase domain containing 3	1.21E-03	2.04E-02
ENSSSCG00000024587	Uncharacterized protein	2.49E-02	2.64E-02
ENSSSCG00000028244	Uncharacterized protein	2.41E-02	4.91E-02
EDNRA	endothelin receptor type A	4.56E-02	2.42E-02
EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	4.86E-02	1.66E-02
EHD2	EH-domain containing 2	9.26E-03	1.20E-02
ENSSSCG00000011239	Uncharacterized protein	3.85E-02	9.09E-03
ENSSSCG00000021406	Uncharacterized protein	1.35E-02	4.85E-02
ENSSSCG00000025934	Uncharacterized protein	2.75E-02	7.72E-03
ENSSSCG00000029074	Uncharacterized protein	1.61E-02	3.58E-02
EPHX1	Sus scrofa epoxide hydrolase 1, microsomal (xenobiotic) (EPHX1)	1.61E-02	1.11E-02
FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	1.66E-02	8.16E-03
FAM213A	family with sequence similarity 213, member A	4.93E-02	1.01E-02
FCGRT	Sus scrofa Fc fragment of IgG, receptor, transporter, alpha	3.42E-02	4.56E-02
FDXR	NADPH:adrenodoxin oxidoreductase, mitochondrial	1.61E-02	1.29E-02
FGFR1	Fibroblast growth factor receptor 1	4.69E-02	1.21E-02
ENSSSCG00000022236	Uncharacterized protein	4.02E-02	1.52E-02

Table 2 Differentially expressed genes common for the two breeds Landrace and Duroc *(Continued)*

Gene symbol	Gene name	FDR L	FDR D
ENSSSCG00000002797	Uncharacterized protein	4.29E-02	2.22E-02
GRK5	G protein-coupled receptor kinase 5	4.60E-02	2.87E-03
GSDMD	gasdermin D	4.11E-02	1.36E-02
ENSSSCG00000000620	Uncharacterized protein	2.01E-02	3.84E-03
ITM2C	Sus scrofa integral membrane protein 2C	4.71E-02	4.75E-02
ENSSSCG00000004207	Uncharacterized protein	3.99E-02	2.36E-02
LAMB2	laminin, beta 2 (laminin S)	1.23E-02	2.81E-02
LAMC3	laminin, gamma 3	1.30E-02	2.15E-02
LIPA	lipase A, lysosomal acid, cholesterol esterase	4.50E-02	3.84E-02
ENSSSCG00000023235	Uncharacterized protein	2.63E-02	1.23E-02
MAOB	monoamine oxidase B	3.62E-02	2.50E-02
NEU1	sialidase 1 (lysosomal sialidase)	4.11E-02	4.56E-02
ENSSSCG00000022516	Uncharacterized protein	2.23E-02	4.97E-02
PGAM1	phosphoglycerate mutase 1 (brain)	1.50E-02	1.29E-02
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	4.06E-02	4.75E-02
PRDX2	peroxiredoxin 2	4.02E-02	4.69E-02
ENSSSCG00000016522	Uncharacterized protein	5.88E-03	1.20E-02
SERTAD1	SERTA domain containing 1	3.22E-02	4.35E-02
ENSSSCG00000011357	Uncharacterized protein	3.87E-02	4.11E-02
SLC1A5	solute carrier family 1 (neutral amino acid transporter), member 5	4.99E-02	3.91E-02
SLC41A1	solute carrier family 41 (magnesium transporter), member 1	4.29E-02	7.39E-03
SLC44A2	Choline transporter-like protein 2	4.02E-02	1.12E-02
SLC44A4	Sus scrofa solute carrier family 44, member 4 (SLC44A4), mRNA	4.98E-02	3.50E-02
TMEM176B	transmembrane protein 176B	4.40E-02	7.46E-03
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	2.64E-02	5.56E-03
TPM4	tropomysin alpha-4 chain	5.77E-03	3.10E-02
VIM	Vimentin	4.02E-02	1.12E-02

Genes differentially expressed in both breeds are presented with gene symbol, gene name and significance level (FDR) for Landrace (L) and Duroc (D)

addition to the annotated genes described below, genes encoding functionally uncharacterized proteins were differentially expressed in both breeds and they are included in the results tables with their corresponding Ensembl ID.

Gene ontology

Functional characterization of differentially expressed genes revealed an overrepresentation of genes with roles in the cellular components "extracellular matrix" and "extracellular region" for both Landrace and Duroc. Results of the GO classification of the differentially expressed genes are shown in Fig. 1. The molecular function "calcium ion binding" was also enriched in both breeds. In addition, "cholesterol metabolic process" and "oxidation-reduction process" were Duroc specific whereas "collagen catabolic process", "hydrolase activity" and "proteolysis" were Landrace specific. Moreover, immune system ontologies were Landrace specific.

Variant calling

Variant detection identified 1501 and 1751 putative polymorphisms in differentially expressed genes in Landrace and Duroc, respectively, out of which 91 and 88% had an existing dbSNP entry [see Additional files 3 and 4]. In Landrace/Duroc, most of the polymorphisms (610/731) in differentially expressed genes were synonymous SNPs (Table 3). Of the polymorphisms in differentially expressed genes, 4/17 in Landrace/Duroc were high impact variants, predicted to cause frameshifts or a change in start or stop codon. 15 of the high impact variants were chosen for validation using the KASP SNP Genotyping system. Five of the SNPs were successfully validated, including four of the ones with previous dbSNP entries (see Additional file 7]. Ten of the detected high impact variants, including one with an existing dbSNP entry, failed validation. When comparing the variants

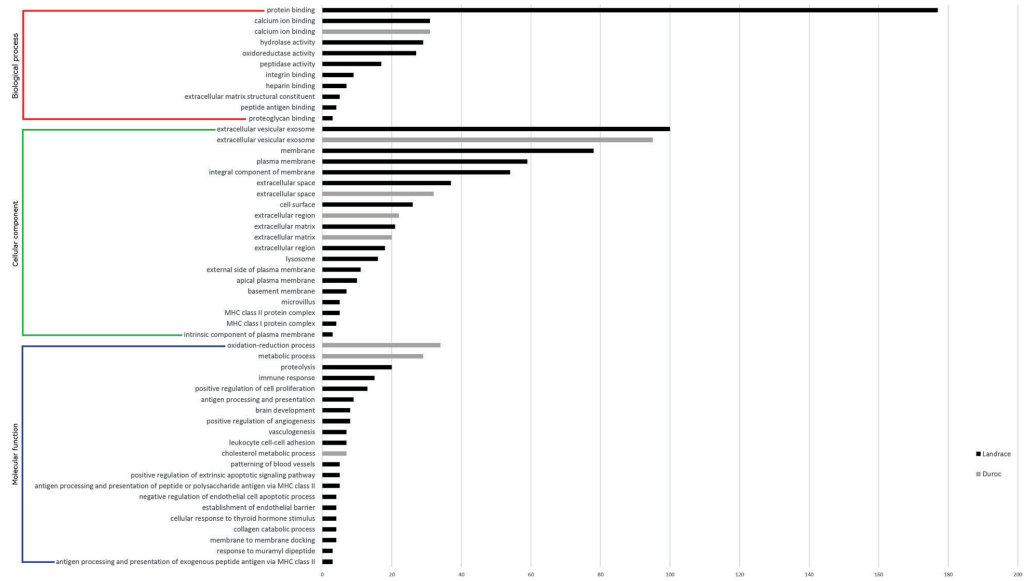

Fig. 1 Gene ontology classification of the differentially expressed genes. The figure shows the GO enrichments of the differentially expressed genes in terms of the biological process, cellular component and molecular function classes

Table 3 Effects of putative SNPs

SNP effect	After filtration		Joined with gene expression results	
	Landrace	Duroc	Landrace	Duroc
3'UTR	17,741	17,715	573	627
5'UTR	3622	3691	62	102
Frameshift	453	516	3	12
Missense	9946	10,424	267	246
Splice acceptor	16	17	0	0
Splice donor	12	12	0	0
Splice site region	338	362	3	7
Start lost	13	8	1	0
Stop gained	68	63	0	3
Stop lost	27	34	0	2
Synonymous	21,060	21,273	610	731
SNP impact				
High	600	663	4	17
Moderate	10,006	10,486	269	250
Low	21,974	22,200	623	752
Modifier	23,251	23,293	676	807

SNP effect according to SnpEff for putative polymorphisms detected in Landrace and Duroc. The results presented are after filtration and joined with differentially expressed genes. Some SNPs have more than one predicted effect

to an independent next generation sequencing dataset, the same result was found. The differentially expressed genes with validated high impact variants were *RAMP2*, *GIMAP6*, *ENSSSCG00000000712*, *ENSSSCG00000009348* and *ENSSSCG00000028326*.

