Institut für Tierwissenschaften, Abt. Tierzucht und Tierhaltung
der Rheinischen Friedrich – Wilhelms – Universität Bonn

Association and expression study of CD9, PLCz and COX-2 as candidate genes to improve boar sperm quality and fertility traits

I n a u g u r a l – D i s s e r t a t i o n
zur Erlangung des Grades

Doktor der Agrarwissenschaft
(Dr. agr.)
der
Hohen Landwirtschaftlichen Fakultät
der
Rheinischen Friedrich – Wilhelms – Universität
zu Bonn

vorgelegt im Februar 2011

von

Kanokwan Kaewmala

aus

Chiangmai, Thailand

Referent:	Prof. Dr. K. Schellander
Korreferent:	Prof. Dr. B. Petersen
Tag der mündlichen Prüfung:	31 March 2011
Erscheinungsjahr:	2011

Dedicated to my beloved family for their supports throughout my Ph.D program. This research is also dedicated to my master's advisor and all friends for their concerns and their encouragements

Assosiations- und Expressionsstudie der Kandidatengene CD9, PLCz und COX-2 zur Untersuchung ihres Effektes auf die Spermaqualität von Ebern und Fruchtbarkeitsmerkmake

In Studien über Ratten, Mäuse und Menschen spielen die Transkriptionsfaktoren Tetraspanin CD9 (CD9), Phospholipase C zeta (PLCz) und Cyclooxygenase-Isoenzym Typ 2 (COX-2) eine wichtige Rolle in der Spermatogenese. Daher war das Ziel dieser Studie die Identifizierung von Polymorphismen und die Erstellung von Expressionsmuster dieser Gene für die Spermaqualität und Fruchtbarkeit, zu untersuchen. Die Assoziationanalysen der SNP mit Spermienqualitätsmerkmalen (Spermienkonzentration [SCON], Spermavolumen pro Ejakulat [VOL], Spermienmotilität [MOT], Plasmatröpfchenrate [PDR] sowie abnormale Spermienrate [ASR]) und den Fruchtbarkeitsmerkmalen (Non Return Rate [NRR] und Anzahl der Ferkel lebend geboren [NBA]) wurde mit 231 Tieren reinrassiger Pietrain (PI) Eber, 109 Tieren einer Pietrain × Hampshire Kreuzung (PIHA) und 340 Eber, die für die Analyse aus PI und PIHA (COMBINED) Ebern kombiniert wurden, durchgeführt. Darüber hinaus wurden die mRNA und Proteinexpressionsmuster. Dafür wurden Eber mit einer höheren Spermienkonzentration, Spermienmotilität und einem niedrigerem Spermienvolumen (G-I Gruppen) und Ebern mit einer geringeren Spermienkonzentration, Spermienmotilität und einem höheren Spermienvolumen (G-II Gruppen) ausgewählt. Das Ergebnis zeigte, dass der SNP im Intron 6 (A>T) von CD9 mit MOT ($p < 0,05$) und PDR ($p < 0,05$) in allen drei Gruppen signifikant assoziiert war aber nur signifikant mit ASR ($p < 0,01$) in der PIHA und in der COMBINED. Außerdem zeigte der SNP im Intron 1 (A>C) von PLCz mit SCON ($p < 0,05$) in der PIHA eine Assoziation. Der SNP im Intron 9 (G>A) von COX-2 zeigte keine Assoziation mit der Spermaqualität und den Fruchtbarkeitsmerkmalen. Eine erhöhte CD9, PLCz und COX-2 Proteineexpression konnte in Leydig-Zellen, Sertoli-Zellen, Epithelzellen, Keimzellen und im Akrosom und in der akrosomalen Membran reifer Spermien gefunden werden. Eine höhere CD9 und PLCz mRNA Expression ($p < 0,05$) wurden in Nebenhoden und Hoden in G-I im Vergleich zu G-II Ebern gefunden. Jedoch war die Expression von COX-2 im Hoden, Kopf und Nebenhodens in G-II ($p < 0,05$) höher als in G-I Ebern. Daher kann die vorliegende Studie zeigen, dass die Gene CD9, PLCz und COX-2 an der Spermatogenese von Ebern beteiligt sind.

Association and expression study of CD9, PLCz and COX-2 as candidate genes to improve boar sperm quality and fertility traits

The transcription factors tetraspanin CD9 (CD9), phospholipase C zeta (PLCz) and cyclooxygenase isoenzyme type 2 (COX-2) are believed to play a key role in spermatogenesis in rat, mouse and human. However, no study was devoted to associate the single nucleotide polymorphism (SNP) and expression pattern of three genes with sperm quality and fertility traits in boars. Therefore, this study aimed to investigate the polymorphism and expressional pattern of these genes with boar sperm quality and fertility. The association of SNP with sperm quality traits (sperm concentration [SCON], semen volume per ejaculate [VOL], sperm motility [MOT], plasma droplets rate [PDR] and abnormal spermatozoa rate [ASR]) and the fertility traits (non return rate [NRR] and number of piglet born alive [NBA]) were analyzed using 231 of purebred Pietrain (PI) boars, 109 of Pietrain × Hampshire crossbred (PIHA) boars and 340 of the combined analysis from PI and PIHA boars (COMBINED). In addition, to study the mRNA and protein expression pattern of those genes in boar reproductive and non reproductive tissue, three boars having higher sperm concentration, sperm motility and lower sperm volume (G-I groups) and three boars having lower sperm concentration, sperm motility, and higher sperm volume (G-II groups) were selected. The result showed that the SNP in intron 6 (A>T) of CD9 was significantly associated with MOT ($p < 0.05$) and PDR ($p < 0.05$) in all groups and only with ASR ($p < 0.01$) in the PIHA group and the COMBINED group. Moreover, the SNP in intron 1 (A>C) of PLCz was associated with SCON ($p < 0.05$) in the PIHA group. However, the SNP in intron 9 (G>A) of COX-2 showed no association with sperm quality and fertility traits. In addition, the mRNA and protein expression of the CD9, PLCz and COX-2 were found to be different between reproductive tissues and non reproductive tissues of boars. Elevated CD9, PLCz and COX-2 proteins expression were found in Leydig cells, Sertoli cells, epithelial cells, germ cells and the acrosome and acrosomal membrane of mature spermatozoa. Importantly, higher CD9 and PLCz mRNA ($p < 0.05$) was found in body of epididymis and testis in G-I compared to G-II boars. However, the expression of COX-2 in testis, head and body of epididymis was higher ($p < 0.05$) in G-II compared to G-I boars. Therefore, the present study may indicate that the CD9, PLCz and COX-2 genes are involved in the spermatogenesis of male pig.