Discussion

Chromatin condensation and DNA packaging in sperm cells occur during testicular spermatogenesis, and altered chromatin structure is associated with sperm DFI. High levels of sperm DFI has been associated with decreased fertility, however, the molecular mechanisms contributing to alterations in sperm DFI is not clear. In the present study, we explored gene expression differences in testis between groups of boars with high and low sperm DFI and investigated the gene enrichments associated with the results. The experiment was performed in two different breeds, Landrace and Duroc, and 308 and 374 genes were found differentially expressed in Landrace and Duroc, respectively. Of these genes, 71 were found to be common for the two breeds, which means they are likely to be essential for alterations in sperm DFI. The Landrace specific and Duroc specific differentially expressed genes might reflect breed specific mechanisms in chromatin condensation and DFI level with regards to these two breeds. Breed differences in DFI have also previously been found in boars as well as bulls [42, 43]. The GO terms "extracellular matrix", "extracellular region" and "calcium ion binding" were significant for both breeds and differentially expressed genes belonging to these pathways are discussed in more detail below. None of the differentially expressed pathways were found to overlap with pathways previously identified for spermatogenesis in Large White, Duroc and Meishan pigs [44, 45], indicating that we have identified pathways related to DFI and not general spermatogenesis.

Genes enriched in "extracellular matrix" and "extracellular region"

The seminiferous tubules in testis contain Sertoli and germ cells and direct progression of spermatogenesis. The "extracellular matrix", an enriched GO term in both Landrace and Duroc, plays a significant role in regulating spermatogenesis because Sertoli and germ cells are structurally and hormonally supported by extracellular matrix during their development in the seminiferous tubules [46]. To complete spermatogenesis, germ cells must migrate across the seminiferous epithelium while still attached to the nourishing Sertoli cells, a process controlled by restructuring events at cell junctions known as ectoplasmic specialization [46, 47]. This is the stage where DNA compaction and chromatin condensation occur [48]. These junctions are located in the "extracellular region" [47], another enriched GO term in both breeds. The results suggest that genes involved in different stages of spermatogenesis affect DNA fragmentation in sperm cells.

Laminins and collagens are important building blocks of the extracellular matrix in testis and they act together with proteases, protease inhibitors, cytokines and focal adhesion components to regulate membrane proteins [46]. Two genes of the laminin family (*LAMB2* and *LAMC3*) and one of the collagen family (*COL3A1*) were found up-regulated in the high DFI group in both breeds in this study. Both pre-collagens and laminins are processed by bone morphogenetic protein 1 (*BMP1*) [49], which was also up-regulated in the high DFI condition in both breeds. Furthermore, genes of the collagen family were exclusively up-regulated in the high DFI group in one of the breeds (*COL1A1* in Duroc and *COL1A2, COL4A1, COL4A2* and *COL14A1* in Landrace). The differential expression of the laminin and collagen genes might suggest that the structure of the extracellular matrix, where the sperm cells are attached during development, could influence chromatin condensation and hence DFI level. This is also supported by the differential expression of genes encoding other components of the extracellular matrix such as the cytokines tumor necrosis factor (TNF) alpha and interleukins. TNFα regulates germ cell apoptosis, Leydig cell steroidogenesis and junction dynamics in the testes [46] and it has also been shown to induce sperm damage such as DNA fragmentation [50]. TNF member *TNFAIP3* was up-regulated in the high DFI group in both breeds in this study. Additionally, breed specific up-regulation in the high DFI group was found for genes of this family (*TNFSF10* and *TNFRSF12A* in Landrace and *LITAF* in Duroc). Interleukin *IL1R1* was up-regulated in the high DFI group in Landrace. This is in agreement with previous findings, where IL1R1 protein was associated with DFI in human sperm and seminal plasma [18, 19].

Genes encoding fibulins, proteases, protease inhibitors and cathepsins, all interacting with components of the extracellular matrix, were also differentially expressed in this study. Fibulins are extracellular matrix glycoproteins that modulate cellular behavior and function and are involved in binding of laminin and calcium [51, 52]. In this study EGF containing fibulin-like EM protein 2 (*EFEMP2*, also known as *FBLN4*) was up-regulated in the high DFI group in both breeds whereas fibulins *FBLN5* and *EFEMP1* (also known as *FBLN3*) were up-regulated in Duroc. Furthermore, extracellular matrix protein 1 (*ECM1*), known to interact with fibulins and laminins [53], was up-regulated in Duroc. The ECM1 protein has previously been found associated with sperm DNA fragmentation in human seminal plasma [19], supporting the findings of this study. Matrix

metallopeptidases (MMPs) and MMP inhibitors (TIMPs) are proteases and protease inhibitors, respectively. They are capable of degrading different components of the extracellular matrix, like laminins and collagen, and thereby regulate spermatogenesis [46, 54]. A disintegrin and metalloproteases (ADAMs) regulate spermatogenesis by cleaving growth factors and cytokines from the extracellular matrix [54]. In this study, *MMP2*, *MMP19*, *TIMP1* and *ADAMTS9* were up-regulated in the high DFI group in Landrace whereas *TIMP3*, *ADAM33* and *ADAMTS4* were up-regulated in Duroc. *ADAMTS4* was the most up-regulated gene in Duroc in this study indicating an important role for proteases in DNA fragmentation of sperm cells, possibly by interrupting with the testicular extracellular matrix stability. Cathepsins contribute in protein degradation in the extracellular matrix by cleaving collagens and laminins [55]. The cathepsin members *CTSA*, *CTSB* and *CTSH* were found up-regulated in the high DFI group of both breeds. Additionally, *CTSC*, *CTSL* and *CTSS* were up-regulated in Landrace. Interestingly, *CTSL* has been linked to chromatin decondensation in sea urchin embryos [56] and CTSA has been shown to affect sperm motility in rats [57]. Moreover, CTSB, CTSC, CTSD, CTSL and CTSS are all involved in testis tissue restructuring during spermatogenesis in rats [58].

Peroxiredoxins are located in the ectoplasmic specialization and encode redox proteins, which protect sperm cells from oxidative stress that cause DNA damage such as DNA fragmentation [59]. In this study, peroxiredoxin *PRDX2* was up-regulated in the high DFI group in both breeds whereas *PRDX3* was up-regulated in the high DFI group in Duroc. Furthermore, glutathione peroxidase *GPX3* was up-regulated in the high DFI group in Landrace. These results are supported by previous findings in human, where levels of peroxiredoxin members PRDX1 and PRDX6 have been associated with sperm DNA integrity [60]. The differentially expressed gene *GPX3* is interesting since glutathione peroxidases can work both as redox proteins and to mediate disulfide bridging, which stabilizes sperm chromatin [61].

Actins are important components of the ectoplasmic specialization of the seminiferous tubules [46, 47] and are involved in the development of mature sperm through several processes, including chromatin remodeling [62, 63]. The *ACTN4* was up-regulated in high DFI boars of both breeds. In Landrace, three additional actin and actin-binding proteins were found to be differentially expressed (*ABLIM1*, *ACTA1* and *ACTA2*). *ACTA1* was down-regulated in the high DFI group, whereas the other actin members were up-regulated, indicating different functions of these actin members when developing DFI in the testis. It was the most down-regulated of the differentially expressed genes in Landrace, suggesting an important role for this gene in DFI levels of this breed. In Duroc, coronin acting binding protein 1B (*CORO1B*) and demantin actin binding protein (*DMTN*) were up-regulated whereas capping protein (actin filament) muscle Z-line, alpha 3 (*CAPZA3*) was down-regulated. The significance of different actin genes between the two breeds could imply breed specialized mechanisms, however, this needs to be further investigated.