Contents Page

List of abbreviations

A	Adenine
A280	Absorbance at 280 nm wavelength (UV light)
AI	Artificial insemination
ATP	Adenosine triphosphate
bp	Base pairs
BLAST	Basic local alignment search
BSA	Bovine serum albumin
C	Cytosine
CD9	Cluster of differentiation 9
cDNA	Complementary deoxyribonucleic acid
COX-2	Cyclooxygenase isoenzyme type 2
CT	Threshold cycle
Cy3	Cyanine 3 fluorescent dye
Cy5	Cyanine 5 fluorescent dye
ddH_2O	Distilled and deionised water
DEPC	Diethylpyrocarbonate
DNA	Deoxynucleic acid
dNTP	Deoxyribonucleoside triphosphate (Usually one of dATP, dTTP, dCTP and dGTP)
DTT	Dithiothreitol
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid (powder is a disodium salt)
EtBr	Ethidium bromide
EtOH	Ethanol
FSH	Follicle stimulating hormone
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
GnRH	Gonadotropins releasing hormone

LH	Luteinizing hormone
min	Minute
$MgCl_2$	Magnesium Chloride
mRNA	Messenger RNA
MW	Molecular weight
NaCl	Sodium chloride
OD260	Optical density at 260 nm wavelength (UV light); = A260
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Prostaglandin (PGF2α)
PLCz	Phospholipase C zeta
qPCR	Quantitative polymerase chain reaction
rpm	Revolution per minute
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAS	Statistical Analysis System
SDS	Sodium dodecyl sulfate
SSC	Sodium chloride – sodium citrate buffer
SNP	Single nucleotide polymorphism
TAE	Tris-acetate buffer
TBE	Tris- borate buffer
TE	Tris- EDTA buffer
TEA	Triethanolamine
Thr	Threonine
tRNA	Transfer RNA
UV	Ultra-violet light
V/V	Volume per volume
W/V	Weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

List of tables Page

List of figures Page

List of appendices Page

1 Introduction

The fertility and sperm quality are important parameters for the selection of breeding boars in the pig breeding industry. The association and gene expression studies are important way to investigate genetic merits of animals for specific traits and to focus on speculated candidate genes. The phenotype expression of fertility traits depends not only on the underlying genotype but is also modulated by environmental deviations. Due to that, the detection of major genes within the reproductive metabolism is a great concern. The economical importance of traits of the boar fertility is the reason for improving selection of boar. Sperm quality will be first determined in the adult boar, whereas the fertility, measured as the non-return rate of the mated sows will be observed even later.

The study of reproductive tissue including non-reproductive tissue is important to predict gene functions. Sperm is produced through complex processes within the testis, epididymis and other parts of male reproductive tract. During the journey through male reproductive tract, sperm compromise to different developmental and maturation stages (Borg et al. 2010). In the epididymis, the head of the sperm incorporates proteins into the membrane, which are essential to fertilize the egg and at the final phase of spermatogenesis, the prostate gland secrete prostate fluid which helps prolonging the life span of sperm (Primakoff and Myles 2002). In the mouse, spermatogenesis is the establishment of a stem cell population, mitosis, meiosis and the morphogenesis of the haploid germ cell, which involve the coordinated expression of more than 2300 different genes (Schultz et al. 2003). That so many germ cell-specific genes are expressed in development provides a large number of potential participants in the process of fertilization. Despite that, there are still many uncertainties in the processes of spermatogenesis, sperm development, sperm maturation and fertilization. Functional male gametes are produced through complex processes within the testis, epididymis and other male reproductive tract. Failure of any of these events leads to disturbances of male fertility.

In this thesis three candidate genes have been identified via their involvement in key processes of reproduction in pigs and in other species such as human, cattle, rat, hamster. Cluster-of-differentiation antigen 9 (CD9) is a member of the tetraspanin or

transmembrane 4 (TM4) superfamily (Horejsi and Vlcek 1991) and is expressed in many cell types including spermatogonial stem cells (Klassen et al. 2001, Oka et al. 2002). CD9 participates in several cellular processes such as cell migration and adhesion, malignant metastasis (Boucheix et al. 2001) and is reported to be a surface marker on mouse and rat male germline stem cells (Kanatsu-Shinohara et al. 2004). The phospholipase C zeta (PLCz) is a mammalian phospholipase C (PLC), an essential fatty acids for arachidonic acid synthesis which is the precursor for inflammatory prostaglandins (Walter 2003). Several adaptor molecules are thought to be associated with PLC and the prostaglandin is synthesized from arachidonic acid by cyclooxygenase isoenzyme type 2 (COX-2). Polymorphisms within COX-2 gene are reported to have significant association with litter size through the effects of prostaglandins production in Landrace pigs (Sironen et al. 2009). Moreover, several studies showed that prostaglandin plays important roles in the spermatogenesis process in the male reproductive tracts in many species for instance in hamster Leydig cells (Frungieri et al. 2006, Matzkin et al. 2009), rat testis (Winnall et al. 2007, Yamaguchi et al. 2008), human Leydig cells (Sirianni et al. 2009), rat vas deferens (McKanna et al. 1998, Ruan et al. 2008), and human prostate gland (Rubio et al. 2005). Since both genes are involved in prostaglandin production, they might be correlated for their functions in spermatogenesis. Therefore, our attempt was to contribute to overcome these disturbances through the association study and identification of potentially expressed candidate genes in male gametes as well as in reproductive tissues in pig. This study focuses also on the association of these genes with boar fertility and sperm quality traits and the differential expression of CD9, PLCz and COX-2 mRNA and protein in different reproductive tissues from boars from divergent phenotype.

2 Literature review

2.1 Boar reproductivity

The reproductive system is the interactions of organs or substances within an organism that strictly pertain to reproduction. From anatomical and morphological assessment, puberty in entire male pigs is characterized by increased size of reproductive organs such as testis and bulbourethral gland, and proliferation of Leydig and Sertoli cells in the testis (FranÃ§a et al. 2000, Harder et al. 1995, Lunstra et al. 1986). In the testes, LH binds to receptors on Leydig cells, inducing steroidogenic enzymes and increasing gonadal hormone secretion (Zamaratskaia et al. 2005). In steroid-treated pigs, the much higher levels of fructose and zinc found in the seminal vesicles (Booth 1980). In the epididymis, the head of the sperm incorporates proteins into the membrane, which are essential to fertilize the egg and at the final phase of spermatogenesis, the prostate gland secrete prostate fluid which helps prolonging the life span of sperm (Primakoff and Myles 2002). The distribution of some glycoconjugates has been studied in boar testis and epididymis. The presence and distribution of galectin-3, a β-galactoside-binding protein, in boar testis and epididymis was studied (Kim et al. 2006). The galectin-3 plays an important role in the mucosal epithelium, as well as in the maturation of sperm in the lumen, as do epididymal secretory proteins in boar (Jean-Louis et al. 2003, Jean-Louis et al. 2005). During the journey through male reproductive tract, sperm compromise to different developmental and maturation stages (Borg et al. 2010). Beside reproductive tissues, some other accessory glands and tissues are also involved (Frungieri et al. 2006). The seminal plasma contains factors, mostly proteins, which influence the spermatozoa, the female genital tract, and the ovum (Caballero et al. 2008). It is well established that seminal plasma contains factors that influence both the spermatozoa and the female genital tract during sperm transport (Johnson et al. 2000, Rozeboom et al. 2000). Therefore, failure of any of these events leads to disturbances of male fertility.