In this study, genes encoding extracellular matrix compounds such as collagens, laminins, fibulins and cytokines were differentially expressed. Moreover, peroxiredoxins and actins of the ectoplasmic specialization were up- and down-regulated. Genes involved in regulation of these compounds, like proteases, protease inhibitors and cathepsins, were also differentially expressed. The results confirm previous findings, as well as reporting a number of new genes, highlighting the importance of testicular steroidogenesis in the outcome of sperm DFI. In this study, a major part of the differentially expressed genes were up-regulated. A hypothesis explaining this could be that deficiencies of the extracellular matrix makes the cell compensate by up-regulating gene expression.

Genes enriched in "calcium ion binding"

The GO term calcium ion binding was significantly enriched in both breeds and calcium uptake in sperm is known to be important for the regulation of fertility by affecting sperm maturation, motility, capacitation and the acrosome reaction [64, 65]. A role for calcium in chromatin condensation and DFI is less described, however, the calcium permeable ion channels proteins VDAC2 and VDAC3 have previously shown significant association with DFI in human sperm [18] and fertility in boars [24]. Moreover, along with chromatin condensation in spermatogenesis, the sperm cell redundant nuclear envelope evolves, which has been proposed a role in calcium ion storage [64]. This could explain the significance of the "calcium ion binding" enrichment in both breeds of this study.

The up-regulation of voltage-dependent anion channel gene *VDAC1* in the high DFI group in Duroc is interesting as the proteins VDAC2 and VDAC3 has been associated with DNA fragmentation in human sperm [18]. Moreover, abnormal regulation of different calcium channels has previously been shown to negatively affect sperm function [66]. A number of other genes involved in reproduction related processes where calcium influx plays a role, like hyperactivation, capacitation, the acrosome reaction and fertilization [63], were differentially expressed in this study (*PLCB1* in both breeds, *PLCZ1*, *DLD* and *PLD1* in Duroc, and *PDGFRB*, *CAPN1*,

PLA2G4A, *NPR1* and *RAPGEF3* in Landrace). The up-regulation of all these genes in boars with high sperm DFI could imply an interrupted function of calcium mediated regulation, which would affect the fertilizing capability of these sperm cells after being ejaculated. Further studies are needed, however, to clarify the role of testicular calcium signaling in sperm DFI levels.

Variant detection

An advantage of calling genomic variants from transcriptome sequencing data is that it directly allows for detection of polymorphisms in transcribed regions and is an efficient way to discover putative causative SNPs. Variant detection requires sufficient coverage with high quality sequence reads in order to distinguish true polymorphisms from sequencing errors. Filtering on sequencing depth might have removed polymorphisms in low expression genes, however, visualization by IGV showed likely false positive variants if this filtering was not done. This is in agreement with another study showing that the majority of false positive SNPs occur at sites with less than 10X coverage [67]. Comparing our detected polymorphisms with variants in dbSNP showed that 91 and 88% of our putative polymorphisms in Landrace and Duroc had a corresponding dbSNP entry, respectively. However, only five of the predicted high impact variants had an existing dbSNP entry and a validation study was therefore conducted to test 15 of the putative high impact SNPs. The results showed that high impact variants in the differentially expressed genes *RAMP2*, *GIMAP6*, *ENSSSCG00000000712*, *ENSSSCG00000009348* and *ENSSSCG00000028326* are particularly interesting for sperm DNA fragmentation in boars. Failure to validate ten of the variants shows that SNP detection in short read sequencing data can produce false positives. It has been shown that a number of factors can contribute to false positive SNPs in sequence data, including quality of the reference sequence, read length, choice of mapper and variant caller, mapping stringency and filtering of SNPs [68]. The importance of a high quality reference genome was highlighted in Ribeiro et al. (2015) [68] and we know that the reference genome used in this study has its limitations [69]. Approximately 90% overlap of our identified SNPs and previously identified SNPs does however indicate that our pipeline works, but that caution should be taken especially for variants with no supporting evidence. The identical results of validation using a PCR-based method (KASP) and in silico in an independent dataset could suggest that the latter is equally good in those cases where datasets are available.

Although many of the putative polymorphisms identified are located outside open reading frames or cause synonymous changes, they may be in linkage disequilibrium to other causative mutations. Moreover, studies have also shown that synonymous SNPs may have functional effects by affecting mRNA stability or by translation suppression [70, 71].

Conclusions

The present study identified whole genome expression differences in testis tissue between boars with high and low levels of sperm DFI. Moreover, putative polymorphisms were detected in the differentially expressed genes. The results of this study show that differentially expressed genes of steroidogenic pathways, where the chromatin condensation and DNA packaging occurs, are important for the outcome of DFI levels in ejaculated spermatozoa. Transcriptome sequencing analysis showed that the major changes at transcription level in the testicle of pig concerning sperm DFI were related to the functional categories "extracellular matrix", "extracellular region" and "calcium ion binding". Variant detection showed that predicted high impact SNPs in *RAMP2*, *GIMAP6* and three uncharacterized genes are particularly interesting for the trait. The candidate genes identified in this study provide a valuable resource to identify molecular markers for sperm DFI, for use in selection towards improved sperm quality.

Additional files

Additional file 1: Differentially expressed genes for DFI in Landrace. The results are presented with Ensembl gene id, gene symbol, gene name, fold change and significance level (FDR). (XLSX 31 kb)

Additional file 2: Differentially expressed genes for DFI in Duroc. The results are presented with Ensembl gene id, gene symbol, gene name, fold change and significance level (FDR). (XLSX 34 kb)

Additional file 3: High quality SNPs occurring in differentially expressed genes in Landrace. The SNPs are presented with Ensembl gene id, gene name, FDR value of differentially expressed gene, chromosome (SSC), position, reference allele and alternate allele, as well as effect, impact according to SnpEff and dbSNP ID. (XLSX 100 kb)

Additional file 4: High quality SNPs occurring in differentially expressed genes in Duroc. The SNPs are presented with Ensembl gene id, gene name, significance level (FDR) of differentially expressed gene, chromosome (SSC), position, reference allele and alternate allele, as well as effect, impact according to SnpEff and dbSNP ID. (XLSX 117 kb)

Additional file 5: Heatmap of the differentially expressed genes for DFI. The differentially expressed genes in testis of A) Duroc and B) Landrace boars with high (bad) and low (good) sperm DFI ordered by hierarchical clustering show higher (red) and lower (yellow) expression of genes in the two DFI groups. (TIFF 59 kb)

Additional file 6: The edgeR source code used for testing for differential expression. (TXT 2 kb)

Additional file 7: Putative high impact SNPs in differentially expressed genes. Putative high impact SNPs in differentially expressed genes presented with breed, gene name, position, FDR and log fold change. Validation by KASP SNP Genotyping System (N.A. is for SNPs not tested). (DOCX 12 kb)

Abbreviations

ABLIM1: Actin binding LIM protein 1; ACTA1: Actin, alpha 1, skeletal muscle; ACTA2: Actin, alpha 2, smooth muscle, aorta; ACTN4: Actinin alpha 4; ADAM33: ADAM metallopeptidase domain 33; ADAMs: A disintegrin and metalloproteases; ADAMTS4: ADAM metallopeptidase with thrombospondin type 1 motif, 4; ADAMTS9: ADAM metallopeptidase with thrombospondin type 1 motif, 9; AI: Artificial insemination; AO: Acridine orange; BMP1: Bone morphogenetic protein 1; CAPN1: Calpain 1; CAPZA3: Capping actin protein of muscle Z-line alpha subunit 3; COL14A1: Collagen type XIV alpha 1 chain; COL1A1: Collagen type I alpha 1 chain; COL1A2: Collagen type I alpha 2 chain; COL3A1: Collagen type III alpha 1 chain; COL4A1: Collagen type IV alpha 1 chain; COL4A2: Collagen type IV alpha 2 chain; CORO1B: Coronin 1B; CTSA: Cathepsin A; CTSB: Cathepsin B; CTSC: Cathepsin C; CTSH: Cathepsin H; CTSL: Cathepsin L; CTSS: Cathepsin S; DCT: Dopachrome tautomerase; DFI: DNA fragmentation index; DLD: Dihydrolipoamide dehydrogenase; DMTN: Dematin actin binding protein; ECM1: Extracellular matrix protein 1; EFEMP1: EGF containing fibulin-like extracellular matrix protein 1; EFEMP2: EGF containing fibulin-like extracellular matrix protein 2; ERCC1: Excision repair cross-complementing gene 1; EV: Electronic volume; FBLN4: Fibulin 4; FBLN5: Fibulin 5; FDR: False discovery rate; GEO: Gene expression omnibus; GIMAP6: GTPase, IMAP family member 6; GO: Gene ontology; GPX3: Glutathione peroxidase 3; IGV: Integrative genomics viewer; IL1R1: Interleukin 1 receptor type 1; Indel: Insertion/deletion; LAMB2: Laminin, beta 2; LAMC3: Laminin, gamma 3; LITAF: Lipopolysaccharide-induced TNF; logFC: Logarithmic fold change; MMP19: Matrix metallopeptidase 19; MMP2: Matrix metallopeptidase 2; MMPs: Matrix metallopeptidases; NPR1: Natriuretic peptide receptor 1; NXPH2: Neurexophilin 2; PDGFRB: Platelet-derived growth factor receptor beta; PLA2G4A: Phospholipase A2 group IVA; PLCB1: Phospholipase C beta 1; PLCZ1: Phospholipase C zeta 1; PLD1: Phospholipase D1; PRDX2: Peroxiredoxin 2; PRDX6: Peroxiredoxin 6; RAMP2: Receptor activity modifying protein 2; RAPGEF3: Rap guanine nucleotide exchange factor 3; RIN: RNA integrity number; SAA4: Serum amyloid A4, constitutive; SCSA: Sperm chromatin structure assay; SD: Standard deviation; SNP: Single nucleotide polymorphism; SS: Side scatter; TIMP1: TIMP metallopeptidase inhibitor 1; TIMP3: TIMP metallopeptidase inhibitor 3; TIMPs: MMP inhibitors; TNF: Tumor necrosis factor; TNFAIP3: Tumor necrosis factor, alpha-induced protein 3; TNFRSF12A: TNF receptor superfamily member 12A; UTR: Untranscribed region; VDAC1: Voltage dependent anion channel 1; VDAC2: Voltage dependent anion channel 2; VDAC3: Voltage dependent anion channel 3

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Availability of data and materials

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) [29] and are accessible through GEO Series accession number GSE74934.

Authors' contributions

MvS conducted RNA extraction, analyzed the data and drafted the paper. NHT was involved in the DFI analysis, the SNP detection analysis and contributed to writing the paper. AHG was involved in planning the project, organizing the DFI analysis and contributed to writing the paper, FDM was involved in planning the project, organizing the DFI analysis and contributed to writing the paper, EBS and TTZ conducted DFI analysis, DIV was involved in supervision and contributed to writing the paper, EG was involved in planning the project, supervision and contributed to writing the paper. All authors read and approved the final manuscript.

Ethics approval

All animals were cared for according to laws, internationally recognized guidelines and regulations controlling experiments with live animals in Norway according to the rules given by Norwegian Animal Research Authority (The Animal Protection Act of December 20th, 1974, and the Animal Protection Ordinance Concerning Experiments with Animals of January 15th, 1996). The animals used in this study were AI boars kept as a routine by Norsvin's breeding program. The semen samples were standard procedure whereas tissue samples were taken after slaughter, and no ethics committee approval was needed. Norsvin's trained technicians obtained all the semen samples and BioBank AS (Hamar, Norway) obtained tissue samples, following standard routine monitoring procedures and relevant guidelines. No animal experiment has been performed in the scope of this research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Paper IV

Association between SNPs within candidate genes and fertility in Landrace and Duroc pigs

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Summary

Finding effective predictors of relative boar fertility is essential for increasing the efficiency of artificial insemination (AI) systems in pig breeding. The main objective of this study was to find associations between SNPs within candidate genes and fertility in two pig breeds; Landrace and Duroc. The selected candidate genes were either previously reported to be associated with reproduction traits or are involved in pathways related to reproduction. Animals with contrasting breeding values for fertility were re-sequenced to detect genetic variants. A total of 619 Landrace boars and 513 Duroc boars were genotyped for the detected

candidate gene SNPs. Two SNPs in *BMPR1* and one SNP in *COX-2* in Landrace were found significantly associated with the total number of piglets born. In Duroc, two SNPs in PLC_z, one SNP in *VWF* and one SNP in *ZP3* were found significantly associated to total number of piglets born. These SNPs explained between 0.27% and 1.18 % of the genetic variance. These effects are too low for being used directly for selection purposes, but the associated variants can be of interest in SNP-panels used for genomic selection.

Introduction

The traditional measurements of boar fertility are pregnancy rate and litter size (number of piglets born). However, these measurements are retrospective and highly influenced by the breeding management and the genetic potential of the sows. Some semen quality parameters, such as motility (Flowers, 1997) and morphology (Xu et al., 1998) have been found to be correlated to boar fertility. Combining physical examination of the boar and conventional semen analysis (concentration, morphology and motility) may identify sub-fertile and infertile males, but cannot predict the relative fertility of boars. Finding effective predictors of relative boar fertility would make it possible to exclude the less fertile boars from pig production. The result would improve the AI system, as the number of sperm cells in each dose could be reduced.

Most of the economically important traits in pigs are quantitative, and are influenced by multiple genes as well as environmental effects. It is therefore challenging to identify genetic variants underlying these traits. This is also the case for spermatogenesis, which is a highly specialized cellular process with a number of genes involved (Lin et al., 2006, Chalmel and

Rolland, 2015). Two principal approaches can be used to identify genetic variants affecting quantitative traits, either a genome wide association study (GWAS), or a more restricted analysis of association to selected candidate genes. The GWAS method has its obvious strength in covering the whole genome without any prior assumptions about involved genes. On the other hand, sequencing candidate genes may reveal causative SNPs or variation located very close to causative SNPs, which potentially might show stronger associations than SNPs on a commercial chip, which are selected to be evenly spaced across the whole genome. Several genes have previously been proposed as candidate genes for male fertility, including phospholipase C zeta (*PLCz),* cyclooxygenase isoenzyme type 2 *(COX-2)* and estrogen receptor 1 and 2 *(ESR1* and *ESR2)* (Kaewmala et al., 2012, Gunawan et al., 2012, Gunawan et al., 2011). Furthermore, associations between candidate gene SNPs and boar taint and reproduction have been reported earlier (Moe et al., 2009).

Also when it comes to female reproduction, several genes are known to play an important role both in pigs and in other mammals. The most well-known genes are probably the genes related to ovulation rate and oocyte quality, like bone morphogenic protein 15 (*BMP15)*, bone morphogenic protein receptor 1B (*BMPR1B),* and growth differentiation factor (*GDF9*) (de Castro et al., 2016, Paradis et al., 2009, Juengel et al., 2004, Våge et al., 2013). Several genes have proved to be involved in both male and female reproduction, such as *ESR1* and *ESR2* (Gunawan et al., 2011, Gunawan et al., 2012). Sows were not included in the current study, but genes linked to female reproduction were also included to investigate the boars as maternal grandsires.

The aim of this study was to find associations between SNPs within selected candidate genes and fertility in Landrace and Duroc boars, based on their own performance as well as the performance of their daughters.

Material and Methods

Breeding values

Two separate studies were performed. One focused on male fertility of the boars as sire of their litters and the other focused on female fertility, measured as fertility of the boars' daughters. The estimated variance components and estimated breeding values (EBVs) for total number of piglets born (TNB) were calculated by using a univariate animal repeatability model and included both the direct genetic effect (male fertility) and maternal genetic effect of the sow (female fertility). In addition, herd X year, parity to the mother of the sow, parity of the sow, season as fixed effects, age within parity as fixed regression effects and litter and animal as random effects were included in the model. Only results from purebred litters were used to estimate variance components and EBVs. In total, 86,539 Landrace and 16,819 Duroc had records on TNB. Pedigree for the sows were traced back seven generations and included 61,293 and 13,480 animals for Landrace and Duroc, respectively. The EBVs of direct genetic effect (male fertility) and EBVs of maternal genetic effect (female fertility) are the phenotypes used in the present study.

Animals and sample preparation

Based on their EBVs, 16 Landrace and 16 Duroc boars were selected to be re-sequenced to identify genetic variation in candidate gene regions. Eight Landrace boars and eight Duroc

boars (four with high breeding values and four with low breeding value for each breed) were selected based on male fertility. The other group (with equal number of animals) was selected based on female fertility. The DNA was isolated from sperm cells at BioBank, Hamar, using the Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, Wisconsin, USA). Nucleic acid concentration was measured using Nanodrop 8000 (Thermo Scientific, Waltham, Massachusetts, USA).