2.1.1 Boar sperm quality traits

The semen evaluation could be used as an indicator of fertility in boars (Malmgren and Larsson 1984). Sperm concentration, motility and normal sperm rate have usually been used as criteria for semen quality evaluation (Colenbrander et al. 1993). The only measure of semen viability is to observe the fertility of inseminated females. The management of AI boars plays such an important role in efficient semen production (Flowers 1997). In this particular situation, the physical environment in terms of housing, ventilation systems and collection pens were identical between the boars, as were standard operating procedures associated with herd health, nutrition, collection procedures and routine animal care. Collection technicians did differ, but each one was required to complete a standardized training program that was overseen by the same individual. A goal of large swine companies is to standardize management conditions across farms so that production is uniform throughout the entire system.

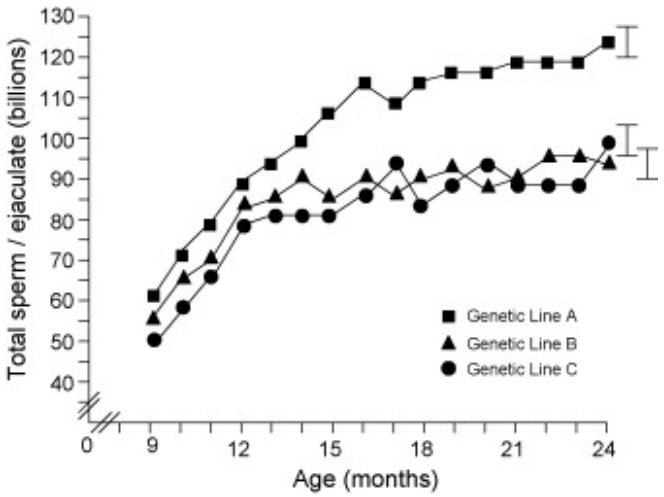

Figure 1: Mean (±SEM) age-related changes in total spermatozoa per ejaculate for three genetic lines of boars (Flowers 2008).

Based on the lack of an effect of boar, it appears that this was achieved for semen production in this situation. There was a genetic line by age interaction ($p < 0.01$) for total spermatozoa per ejaculate (Figure 1). Sperm production was similar and increased over time in all genetic lines between 9 and 12 months of age. At this point, sperm numbers continued to increase in Line A over the next 4 months, eventually reaching a

plateau of approximately 120×10^9 spermatozoa per ejaculate. In contrast, sperm production did not increase appreciably after 12 months of age in Lines B or C and remained fairly constant at $\approx 88 \times 10^9$ (Flowers 2008). Based on these data, there was considerable variation in sperm production among modern genetic lines of terminal sires. The superiority of 30×10^9 sperm cells in Line A was considerably larger than what has been reported previously (Kennedy and Wilkins 1984). This was surprising, since essentially no selection pressure for sperm production was placed on these lines. Perhaps sperm production was positively correlated with other traits used in the selection index. As mentioned previously, these genetic lines were all selected for growth and average daily gain. Previous studies have reported positive correlations between body weight and testis size and selection for testis size increases daily sperm production (Harder et al. 1995, Huang and Johnson 1996, Rathje et al. 1995). It is possible that the differences in total spermatozoa simply reflected differences in growth rate and mature size. Accordingly, it seemed unlikely that the observed differences in sperm production were due to differences in growth rates.

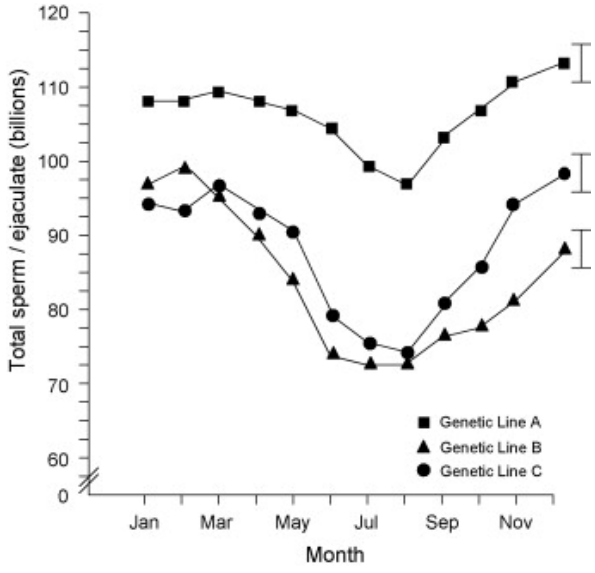

Figure 2: Mean (±SEM) seasonal changes in total spermatozoa per ejaculate for three genetic lines of boars (Flowers 2008).

From a biological perspective, one way to increase sperm production is to increase the number of Sertoli cells in the testes. In the boar, there are two periods of active Sertoli

cell proliferation. One occurs during the 3-week period immediately after birth and coincides with lactation, while the other is believed to take place between 28 and 50 days of age (McCoard et al. 2001). The recently demonstration reported that reducing the number of littermates during lactation increased preweaning growth of replacement boars and had a significant, positive effect on their adult sperm production (Griffin et al. 2006). One interpretation of these findings is that differences in growth rates of boars are more likely to impact sperm production if they occur coincident with periods during which Sertoli cells are actively undergoing mitosis. As was the case with the previous analysis, a time by genetic line interaction was present ($p < 0.05$) (Figure 2). The general pattern for each line was for the total number of spermatozoa to decrease after May and then began to increase. The interaction was the result of differences among lines in the magnitude of these changes (Flowers 2008). The detrimental effects of elevated ambient temperatures on boar reproduction are well documented and although in this trial changes in daylength were confounded with temperature, it is likely that the changes observed were due mostly to temperature. It is generally assumed that the negative effects of heat stress on pigs tend to be universal and affect most genetic lines equally. In cattle, genetics definitely affect heat tolerance (Hammond et al. 1996), so it is reasonable to assume the same is true for swine. Decreases in sperm production associated with high ambient temperatures are a universal problem for the swine industry. Development of genetic lines of “heat tolerant” boars based on the phenotypic variation that, apparently, is currently present in modern terminal sire lines would substantially enhance reproductive efficiency. However, a number of semen characteristics can be used to evaluate the general quality of the sample and to estimate the extent to which it may be extended. The evaluation of the ejaculate is an important part of examination a male.

2.1.1.1 Appearance of semen

A properly collected semen sample should be near the characteristic volume. The colour should be opaque in appearance, indicating a high sperm concentration and nothing indicative of contamination. Semen with a crude appearance, containing chunks of material, like the gelatinous fraction of boar’s semen, should not be used. Boar seminal plasma is a complex mixture of secretions from the testes, epididymis, and the male

accessory reproductive organs of the spermatozoa at ejaculation (Caballero et al. 2008). Occasionally small amounts of blood, usually originating from the urethra, may be present in the ejaculate, which gives the semen a pinkish hue. The emitting odor is most likely reflective of prepuce fluids (urine) which are generally heavily laden with bacterial and foreign contaminants. Furthermore, routine periodic semen culturing is becoming standard operating procedure in most boar studs and should be conducted in on farm to monitor facility sanitation and hygiene processing procedures (Rozeboom 2000). Bacteriology cultures are fairly inexpensive, and are performed at all diagnostic laboratories. Even though contamination of semen with bacteria is almost inevitable, there is very little scientific information regarding interactions between type and level of bacteria and fertility. However, since bacterial contamination has been associated with decreased storage life, clumping, and persistent uterine infections, it is necessary to periodically test ejaculates for bacterial contamination, identify the source of contamination, and take proactive measures to reduce contamination (Althouse 1997).

2.1.1.2 Sperm concentration

One of the most important measurements taken on an ejaculate is sperm concentration. An accurate estimate of sperm concentration to a large degree is pivotal in determining both the reproductive success of the AI program and the efficiency of operation of the stud. The goal is to extend semen with precisely the desired number of motile sperm per dose, which will result in optimum reproductive performance under a given set of conditions (specific extender, storage time, and individual boar differences) (Foote 1968). Sperm concentration in semen is estimated using a calibrated electronic cell counting equipment, spectrophotometer or haemocytometer. Accurate determination of concentration, semen volume, and percentage of living cells is essential for estimating the maximum dilution of sperm which can be prepared for artificial insemination and how many females can be inseminated. Sperm concentration of AI must be 2×10^9 or greater per insemination (Xu et al. 1998). Techniques most commonly used for routine boar semen processing will be reviewed and discussed. When one considers that a typical ejaculate may contain from less than 10 to over 100 billion sperm cells it should be apparent that all techniques provide only an estimate of the actual number of sperm. Therefore, regardless of the method used, it is important to fully understand the basis

for the technique, and to gain an appreciation of factors, which can result in to inaccurate estimates (Foote 1968).

2.1.1.3 Sperm motility

Sperm motility is one of the most widely used tests for semen quality. Motility is essential for fertility. However, motility, while it is an essential feature of healthy spermatozoa, is not necessary indicative of fertilizing capacity (Vyt et al. 2004, Xu et al. 1998). Sperm motility is estimated visually using light microscopy. In measuring this, two aspects are usually considered, the percentage of motile sperm and the motility quality of individual spermatozoa by subjective judgement. Other ways of assessing motility include computer assisted image analysis and the swim-up technique. Visual estimates of the percentage of motile spermatozoa by light microscopy are the most widely used and acceptable method. Technician skill and experience greatly influences the relative accuracy of this procedure. Briefly, a very small drop of diluted spermatozoa (dilution rate must be standard for all evaluations) is placed on a warmed microscopic slide and overlaid with a cover slip. The sample should be dilute enough to view individual sperm cells at 400 X power (Caballero et al. 2008).

2.1.1.4 Semen volume

This is measured in a warm, dry calibrated flask. Semen volume varies between 100 and 500 ml, a large volume does not mean the total spermatozoa content is greater than that from smaller ejaculates. If the semen is to be used undiluted, its volume determines the number of inseminations possible from each collection. The dose per insemination is between 50 and 100 ml. Semen volume decrease with age, possibly due to a seminal vesicle insufficiency, since the seminal vesicle contributes most to ejaculate volume (Rolf et al. 1996).

2.1.1.5 Plasma droplets rate

In most species studied there is convincing evidence that it migrates along the midpiece from neck to annulus during proximal epididymal transport and remains on the majority

of cells in the epididymis (Cooper and Yeung 2003). The mechanism of transport has not been elucidated but migration of droplets from the neck of caprine and porcine testicular spermatozoa can be achieved by the application of repeated or sustained centrifugation (Kato et al. 1984). Peristaltic motions of the epididymal tubule acting on high concentrations of spermatozoa within the lumen could thus contribute to the migration of the droplet along the tail.

2.1.1.6 Sperm morphology

Sperm morphology and acrosome integrity are also effect tools to estimate semen viability and can also provide more information about the ejaculate in terms of its quality than is possible with just a motility evaluation. Both of these criteria are important to use, along with motility, as a determinant for keeping or discarding ejaculates. Stained slides are used to examine the morphology of sperm and the ratio of living to dead cells, using the eosin-negrosin stain. Abnormalities of the head, midpiece, and tail may be due to defective mechanisms in the reproductive tract of the male or to changes caused during collection and processing. These are usually not associated with lower fertility rates until the proportion of abnormal sperms exceeds about 20%. Morphological evaluation is done by light microscopy. Nevertheless, while acknowledging the evident limitations of semen analysis for estimation of fertility, it should be remembered that a number of insemination trials using commercial doses of $2–3 \times 10^9$ sperm have shown significant relationships between the incidence of morphological abnormalities and fertility in pigs (Alm et al. 2006, Waberski et al. 1994).

2.1.1.7 Acrosome integrity

Acrosome integrity is regarded as an important semen quality parameter (Zou and Yang 2000). The acrosome is an essential part of the sperm cell and it contains enzymes which play important roles in the penetration of the zona pellucida and therefore fertilization. Acrosome damage removes the chance to fertilize an egg. The new sperm assays try to explore the functional capacity of the spermatozoa. The binding and penetration of the zona pellucida, the interaction with the oocyte plasma membrane, the

relationship between sperm factors and in vitro fertility may be a good strategy and assays that may lead to predict male fertility (Gadea 2005). A sperm function test as a laboratory analysis of the cellular processes exhibited by spermatozoa between the time they leave the seminal fluid and the final step of fertilization. These tests attempt to determine the ability of sperm to capacitate and fertilize (Muller 2000). Diverse fluorescent dyes (Johnson et al. 1995), modern flow cytometry that allows rapid counting of large numbers of cells (Gillan et al. 2005), a biochemically active membrane by the hypoosmotic swelling test (Correa et al. 1997) and the motility and plasma membrane integrity (Gadea et al. 1998, Pérez-Llano et al. 2001, Zou and Yang 2000). Finally, some other molecules are implicated in the fertilization process, such as seminal plasma proteins, heat shock proteins, etc. In the near future, assays of these molecules may lead to the identification of differences that could be associated with fertility.

2.1.2 Boar fertility traits

In commercial production, boar fertility is very difficult to evaluate for several reasons. From a practical perspective, pooling ejaculates from several boars is common (Singleton 2001). This technique has gained widespread acceptance, because it has been reported to improve reproductive performance (Alm et al. 2006) and increase the efficiency of producing insemination doses. Obviously, its use prevents the use of litter size and farrowing rates to assess the fertility of individual males. From a physiological perspective, boar semen cannot be stored for long intervals without reductions in fertility (Johnson et al. 2000) and numbers of spermatozoa required to achieve acceptable reproductive performance are higher for pigs than other species (Flowers 1998). Both of these effectively limit the number of sows that can be inseminated from a single ejaculate, which, in turn, makes it difficult to evaluate boar effects independently from those of the sow, production environment, and their interactions.