For the association study, a total of 619 Landrace boars and 513 Duroc boars were used, for both the investigation of male and female fertility traits. All the boars in this study have been used in AI during the last ten years, with 107 640 litters in Landrace (mean TNB = 13.7 ± 3.6) and 16 849 litters in Duroc (mean TNB = 9.3 ± 2.9). Genomic DNA was extracted from blood or semen using BioSprint DNA Kit (Qiagen, Hilden, Germany). DNA concentration and quality was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA).

Candidate genes

The candidate genes were chosen based on previous reports on involvement in reproduction or having a key role in pathways related to reproduction. Both genes specific to male and female fertility were included. In total, 14 candidate genes were selected for further studies, seven specific for male fertility, five specific for female fertility and two genes important for both male and female fertility. The candidate genes and their known functions are listed in Table 1.

PCR amplification and sequencing

PCR-primers were designed to amplify the coding regions of the selected genes, based on the pig sequences available in GeneBank (*Sscrofa10.2)*, using Primer3 (Untergasser et al., 2012). The primers were purchased from Metabion International AG (Steinkirchen, Germany), and the primer sequences are listed in Supplementary Table 1. PCR was performed using HOT FIREPol® DNA Polymerase (Solis BioDyne, Tartu, Estonia), and the resulting fragments were controlled running a gel stained with ethidium bromide containing a 100kb ladder (New England BioLabs Inc, Ipswich, MA, USA). The PCR products were treated with EXO I (New England BioLabs Inc) and sequenced using a previously described Step method (Platt et al., 2007) with the BigDye® Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA). The sequencing reaction was cleaned up using sodium acetate (NaOAc), EDTA and ethanol (EtOH). The pellets were re-suspended in deionized formamide and analyzed on ABI 3130XL (Applied Biosystems).

SNP detection and SNP genotyping

Sequences were aligned and screened for SNPs using the programs phred, phrap and consed (Gordon et al., 1998, Ewing and Green, 1998). The assay design was done using the software Assay Design Suite (Agena Biosciences, San Diego, CA). The SNPs were genotyped using the Sequenom massARRAY platform (Agene Biosciences, San Diego, CA) at the Centre for Integrative Genetics (CIGENE), Norwegian University of Life Sciences, Ås, Norway. A total of 619 Landrace boars and 513 Duroc boars were genotyped for the candidate-gene SNPs. Genotype clustering and individual sample genotype calls were generated using Sequenom TyperAnalyzer (v.4.0). Newly detected SNPs were submitted to the European Variant Archive (EVA) (https://www.ebi.ac.uk/eva) and are available PRJEB23828.

Statistical analysis

SNPs were filtered based on call rate (> 0.97) and minor allele frequency (MAF) (>0.01). In addition, Plink 1.9 (Purcell et al., 2007) was used to test the deviation from Hardy-Weinberg equilibrium (HWE), and SNPs with HWE < 0.0001 were excluded from further analysis.

Genotype effects were estimated using the following animal model in ASReml (v. 3.0.22.2):

$$y = \mu + SNP + ID$$

where y is EBV for TNB (male or female fertility), SNP genotype (AA, AB or BB) was fitted as a fixed effect and animal ID was fitted as a random effect. A pedigree based relationship matrix was used, and a p-value < 0.05 was considered significant. The genetic variance explained by a SNP was calculated in ASReml as $2p(1-p)\alpha^2$, where α is the allele substitution effect, divided by the additive genetic variance.

Results

Resequencing of candidate genes

Out of the 13 re-sequenced genes, 57 SNPs were found in eight different genes (Supplementary Table 1). Due to difficulties in designing primers for SNPs located physically too closely to each other, only primers for 52 SNPs were designed. In addition to these, one extra SNP (*VWF*) was added to the analysis based on previous results (van Son et al., 2017). Out of these 53 SNPs, 14 did not work in the assay or were monomorphic. These were excluded from further analysis. The complete list of SNPs used for genotyping are presented in Table 2.

Effects of SNPs on fertility

After filtration on MAF > 0.001, HWE > 0.0001 and call rate > 0.97, there were 25 and 21 SNPs left for association analyses in Landrace and Duroc, respectively. There were slightly different results for TNB when the boar was analyzed as father of the litters (male fertility) or father of the sow (female fertility). In Landrace, the analyses showed two significant SNPs, *rs331082315* in *BMPR1* and *rs337596396* in *COX-2* (male fertility), and two significant SNPs, *rs45435443* in *BMPR1* and *rs337596396* in *COX-2* (female fertility) (see details in Table 3). In Duroc, the analyses resulted in three significant SNPs, *rs338483233* in PLC_z, *rs328291649* in *VWF* and *rs339149260* in *ZP3* (male fertility) and two significant SNPs, *rs338483233* and *rs196952431* in PLC_z (female fertility) (see details in Table 4). All the significant SNPs have previously been annotated in the pig genome.

Discussion

In the present study we found SNPs in *BMPR1* and *COX-2* that were significantly associated with male fertility in Landrace. The same SNP in *COX*-2 and another SNP in *BMPR1* were significantly associated to female fertility in Landrace. In Duroc, SNPs in PLC_z, *VWF* and *ZP3* were significantly associated to male fertility, and SNPs in PLC_z were significantly associated to female fertility.

For Landrace, the SNP in *BMPR1B* was found significant in both groups, meaning that this gene might be important for both male and female fertility traits. The gene family member *BMP1* have previously been found differentially expressed in both the Norwegian Landrace

and Duroc boars with high/low levels of sperm DNA fragmentation (van Son et al., 2017). Thus, this study suggests that BMPs have an impact on fertility as reported earlier (Shimasaki et al., 1999).

The significant SNPs found in *PLC$_Z$* in the current study supports the possible importance of the gene involvement in porcine fertility. Both *PLC$_Z$* and *COX-2* are suggested to be important in spermatogenesis through the prostaglandin production. In addition, the relationship between these genes in the spermatogenesis in pigs are reported in an earlier study (Kaewmala et al., 2012). A significant association between polymorphisms within the *COX-2* gene and litter size has previously been reported, while no association of *PLC$_Z$* has been reported (Kaewmala et al., 2012, Sironen et al., 2010). A significant SNP within the *PLC$_Z$* gene was found in Duroc pigs, related to both male and female fertility. This supports the earlier studies reporting the role of this gene in both the egg activation and embryonic development, and in prostaglandin production in males. This SNP was not significant in Landrace. However, a SNP within the *COX-2* gene was found significant in Landrace pigs, both for male and female fertility, suggesting that this gene also might be important in both males and females.

The zona pellucida protein 3 (*ZP3*) have been reported to initiate the sperm binding and to induce the acrosome reaction in sperm cells in humans (Chiu et al., 2008). The acrosome reaction is suggested to be needed for the sperm cells to penetrate the zona pellucida (Chiu et al., 2008). In this study, a significant SNP within the *ZP3* gene was found associated with female fertility in Duroc. This supports the previous findings of the gene's involvement in

reproduction. It makes sense that this SNP is related to female fertility, since this protein exert its function in females (sows).

A significant SNP was found associated to female fertility in the gene encoding Von Willenbrand factor (*VWF*) in Duroc. *VWF* has been suggested to have an effect in the angiogenesis in the porcine oviduct in response to seminal plasma (Krawczynski and Kaczmarek, 2012). *VWF* has also been suggested to be involved in the early stage of endothelium activation and to be sign of vascular bed remodelling in the oviduct (Zanetta et al., 2000, Krawczynski and Kaczmarek, 2012). This could explain the observed association of the *VWF* to female fertility in this study. No association with male fertility was found, despite the previously differential expression observed for this gene in Landrace boars with high/low levels of sperm DNA fragmentation (van Son et al., 2017).

The significant SNPs found in this study were in different genes in the two breeds. This supports earlier findings of breed differences when it comes to reproduction traits (e.g. van Son et al., 2017).

The SNP with largest effect explains 1.18 % of the genetic variance, while the SNP with the lowest effect explains 0.27 % of the genetic variance. The rather low genetic effects for TNB is supported by other association studies for litter size in pigs (Sell-Kubiak et al., 2015, Bergfelder-Druing et al., 2015). In highly selected breeds such as Landrace and Duroc, any gene variant with a major effect on fertility is expected to be fixed. Also, the moderate heritability for litter size (e.g. Holm et al., 2005) indicate a true quantitative nature of these

traits, with a number of "low effect" genes involved. Only a subset of possible candidate genes were included in the current study, so there might of course be other genes with larger effects. However, GWAS-studies in pigs have so far not identified any major QTL related to fertility traits (Trenhaile et al., 2016). The genetic effects obtained in this study are too low to be used for selection purposes directly, but might be useful in a whole genome SNP-panel for genomic selection, since these SNPs are located closer to causal variants than SNPs mainly selected to be evenly spaced SNP across the genome. With the current result and the quantitative nature of the litter size in mind, genomic selection would most likely be the most efficient strategy for breeding of increased litter size.

Conclusion

The current study suggests a role of the genes *BMPR1*, *COX-2, PLC$_z$, VWF* and *ZP3* in total number of piglets born. The SNPs explain between 0.27 and 1.18 % of the genetic variance for TNB, suggesting a limited contribution to the total genetic variation of the trait. Further studies would be needed to discover the functional mutations and to find other genes contributing to the genetic variation of litter size. The SNPs obtained might, however, be of interest in SNP-panels used for genomic selection, since they most likely are more correlated to these traits than randomly selected SNP-markers.

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Table 1 An overview over the candidate genes sequenced in the current study and their functions related to reproduction. The phenotypes used in this study were the boars' property as being a father to the litter (male fertility) and the boars' property as being father to the sow (female fertility).

Gene name	Function	Phenotype Male/Female	Reference
SRD5A	Involved in steroid hormone production Associated with semen quality (semen volume and motility)	M	(Zhao et al., 2012)
PLCz	Important in spermatogenesis, involved in acrosome reaction, induces activation of oocyte during fertilization SNP in non-coding area found associated to sperm concentration	M	(Kaewmala et al., 2012, Fukami et al., 2003, Yoneda et al., 2006)
COX-2	Polymorphism within gene is found associated with litter size through synthesis of prostaglandin	M	(Kaewmala et al., 2012)
ACTB	Actin polymerization during capacitation and acrosome reaction important for the fertilization process	M	(Lin et al., 2006)
CATSPER	Main Ca2+ channel in spermatozoa Essential for sperm hyperactivation and male fertility	M	(Carlson et al., 2003)
AR	Essential for male sexual differentiation and maturation and spermatogenesis	M	(Dirac and Bernards, 2010)
PRM1	Condenzation of sperm chromatin	M	(Domenjoud et al., 1991)
ESR1	Binding of estrogens Spermatogenesis Lack of ESR1 impair male fertility	M/F	(Gunawan et al., 2011)
ESR2	Binding of estrogens SNP associated with several sperm quality and boar fertility parameters	M/F	(Gunawan et al., 2012)
GDF9	Growth factor essential for folliculogenesis A functional SNP strongly associated to litter size in sheep	F	(Våge et al., 2013, Zhang et al., 2008, Juengel et al., 2004)
ZP3	Triggers sperm acrosome reaction Sperm receptor function	F	(Chiu et al., 2008)
CD9	Fusion of sperm to egg plasma membrane	F	(Inoue et al., 2011)
BMP15	Involved in regulation of folliculogenesis	F	(Juengel et al., 2004, Li et al., 2008, Paradis et al., 2009)
BMPR1B	Involved in regulation of folliculogenesis	F	(Paradis et al., 2009)

Table 2 The candidate gene SNPs used for genotyping of 619 Landrace and 531 Duroc boars.

ID	Gene	Chromosome	Position	Allele 1	Allele 2	Concequence
rs692771880	BMPR1	8	133743078	C	T	3 prime UTR variant
rs337443299	BMPR1	8	133743715	A	T	3 prime UTR variant
rs320933602	BMPR1	8	133743777	C	T	3 prime UTR variant
rs332006607	BMPR1	8	133743817	A	T	3 prime UTR variant
rs331082315	BMPR1	8	133743941	T	C	3 prime UTR variant
rs318687807	BMPR1	8	134040860	C	T	5 prime UTR variant
rs319730805	BMPR1	8	134040955	C	A	Upstream gene variant
rs329243677	BMPR1	8	134041001	A	G	Upstream gene variant
rs45435443	BMPR1	8	133765484	G	A	Synonymous variant
rs45435442	BMPR1	8	133765592	G	A	Synonymous variant
rs45435441	BMPR1	8	133765640	G	C	Synonymous variant
rs340645867	BMPR1	8	133865588	C	T	Intron variant
PRJEB23828_1	CD9	5	66893142	C	G	Intron variant
PRJEB23828_2	CD9	5	66892939	G	A	Intron variant
PRJEB23828_3	CD9	5	66883110	C	T	Intron variant
rs337596396	COX-2	9	140252549	C	T	3 prime UTR variant
rs318653228	COX-2	9	140252612	G	A	Non coding transcript exon variant
rs328855871	COX-2	9	140253513	C	T	Non coding transcript exon variant
rs692715832	COX-2	9	140254511	C	T	Splice donor variant
rs345073121	COX-2	9	140254614	G	A	Non coding transcript exon variant
rs328234523	COX-2	9	140254638	C	T	Non coding transcript exon variant
rs333336289	COX-2	9	140258830	C	T	Intron variant
rs322393640	ESR1	1	16577100	C	T	Synonymous variant
rs81219139	ESR2	1	215750855	A	G	Missense variant
rs338483233	PLCz	5	57682420	G	A	Missense variant
rs196952431	PLCz	5	57693191	T	C	Synonymous variant
rs342130351	PLCz	5	57700340	C	T	Synonymous variant
rs45435460	SRD5A	3	114655903	C	T	3 prime UTR variant
rs692885360	SRD5A	3	114655905	T	C	3 prime UTR variant
rs326135378	SRD5A	3	114656677	A	G	3 prime UTR variant
rs328291649	VWF	5	67005497	C	T	Missense variant
rs331519492	ZP3	3	9837795	G	A	Upstream gene variant
rs321573987	ZP3	3	9840472	C	T	Intron variant
rs339149260	ZP3	3	9840522	G	T	Intron variant
PRJEB23828_4	ZP3	3	9840611	C	T	Intron variant
rs337462558	ZP3	3	9848217	G	T	Downstream gene variant

Table 3 The significant SNPs for Landrace (NL) and Duroc (ND) boars based on their performance as father (male fertility). Positions are given according to pig genome build *Sscrofa10.2* obtained from the ENSEMBL web site (http://www.ensembl.org).

Breed	Gene	Genbank	Chromosome	Position	Genotype	SNP database	Genetic variance (%)	p-value	MAF	HWE	Consequence
NL	BMPR1	NW_003610967.1	8	133743941	[C/T]	rs331082315	0.92	0.010	0.21	0.58	3 prime UTR variant
NL	COX-2	NW_003300644.3	9	140252549	[C/T]	rs337596396	1.18	0.010	0.48	0.78	3 prime UTR variant
ND	ZP3	NM_213893.1	3	9840522	[G/T]	rs339149260	0. 55	0.018	0.046	0.19	Intron variant
ND	PLCz	NM_214350.1	5	57682420	[A/G]	rs338483233	0.71	0.025	0.44	0.89	Missense variant
ND	VWF	NM_001246221.1	5	67005497	[C/T]	rs328291649	0. 91	0.010	0.43	0.74	Missense variant

Table 4 The significant SNPs for Landrace (NL) and Duroc (ND) boars based on the performance of their daughters (female fertility). Positions are given according to pig genome build *Sscrofa10.2* obtained from the ENSEMBL web site (http://www.ensembl.org).

Breed	Gene	Genbank	Chromosome	Position	Genotype	SNP database	Genetic variance (%)	p-value	MAF	HWE	Consequence
NL	BMPR1	NW_003610967.1	8	133765484	[A/G]	rs45435443	0.27	0.039	0.046	0.82	Synonymous variant
NL	COX-2	NW_003300644,3	9	140252549	[C/T]	rs337596396	0.67	0.042	0.48	0.78	3 prime UTR variant
ND	PLCz	NM_214350.1	5	57682420	[A/G]	rs338483233	0.82	0.016	0.44	0.89	Missense variant
ND	PLCz	NM_214350.1	5	57693191	[C/T]	rs196952431	0.71	0.030	0.43	0.62	Synonymous variant

Supplementary Table 1 The primers used for re-sequencing the candidate genes.