The most commercial boar do not routinely collect data that can be used to assess individual male fertility. A recent study conducted in a 200 boars provide some insight into the phenotypic variation in fertility among commercial AI boars (Flowers 2002). This applications of molecular genetics is the case for traits that are age-linked (measurable only in animals of a certain age), sex-limited (measurable only in one sex),

and/or of low heritability, such as litter size, but also for traits such as disease resistance that are difficult to improve through conventional means because of the difficulty and expense of recording phenotype (Spotter et al. 2005). The use of multivariate analysis would help to discriminate potential fertility because this combines the functional information regarding different capacities of the spermatozoa (Gadea 2005). Farrowing rate and litter size are of enormous commercial importance and, while linked, are arguably best examined separately. There is a dearth of conclusive studies on the boar's impact on these important economic measures, and little more on males from other species (Robinson and Buhr 2005). Boars are a significant source of variation with regards to the success of both in vivo (Long et al. 1999) and in vitro fertilization in swine. However, only a few studies have attempted to investigate whether there is a genetic component to these differences (Swierstra and Dyck 1976, Wilson et al. 1977). The general conclusion from these studies was that crossbred boars were more likely to produce more pigs than purebred boars.

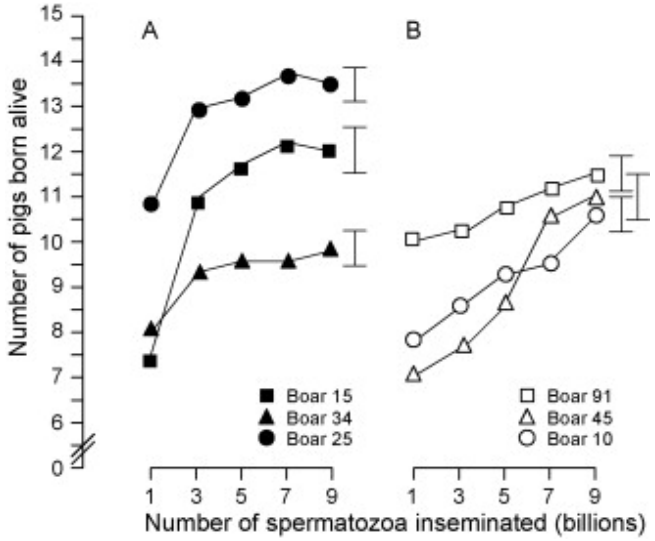

Figure 3: The different relationships between number of pigs born alive (mean ± SEM) and number of spermatozoa inseminated for boars in a commercial stud. Panel A illustrates asymptotic relationships. Panel B illustrates linear relationships (Flowers 2008).

Boars varied in terms of the insemination dose required to reach the plateau, and the number of pigs born alive at the plateau. The second pattern was linear and variation

among boars was also present. For some boars, there was a robust response in litter size as sperm numbers in the insemination dose increased, whereas for others, the change was less pronounced (Flowers 2008). The proportion of boars that exhibited asymptotic and linear responses to increases in sperm numbers and the overall number of pigs born alive was similar between lines. An interesting observation concerning these data involves boars that have a fertility pattern similar that of Boar 25 (Figure 3). There are boars currently being used in the industry that are capable of producing exceptional fertility results with low numbers of sperm (Flowers 2008).

2.1.2.1 Non return rate

Fertility was determined as non return rate, which was observed as the proportion of females that conceived, divided by the total number in a herd of sows that were artificially inseminated. Fecundity was measured in terms of the percentage of sows that farrowed and the total number of piglets (litter size) born per litter. Results from previous study indicated that 5 min of sexual restraint was necessary to achieve the highest sexual drive at the shortest reaction time of 2.8 min, suggest that sexual stimulation of boars significantly enhanced non return rate of AI sows (Umesiobi 2008).

2.1.2.2 Number of piglet born alive

Number of piglets born alive, number of survived animal born at the day after farrowing (% per litter) were available as the deviation from the population means within sow breed, parity of sow, farm and season classes. Selection for total number of piglets born since 1992 has led to an increase in this trait in Danish Landrace and Danish Yorkshire, but also to an increase in piglet mortality, and it has been further observed that most cases of death occurred at birth and during the following 5 day (A. Vernersen, DanBred International, Copenhagen, Denmark, personal communication). Thus, it is expected that selection for litter size at day 5 will capture a large part of the genetic variance for piglet survival, and thereby will be more effective than selection for total number born with regard to genetic improvement of litter size at weaning and piglet survival (Su et al. 2007).

2.2 Heritability of boar reproductivity

The genetic evaluation procedures to select the top boars for AI are commonplace, attention still needs to be addressed on levels of inbreeding in boar populations and the cost of boar production relative to desired selection intensity. Currently boars selected as AI quality are indexed and selected strictly on performance. The following is a review of the genetic control of male reproductive traits with discussion towards the opportunity of selecting boars that would be optimal for boar sperm quality as well as boar fertility traits.

2.2.1 Heritability of boar sperm quality traits

The number of spermatozoa a boar produces per month is limited by the testicular capacity, libido and physical soundness (feet, legs, back); monthly production is affected by collection (frequency and animal handling) (Rutten et al. 2000) health, season, age, feeding (Wilson 2002) and other factors (Mathevon et al. 1998a, Mathevon et al. 1998b). The semen traits that affect AI centre profitability are volume, sperm concentration and gross sperm morphology. Semen volume and sperm concentration have been thought to be heritable traits, but estimates of heritabilities and repeatabilities for these traits varied widely (Humblot et al. 1993, Taylor and Everett 1985). A carefully designed study analyzing the wealth of data available in large boar for heritability and correlations of boar sperm characteristics and fertility would be of immense value to the swine AI industry (Robinson and Buhr 2005). Commercially important sperm quality measures (morphology, motility, longevity in extender, fertilizing ability) are affected by maturation in the male tract, collection and processing, and the dam. Therefore, effects of single loci are expected to be low and require a higher number of animals to be identified. In contrast, sperm quality traits have moderate to medium heritability (h^2 = 0.19–0.37) (See 2000). All these contributing factors make it difficult to determine if there is a genetic basis for poor semen, and so it is extremely easy for such genes to be maintained and even deliberately or unintentionally propagated in the breeding herd, if the male carrying them has good production traits. Female pigs respond similarly and taken together, the evidence clearly suggests that current selection criteria could certainly ultimately cause a reduction in

porcine semen quality and fertility (Merks et al. 2000). Certainly factors that a manager can control will affect semen quantity and quality, but a good manager must be aware of the potential genetic basis behind poor semen and consider screening boars for relationship to boars known to be poor semen producers.

Heritability estimates for semen traits are summarized in Table 1 (See 2000). Sperm quantity has moderate to high heritabilities and would therefore be expected to respond to selection. Sperm cell motility, morphology and concentration (count) are moderate in heritability. Genetic control of sperm cell morphology is supported by Wekerle (1982) who reported that when sires had an increased incidence of morphological abnormalities (> 30%) their sons also had an incidence of abnormalities greater than 30%. While of increasingly less importance, halothane positive boars have been reported to have significantly lower sperm cells per ejaculate (Hillbrand and Glodek 1984, Schlenker et al. 1984) lower sperm volume (Schlenker et al. 1984) reduced forward motility (Schlenker et al. 1984) and increased percentage of abnormal sperm cells (Hillbrand and Glodek 1984, Schlenker et al. 1984).