Gene	Exon	F/R	Sequence	Tm	% GC	Lenght	ANY	SELF	3' stability	F/R	Sequence	Tm	% GC	Lenght	ANY	SELF	3' stability
ZP3	Ex1	F	TGGCCTCTAACATTCCCTGT	59.5	50	20	4	1	7.9	R	CCCACCTCCAACAGCTACAT	60	55	20	4	2	5.6
	Ex2	F	CCCAGTGTCTCTGGTCCATT	60	55	20	7	1	8.4	R	AGCTGTGGGCTGAACAGATT	59.9	50	20	4	1	6.6
	Ex3-5	F	GGTCAGGCTGAGACTTGAGG	60	60	20	7	1	8.2	R	TACCAAGTCGCCGAATAAGG	60.1	50	20	5	3	7.5
	Ex6	F	ACCACTGCACCACCTAGGAC	60	60	20	6	2	7.6	R	GTCAGCTTTTCCACCCAGTC	59.7	55	20	4	1	6.4
	Ex7	F	GACCCTGGTCCTTCCTTCTC	60	60	20	6	0	6.7	R	GACTGCATCCCCCAGAGTAA	60.1	55	20	4	1	5.7
	Ex8	F	AGTTCTGACCCGTCATCAGG	60.1	55	20	6	2	8.2	R	AAATGCAGGCCTGTCTTCAC	60.3	50	20	8	2	6.7
ESR2	Ex1	F	TGTCCCTTTGTGCCTCTTCT	59.8	50	20	2	0	6.7	R	TGATTTGAGTAATGCCCATGAA	60.3	36.4	22	4	2	6.9
	Ex2	F	GCTTGATCGGATTTCTTGGA	60.2	45	20	5	1	8.5	R	GGTCACCTGCGAATTTGTTT	60	45	20	5	0	7
	Ex3	F	TCCCCTGTCTCTCAGCTTGT	60	55	20	4	0	6.7	R	TCCCATGTCTCCTCTCATCC	60	55	20	4	0	8.1
	Ex4	F	AGTGACTGGGGGAACTTGTG	60	55	20	5	0	7	R	CTGGTGGACCTCCATCTTGT	60	55	20	7	0	6.7
	Ex5	F	CTTCCTTGGCAGCATTTAGC	60	50	20	4	2	7.5	R	ATGCTCTCCTTCTTCGGTGA	60	50	20	3	2	7.9
	Ex6	F	TTCTGGCTGTGGTCTCATTG	59.8	50	20	3	3	7.2	R	GAAAGGCATTTTCCCCTTTT	59.4	40	20	6	1	7.3
	Ex7	F	CTGAACAACCCAGGTCCACT	60	55	20	4	1	7.9	R	AAACTTGCACAATCCGTTCC	60	45	20	4	0	7.9
	Ex8	F	GTGAACAGCAAGACCCCATT	60	50	20	2	1	8.4	R	AGTCCACCCACTGCTTATGG	60	55	20	3	0	7.4
GDF9	Ex1	F	GAGGCTGAACAGGCGATAAA	60.4	50	20	4	0	6.2	R	TCAAGTACCGCATTTGCTTG	59.9	45	20	5	2	8.5
	Ex2	F	AGAAAGGGTTCACGAAAGCA	59.9	40	20	4	0	8.5	R	CCATTGAAGGAGCAGGGTTA	60.1	50	20	3	2	7.2
PLCz	Ex1	F	GGTGTTCAGACCGAAAGGAA	60.1	50	20	5	0	8.2	R	CAGTGGGTTGGAGGAAGCTA	60.2	55	20	4	2	7.2
	Ex2	F	TGCAGGGACCACATCTTACA	60.1	50	20	4	1	6	R	TCAGGGCCTTTCAAAAAGAA	59.8	40	20	5	1	7
	Ex3	F	CATGAGATAGACTGCCCTCTGA	59.5	50	22	4	3	6.7	R	TCCTTCCTAGGGTGGAGTGG	61.4	60	20	8	0	7.9
	Ex4	F	TTCACAATATGAGCCCACCA	59.9	45	20	5	0	8.2	R	CCAGAAGAAATGTGACCCTGT	59.1	47.6	21	3	1	7.9
	Ex5	F	GTCTTCTGGGGTGCTGTCAT	60.1	55	20	3	2	6.3	R	GAGGGTGGCAGACAGGATTA	60.1	55	20	3	2	5.9
	Ex6	F	GAAGAAGAGGGAGGGAGGAA	60.1	55	20	0	0	8.2	R	GGCCAAGCATTACAGGATTT	59	45	20	4	0	6.9
	Ex7	F	TGCTCTACCTCCTTTGTGACAG	59	45	20	5	3	6.4	R	TTCAGCTGAGCTTCTCAGACA	59	47.6	21	8	3	6.4
	Ex8	F	GCTTTGGGCAAATTGAAAAT	59.1	35	20	6	3	7.2	R	TTAGACACCAGCCTGGGAAC	60.1	55	20	7	3	7.9
	Ex10	F	TGTGAACCATAAAGGCATGAA	59	38.1	21	4	0	6.9	R	CAAAACACCTAGGGCCAAAA	60	45	20	6	0	7.6
	Ex11	F	GACATGGCAGAAGGAAATGTT	59	42.9	21	4	2	6.6	R	TGCTGAAATACCACCCACCT	60.4	50	20	2	0	7.9
	Ex12	F	TTTTGGTGTGTGGGAATTGA	59.8	40	20	4	1	6.9	R	TTGCTGCATATAACACATTGGTC	59.9	39.1	23	5	1	7.9
	Ex13	F	TCCCCTAGGGAGAGTGTTTG	59.1	55	20	8	0	7	R	CCCCAACTCCACCTTCTAAA	59	50	20	2	2	6.3
	Ex14	F	TGGAGATGCTGCATTTTGAG	59.9	45	20	7	1	7	R	CTGGCCTGAGATTTCCCATA	60	50	20	4	2	7.4
SRD5A2	Ex1	F	CCAGTCCTGGGTTTGTAGGA	60	55	20	6	0	7.2	R	AACTGGGTCGTGGTGAGAAG	60.2	55	20	2	0	6.7
	Ex2	F	GCACAAATACGCTTTGCTGA	60	45	20	4	2	8.2	R	CGAGGAAGAGTGGGAATCTG	59.8	55	20	3	1	6.6
	Ex3	F	TTGCTGGTTCTTCTGCACAC	60	50	20	4	2	6.4	R	ACTTGGGTTTGGTTGTGCTC	60	50	20	2	2	8.2
	Ex4-1	F	TGACTGCAGGCTGATTTGTC	60	50	20	6	1	6.7	R	CATCCCTCTGGTTGCAGTTT	60.1	50	20	4	0	6.7
	Ex4-2	F	GCGGTCTTAGTGCCTCTGTC	60	60	20	3	1	6.4	R	GACATCTGAGCTGTGGACGA	60	55	20	7	1	8.1