Table 1: Heritability estimates for semen traits (See 2000)

Trait	Number of estimates	Mean h^2	Range	References
Sperm quantity	3	.37	.31 - .42	Rothschild and Bidanel. (1998)
Sperm cell motility	7	.20	.05 - .55	Brandt and Grandjot. (1998), Oh et al. (2000), Rothschild and Bidanel. (1998)
Sperm cell volume	4	.21	.14 - .29	Brandt and Grandjot. (1998)
Sperm cell count	4	.19	.01 - .26	(Brandt and Grandjot. (1998)
Sperm cell morphology	3	.31	.05 - .62	Brandt and Grandjot (1998), Oh et al. (2000)

The sperm production differs considerably among breeds by the evaluation of semen characteristics of purebred boars housed in Canadian studs over a 10 years period. Yorkshire boars routinely produced $10–12 \times 10^9$ more spermatozoa than Hampshires with Landrace and Durocs producing intermediate amounts (Kennedy and Wilkins 1984). Other studies have reported even greater breed differences in total numbers of

spermatozoa per ejaculate when comparisons were made among European, Chinese and African pigs (Okwun et al. 1996). It is also well established that crossbred boars produce more sperm than their purebred counterparts (Wilson et al. 1977). Consequently, there appears to be a large amount of genetic diversity in semen production among swine breeds. Sperm production also responds to selection for testis size (Harder et al. 1995, Rathje et al. 1995). Boars selected for increased testis size produced 6×10^9 more spermatozoa per ejaculate than their counterparts from a randomly mated, control line, this represents almost a 10% increase in sperm numbers (Huang and Johnson 1996). This response is impressive, but the time course over which it occurred is of equal importance. Daily sperm production increased more rapidly and reached its plateau at younger ages in the lines selected for testis size compared with controls (Rathje et al. 1995). Replacement rates in terminal sire lines are high, so that swine production companies can take advantage of superior genetics. Boars that can produce large quantities of semen at young ages and maintain them over their productive life are of premium value. Selection for testis size appears to be a valid approach for enhancing spermatogenesis and could be applied to any type of genetics.

2.2.2 Heritability of boar fertility traits

Traits related to fertility of boar are of low heritability (h^2 = 0.01–0.06) and are strongly affected by environmental and genetic effects of the boar itself, the dam and the offspring (See 2000). Litter size, however, is low in heritability (about 10%) (Robinson and Quinton 2002) and is not measured on the boar. Until a boar accumulates data on the litter size produced by a number of offspring, this estimated breeding values will be lower in accuracy than those for the growth traits. In addition to quantitative trait selection, specific genetic markers are used in selection programs (Dekkers 2003). No definitive tests were undertaken to compare the boars, so at best this is an indication of possible male genetic impact on porcine pregnancy rates. However, an apparently normal fertile male can be transmitting a genetic cause of subfertility to their female offspring. There is a male genetic influence on pregnancy rate, which warrants definitive study in pigs. Large commercial swine units using AI could, on their own or in collaboration with research institutes, carry out such valuable studies. Reciprocal translocations occur when different chromosomes exchange pieces, and subsequent

segregation during meiosis produces gametes that can be balanced or unbalanced with respect to the chromosomes carrying the translocations, with the unbalanced gametes carrying a chromatid that is either too long or too short. If such a spermatozoon penetrates an egg, the unbalanced chromatid cannot pair properly with the female partner chromatid, resulting in early embryonic death and therefore a smaller litter. Numerous such translocations have been identified in boars (Ducos et al. 1998, Makinen et al. 1999), and a Finnish York boar carrying such a translocation produced an average of two less pigs per litter than the breed average (Makinen et al. 1999). Furthermore, although the offspring that receive the unbalanced chromosome die, half of the living offspring carry the balanced translocation. Although in this case the originating translocation apparently resulted from a spontaneous translocation, the problem can clearly be perpetuated in subsequent generations. It has been strongly recommended that AI stations should not admit boars with genetic abnormalities (Ducos et al. 1998).

2.3 The genetic aspects of boar reproductivity

Selecting boars in an artificial insemination station to use selected for their genetic potential within a swine herd is one of the most important decisions a producer. This decision is the performance of the herd and based on the boar genetic. Molecular genetics and reproductive technologies will initially develop such animals (Robinson and Buhr 2005). Worldwide, male factors are thought to be responsible in 20%–50% of all infertility cases such as azoospermia, the absence of sperm in the ejaculate due to defects in its production or delivery is common in male infertility. Thereby, boar fertility results from a variety of defects in the developmental stages of spermatogenesis, the stage-specific expressions of genes in the testes must be investigated. The method of screening varies by AI unit but normally includes a review of the estimated breeding values (EVB) and any available test results for specific genes. Boar selection decisions are also tied to boar replacement decisions. For maximum genetic progress in economically important traits after the screening and with additional factors used to decide among boars of close to equal merit. Boars are generally replaced when a young boar with better EBV and/or index values is available. There is a paucity of published results from well-designed studies about factors other than EBV (Rutten et

al. 2000, Wilson 2002). The incidence of boars replaced for semen quality and production problems was variable but constituted a significant proportion of the selected population; this involuntary selection obviously interferes with the selection program designed by the geneticists for the station. The management of the AI station develops selection programs in response to market demands, which necessarily vary with the purpose of the unit. Genetic progress, genetic diversity, meeting customer needs for product characteristics and the cost of purchasing replacements are all factors in the decision process. Modern reproductive technologies and gene manipulation tools may well play a prominent role in developing the pigs of the future, creating a few, extraordinarily expensive and valuable founder animals. Testicular transplants (Schlatt 2003), intra-cytoplasmic sperm injection, in vitro fertilization, and embryo transplants are all tools that can be used to promote propagation and decrease generation intervals of animals (Garniera et al. 2003).

The economic reality of an AI centre is that, primarily, a boar's productivity depends on the number of spermatozoa that boar produces per week, or month. Of secondary importance is his fertility, because in practice, fertility is only an issue if a reasonable number of customers complain about one boar's poor pregnancy rates or small litter sizes (Robinson and Buhr 2005). Different boars produced apparently good quality semen which, used in AI under controlled conditions, altered farrowing rates (Foote 2002). The development and use of more sensitive sperm assays to address these problems have been reviewed recently (Holt and Van Look 2004, Petrunkina et al. 2007, RodrÃguez-MartÃnez 2006). Another aspect that may be partly responsible for the apparent failure of semen assessment to predict fertility or infertility concerns the relatively large numbers of sperm normally inseminated in AI practice: the use of such large numbers may mean that certain sperm traits that hinder sperm entry into the oviduct may be compensated for, thereby masking intrinsic differences in sperm quality between individual boars (Saacke et al. 2000).

2.4 Candidate gene analysis

Candidate gene analyses have been employed to investigate a variety of traits whose physiology is well understood. This approach can be very powerful and can detect loci even with small effects provided that the candidate gene is the causative gene. The

representation of the physiology of a few transcription products involved in reproductive processes results of studies analysing the association of alleles of these candidate genes with fertility traits are given.

2.4.1 A genome scan for quantitative trait loci affecting boar reproductivity

The phenotype expression of a trait depends on the underlying genotype that is modulated by environmental influences. Boar fertility is not only dependent on genes controlling the germ line but also on genes of the networks functional for gonad development and somatic development, respectively. Selection of boars has traditionally been done with the objective of producing the most valuable offspring, with little attention given to the reproductive fitness of the individual boar. Although the genetics of male fertility have not received the attention of female fertility, several estimates of repeatabilites/heritabilities suggest that these traits would respond to selection. A greater understanding is needed of underlying genetics of male reproductive traits, as well as their relationship with female reproduction and market progeny performance. Commercial farms will still need to consider health status, genetic improvement program, cost, etc., as they select individual boars (Safranski 2008). Quantitative trait loci (QTL) affecting economically important traits of livestock are of great interest. The usefulness of candidate genes is then examined by association studies between genetic polymorphisms identified in the respective candidate genes and the phenotypic reproductive traits. The discussed uses pre-existing or designed families for linkage analyses in order to map the location of quantitative trait loci for the reproductive trait of interest.

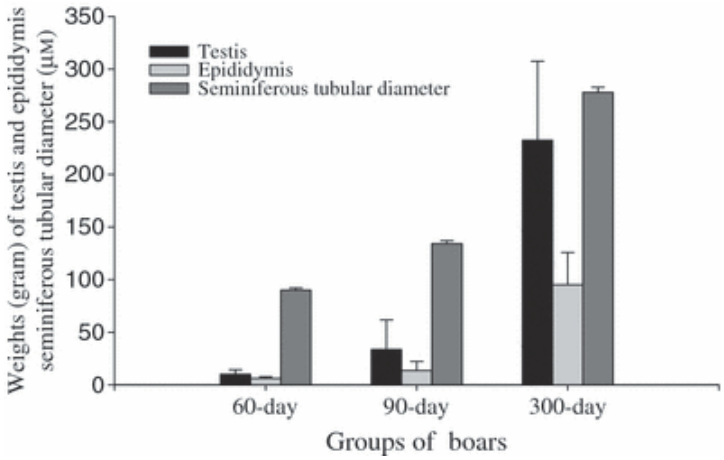

Figure 4: Growths of the testis, epididymis and seminiferous tubule from pre-puberty to maturity (60, 90 and 300 days) in the White Duroc–Erhualian boars. Note that the large standard errors occurred in testis and epididymis weights in the three age groups, indicating very uneven growths of the testis and epididymis in these hybrid boars (Ren et al. 2009a).

A pig F2 resource population by crossing a Meishan sow and a Duroc boar to locate economically important trait loci. The results identified 38 QTL for 28 traits at the 5% genomewise level. Of the 38 QTL, 24 QTL for 17 traits were significant at the 1% genomewise level (Sato et al. 2003). The localization of two testis and one prostate weight-regulating of mice QTLs (Ltw1, Ltw2 and Lpw1), 4 QTLs controlling the sperm nucleus shape (Sh1, Sh2, Sh3 and Sh4), and one QTL influencing sperm survival (Dss1). For instance, Spata1, Capza and Tuba7, are very strong candidates for influencing the shape of the sperm head (Lhote et al. 2007). Identifying new genes implied in mammalian fertility pathways is a necessary prerequisite to clarify their molecular grounds, and to propose in the future efficient diagnostic tools for masculine infertilities. In the White Duroc–Erhualian boars, the remarkable segregations in the traits measured except for the seminiferous tubular diameter and had high ratio (13.9%) of the abnormality of spermatogenesis, providing a good experimental population for detecting quantitative trait loci affecting these male reproductive traits. In general, the gonad development was obviously uneven among the crossbred boars, particularly for the testis and epididymis weights, even though it was not so dramatically uneven for the seminiferous tubular diameters (Figure 4). Furthermore, the correlations among nine

male reproductive traits at 300 days of age indicated that the testis weight and the body weight were strongly correlated with the sperm production, supporting the two traits as important parameters for boar selection to increase sperm production and ultimately improve boar fertility (Ren et al. 2009a). Chinese Erhualian alleles were not systematically favorable for greater reproductive performance. This study confirmed the previous significant QTL for testicular weight on SSCX and for epididymal weight on SSC7, and reported QTL for seminiferous tubular diameter and testosterone concentration at the first time. The observed different QTL for the same trait at different ages reflect the involvement of distinct genes in the development of male reproductive traits (Ren et al. 2009b). The considerable interest has been directed toward finding candidate genes affecting boar reproduction which are often non-reproducible, and effects of candidate markers are sometimes population-specific (Georges 2007). As an initial step to decipher genes underlying complex traits in farm animals, QTL mapping has been widely performed using experimental and commercial pedigrees. So far, only limited QTL affecting boar reproductive characteristics have been mapped. Significant QTL for several male reproductive traits have been identified on porcine chromosomes (Bidanel et al. 2001, Ford et al. 2001, Rohrer et al. 2001, Sato et al. 2003). Additional genetic evidence is required to determine if the His226Asn polymorphism of SERPINA7 is a closely linked marker or the causative mutation underlying the testicular size because of the extensive linkage disequilibrium on SSCX. To our knowledge, this is the first time to identify the QTL for testicular weight on SSC1, though several QTL affecting correlated reproductive traits including age at puberty, gestation length, FSH concentration, and the weights of seminal vesicles, bulbo-urethral glands, and uterine horns were detected at positions near the QTL (Bidanel et al. 2001).

2.4.1.1 QTL affecting boar sperm quality traits

The QTL for semen and ejaculation traits in pigs, providing a start point to decipher the genetic basis of these complex traits for traits related to semen and ejaculation, phenotype data including semen volume, sperm concentration, total sperm per ejaculate, sperm motility, sperm abnormality rate, semen pH value, ejaculation times and ejaculation duration were measured on 206 F_2 boar at 240 days in a White Duroc × Erhualian intercross (Xing et al. 2009). A genome-wide scan was performed

and the entire White Duroc×Erhualian intercross was genotyped for microsatellite markers covering for traits related to semen and ejaculation, phenotype data including semen volume, sperm concentration, total sperm per ejaculate, sperm motility, sperm abnormality rate, semen pH value, ejaculation times and ejaculation duration in the whole pig genome (Xing et al. 2009). Pig is a good choice as animal model for human disease, and the genetic research for boar semen quality can provide referenced information for human infertility study. The limited number of genome-wide significant QTL could be due to the relatively small size of the F2 population. However, no phenotypic correlation was reported among these traits mentioned above. Hence, these traits having overlapping QTL could be affected by different causal genes. The estrogen receptor locus associated with litter size (Rothschild et al. 1996) is located in the QTL for total sperm per ejaculate and near the QTL for sperm motility on SSC1. Borg et al (1993) reported that boars from the Taihu breed had lower total sperm per ejaculate than Duroc boars ($p < 0.05$). However, the Erhualian allele increasing sperm per ejaculatewas observed at the QTL on SSC2, which is in contrast to the breed characteristic difference in this trait. Similar phenomena have also been observed in a Meishan×European intercross (Milan et al. 2002), and the reason for this remains unknown. It has been shown that boars carrying different estrogen receptor 1 (ESR1) genotypes have significant ($p < 0.01$) difference in semen traits including total sperm number per ejaculate and sperm motility (Terman et al. 2006). Moreover, reducing estrogen synthesis in developing boars increases total sperm production (At-Taras et al. 2006) and polymorphisms in the promoter of ESR1 are associated with men's lower sperm count (Guarducci et al. 2006). Wichmann et al (1994) found that semen pH was related to fertility ($p = 0.045$). Salsabili et al (2006) showed that semen pH had a direct relation with sperm motility and an inverse relation with tail defects in humans ($p < 0.05$). However, semen pH did not show a high correlation with sperm motility and sperm abnormality rate in this study. Candidate genes associated with semen pH remain largely unknown. Although low pH value was significantly more frequent in patients with CFTR mutations (Eckardstein et al. 2000), the porcine CFTR gene is not located in the QTL region for semen pH in this study. It has been shown that ACTG2 mutations are significantly associated with semen volume in Pietrain boars (Wimmers et al. 2005).