AR	Ex1	F	GTTGCATTTGCTTTCCACCT	60.1	45	20	4	0	7.9	R	AGGGAGTCACCTCTCTGCAA	60	55	20	5	2	8.8
	Ex2-1	F	ACCCCTTCAAGATTTGAGCA	59.7	45	20	6	2	8.2	R	CGACTCGGATAGGCTGCTAC	60	60	20	5	1	6.9
	Ex3	F	TGGGAGAATTCCAAGGAAAA	59.5	40	20	6	2	7.3	R	GGGGACCAGCAAGTATGAAA	59.9	50	20	3	1	7.3
	Ex4	F	CCTGGGAACCTTCATATGCT	59	60	20	6	2	8.1	R	GGAAAGTAAAGGGGGAAGGA	59.4	50	20	2	0	8.2
	Ex5	F	GCACTTGCCCTTAAAGGAGTT	59.8	47.6	21	7	2	6.4	R	TGGTCCATAGGTGCATTCAA	59.9	45	20	5	3	7.3
	Ex6	F	CACTGCCTCTGCCCTATCTC	60	60	20	3	0	6.3	R	GTCACCCAGGTCTGGACAGT	60	60	20	6	3	6.1
	Ex7	F	CCTGTCTCAGGAAGCAGGAG	60.1	60	20	6	2	7.9	R	AGCTCCCCTTTGATTCCCTA	60	50	20	4	2	8.7
	Ex8	F	CTAGCCTTTTCCCCAAGACC	60.1	55	20	4	0	7.6	R	ACTCAAAGCCAGAGGGGAAT	60.1	50	20	3	2	8.1
CATSPER1	Ex2	F	CTCAGGCAGACCTCAAGTCC	60	60	20	5	3	7.6	R	TCACTCTAAGGCTGGGCAAT	59.8	50	20	3	2	8.4
	Ex8-9	F	CACGAATCCAAAGGAGGGTA	59.9	50	20	3	2	8.4	R	TTTTAGGGCCATACCCACAG	59.8	50	20	6	1	6.7
	Ex10	F	ACTAGTCGGGTTCGTCATCG	60.1	55	20	6	2	8.6	R	GGCACAAGGTGACTCTGGTT	60.2	55	20	5	3	8.2
	Ex11-12	F	ATAATCGTCGGAGGCTGTGT	59.6	50	20	3	1	6.4	R	CAGCAGATCTGGGGACTTGT	60.3	55	20	8	2	6.7
BMP15	Ex1	F	ATTTTGGCTTGTGCTGGAAC	60.1	45	20	2	2	7.9	R	CCCAAGGCAATCTCTCGTTA	60.2	50	20	2	2	7.7
	Ex2	F	GATCCTCAACCCACCGAGTA	59.9	55	20	4	2	5.4	R	CCCATAGATTTGGCAGAGGA	60	50	20	4	0	7.9
ESR1	Ex1	F	AGGGATTCTTGGAACCTTGG	60.3	50	20	3	2	8.5	R	CTTTCTTCCTTGGCCTTCCT	59.8	50	20	4	0	8.2
	Ex2	F	GAGCGGTACTCTGGCATCAG	61	60	20	4	2	6.6	R	CGCTTTCTCACGCTCTCTCT	60	55	20	2	0	6.4
	Ex3	F	TGTGATTGTGCCTTCAGAGC	60	50	20	3	2	7.9	R	ATGAAGAGAATGGGCTGTGG	60.1	50	20	2	0	8.2
BMPR1B	Ex1-1	F	CAGAAACCCTCCATCCTTCA	60	50	20	3	1	7	R	CAGCTTTCAGGGGATATGGA	60	50	20	4	0	8.1
	Ex1-2	F	CCCTCCCTCCCTTAACTCAG	60.1	60	20	4	3	6.7	R	TTGACCATTGGCATGAGAAA	60	40	20	5	2	7
	Ex2	F	TCACAACGACCTGGTGTCAT	60	50	20	5	3	6.3	R	TGGAGCCCAGTTGGTTAGAC	60.1	55	20	4	2	5.4
	Ex3	F	TTTCTGGTCAGCGAGGACTT	60	50	20	4	1	6.4	R	AATCCCCTTGACTGGTCCTT	59.8	50	20	4	0	8.2
	Ex4	F	TTCCTTCTTTTTCCCTGCAA	59.8	40	20	4	2	8.8	R	AACAAGGAGCTCCGAACCTT	60.2	50	20	8	3	7.9
	Ex5	F	CTGTGAGGGCTCCCATAAGA	60.2	55	20	6	2	6	R	TTCAACAAATCTGTCGTGCTG	59.9	42.9	21	6	1	8.5
	Ex6	F	CCTCTGCCTCAGGAGTAGGA	59.5	60	20	7	3	7.2	R	TGGGTTAAGCCACACCTTCT	59.6	50	20	8	0	6.7
	Ex7	F	ATCTGGCCAATGAAAGGTTG	59.9	45	20	6	0	8.2	R	GGTACCTGCGAAAGACCAAA	60.1	50	20	6	0	8.8
	Ex8	F	GTGCGGCTCCATGAGTTAAT	60.1	50	20	4	2	6.2	R	GCTGGGCTATCCCACAGATA	60.1	55	20	8	3	5.6
	Ex9	F	TCCTGATCAGCTTGAACGTG	60	50	20	8	3	8.1	R	AACACAGACCTGTGCCATGA	60.2	50	20	8	2	6.9
	Ex10	F	CTTCACGAGCACCTCCTCTC	60.1	60	20	4	0	6.4	R	TTATGTTGCCTCCAGTCGTG	59.7	50	20	2	1	8.4
COX-2	Ex1-1	F	CAGGGCCAGTTTGGAATAGA	60.1	50	20	4	0	5.6	R	GAACTGAGATCCCCCATCAA	59.9	50	20	5	0	6.9
	Ex1-2	F	CTGCAGCCTTAAACCCAGTT	59.4	50	20	6	3	6.7	R	AGGCTGCCCTTTTACCTCAT	60.1	50	20	5	2	6.6
	Ex1-3	F	CAGCATTTTGCCGTCTTGTA	59.9	45	20	3	2	6	R	TGAGCGGAGGGGTAAAACTA	57.9	50	20	2	2	5.7
	Ex2	F	GCTTTCCTCTCCTGTTTCCA	59.4	50	20	2	0	8.5	R	TCTTTGTACCCAAGGGATGC	59.9	50	20	4	2	8.1
	Ex3	F	CTGACTAAGCCCTTTGTTTCG	59	47.6	21	6	2	9	R	CCCTGGGCTTGATTAGGAAT	60.3	50	20	5	3	8.1

	Ex4	F	TTCCCTGGAGAAGAGCGTTA	59.9	50	20	5	2	7.7	R	CAGCATCATTAACGATTGGAA	58.6	38.1	21	8	2	8.5
	Ex5	F	CAATTTAAACCTCCCCCGATA	60	42.9	21	6	2	7.6	R	TTCATTATTTTGTGGCTGTGAAA	59.5	30.4	23	4	1	7.3
	Ex6	F	TGGCCTTTAATGTTCACACTTC	59.1	40.9	22	4	2	6.4	R	TGACAGCCACTCTCTGCTTC	59.3	55	20	3	0	8.2
	Ex8	F	TTGAGGCAGCTAAAAACCCTA	59	42.9	21	4	2	8.7	R	GCACCCGAACAGGATTCTAC	59.6	55	20	4	0	5.4
	Ex9	F	ATTCCAAACAAGGGCACTCA	60.5	45	20	4	1	6.4	R	CACCACGGGAACTCTGGTAT	59.8	55	20	4	2	6.8
ACTB	Ex3	F	TGTTTCTGCCTGCTTCCTTT	60	45	20	2	0	8.5	R	GAGGTCCTTCCTGATGTCCA	60	55	20	5	2	7.9
	Ex4	F	CTCTTCCAGCCCTCCTTCTT	60	55	20	2	0	7	R	GAGCCGCGTGTGTGTAACTA	59.9	55	20	4	2	5.7
	Ex5	F	ACGTGGACATCAGGAAGGAC	60	55	20	4	1	7.6	R	TGGTTTTCCCAAGTCAGACC	59.9	50	20	5	3	7.6
CD9	Ex2	F	CACGCAGTACCCCTTCTCTC	59.9	60	20	4	0	6.4	R	GGCTTGGTCAACTTGGAAAA	60.1	45	20	5	3	7.3
	Ex3	F	CATCTCCTCGTCCTGTCCTC	59.8	60	20	2	0	7.9	R	GAGACAGAGCCCTGCTCAAG	60.3	60	20	8	3	7
	Ex4	F	ACACCTCACCTCAGCCCTTT	61.1	55	20	3	0	8.5	R	ATTGAAGGCTGACCCAAGTG	60.1	50	20	3	2	6.7
	Ex5	F	GTGGCTGAGCATGAATGAGA	60	50	20	5	0	6.7	R	AAGCAAGGGGAGGAGAGAAG	60	55	20	2	0	6.7
	Ex6	F	TTGTCTCTCTCACCCCATCC	60	55	20	2	0	8.1	R	ATGGTGGGTCCTTCCTGAG	59.9	57.9	19	3	3	6.7
	Ex7	F	CGTGTGTGACCTGGATCTTG	60.2	55	20	4	2	7	R	CCCCAAAACAAAAGCAAAAA	60	35	20	2	0	7.6
PRM1	Ex1	F	CAAAGTTCCACCTGCTCACA	59.9	50	20	3	1	6.7	R	AGTGCGGTGGTCTTGCTACT	59.9	55	20	3	1	5.4
	Ex2	F	TCACCATGGCCAGATACAGA	60.1	50	20	6	0	6.4	R	CAAGGCAAATGAGACCAGTG	59.3	50	20	3	3	6.7

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