2.4.1.2 QTL affecting boar fertility traits

Deciphering the genetic basis of porcine male reproductivity will not only benefit the pig industry but also human medicine. Human infertility is an important reproductive health problem that is implicated in about 50% of sterility cases (Griffin and Finch 2005). About 15% of man infertility cases are attributable to genetic factors (Ørstavik 2008). Overlapping regions between QTL for ovulation rate (Rohrer et al. 1999) and the QTL for pH value detected were also found on SSC2 and SSC12. These indicate that these regions have significant effect on both male and female reproductive traits. However, it is difficult to assure whether these QTL were due to pleiotropic effects of a single locus or effects of closely linked multiple loci. The epididymis plays a critical role in sperm maturity and storage, and greater epididymal weight may result in greater capacity for sperm storage and improve male fertility (Bidanel et al. 2001). The reason for the discrepancy remains unknown. A high-resolution human-pig comparative map of this region (Demars et al. 2006) allows the identification of positional candidate genes.

2.4.2 Polymorphism identification of boar reproductive traits

Polymorphisms identification of the porcine ACTN1 gene and ACTN2 gene by comparative sequencing of animals from the Pietrain (PI) and Hampshire (HA) breed. The study provides evidence for effects of ACTN1 on fertility and of ACTG2 on sperm quality traits (Wimmers et al. 2005). Retinol-binding protein 4 (RBP4), androgen receptor (AR), relaxin (RLN), acrosin (ACR) and osteopontin (polymorphism in intron 6 named OPNin6; polymorphism in promoter region named OPNprom) were addressed as functional candidate genes for sperm quality and boar fertility and investigated for their association with sperm concentration, motility, semen volume per ejaculate, plasma droplets rate, abnormal spermatozoa rate as well as non-return rate and number of piglets born alive (Lin et al. 2006b). The eighteen candidate gene loci FSHB, PRL, PRLR, INHA, RBP4, OPNin6, ACTN1, ACTN4 GnRHR, LHB, RLN AR, FST, INHBA, INHBB, ACR, ACTG2 and OPNpro were investigated. A candidate gene can also be identified by association with the reproductive phenotype and by linkage analysis to a region of the genome, to analyse the expression levels of specific genes in

a case control study (Lin et al. 2006a). The polymorphisms in CYP2E1, CYP21, CYP2D6, CYP2C49, NGFIB and CTNND1 might be used to reduce levels of boar taint without affecting levels of testosterone, estrone sulphate, 17β-estradiol or length of bulbo urethralis gland (Moe et al. 2009). The production and growth trait SNP may serve to assist in selection of young females for superior reproductive performance. The SNP associated with economically important traits such as litter size, growth rate, and feed intake such as; PRLR SNP were associated with total number of piglets born and number of piglets born alive, the PPAR γ coactivator 1 SNP was associated with total number of piglets born and number of piglets born alive, the SNP within ESR1, ESR2, IGF2 SNP, and IGFBP3 SNP were associated with number of piglets born dead. Many of the SNP analyzed are from genes involved in regulation of metabolism, suggesting that there is an important link between physiological events associated with reproduction and energy utilization (Rempel et al. 2010). The polymorphisms in genes encoding porcine adiponectin (ADIPOQ) and its receptors (ADIPOR1 and ADIPOR2) were evaluated for associations with reproductive traits in a Landrace sow population. Therefore, specific SNPs and haplotypes that are associated with large litter size, fewer stillborn and mummified piglets and shorter weaning-to-oestrus intervals. Selection for these SNPs and haplotypes is a strategy to improve reproductive success in pigs (Houde et al. 2008).

2.4.3 Transcriptomic of boar reproductive traits

International cooperative efforts will therefore largely advance the pig transcriptome and functional analyses. The overall objectives are an analysis, assessment and description of the realisation of cellular function from genetic information as well as the understanding of the regulation of the relevant processes. Comparative studies are under way on the epigenetic modulation of the genome, in combination with transcription factor binding assays, measurements of transcript levels and the actual protein expression, the last performed by means of complex antibody microarrays, toward an understanding of biological functions and their cellular consequences. Microarray technologies now allow us the potential to review the transcriptional profile of expressed genes in a spermatogenesis and/or fertility of cattle (Dawson 2006, Evans et al. 2008), human (He et al. 2006, Hoei-Hansen 2008), rat (Daniel et al. 2007) and pig

(Bissonnette et al. 2009, Guyonnet et al. 2009). The differentiation in Gene Ontology terms between fresh and post-swim-up pooled samples from infertile males and donors by microarray technology was employed to study the mRNA profile. These differences could potentially be employed to establish markers of fertility success and to identify cellular processes and complex systems related with male infertility (García-Herrero et al. 2010). Moreover, in mouse microarray analyses performed on Sly-deficient males and on MSYq-deficient males, a remarkable up-regulation of sex chromosome genes in spermatids, suggest that the expansion of sex-linked spermatid-expressed genes is a consequence of the enhancement of postmeiotic sex chromatin repression that accompanies Sly amplification (Cocquet et al. 2009). In transgenic animals, postnatal rats and infant monkeys, their testicular Sertoli cells were cultured and differential gene expression was evaluated by DNA microarray using the Agilent microarray system was developed (Majumdar et al. 2009). In male pig, the microarray analysis can complement QTL analysis to identify systems of regulation of phenotype data (Ponsuksili et al. 2005). The complete transcriptomes of the testis, the epididymis, the vas efferens and the vas deferens on the same species. It described new genes or genes not yet reported over-expressed in these boar tissues, as well as new control mechanisms. It emphasizes and fulfilled the gap between studies done in rodents and human, and provides tools that will be useful for further studies on the biochemical processes responsible for the formation and maintain of the epididymal regionalization and the development of a fertile spermatozoa (Guyonnet et al. 2009). Microarray analysis of pig limbal SP cells yielded a molecular signature underscoring a phenotype characterized by slow cycling and low metabolic activity. The results provide valuable insights for the preservation and/or replication of epithelial stem cells (Akinci et al. 2009). The earliest comprehensive microarray expression analysis of purified presumptive pre-Sertoli cells, the male genital ridge is the first cell type to display sex specific differentiation and differential gene expression, used to generate molecular probes that were hybridized onto Affymetrix Mouse Genome 430 2.0 micro-arrays (Aron et al. 2007).

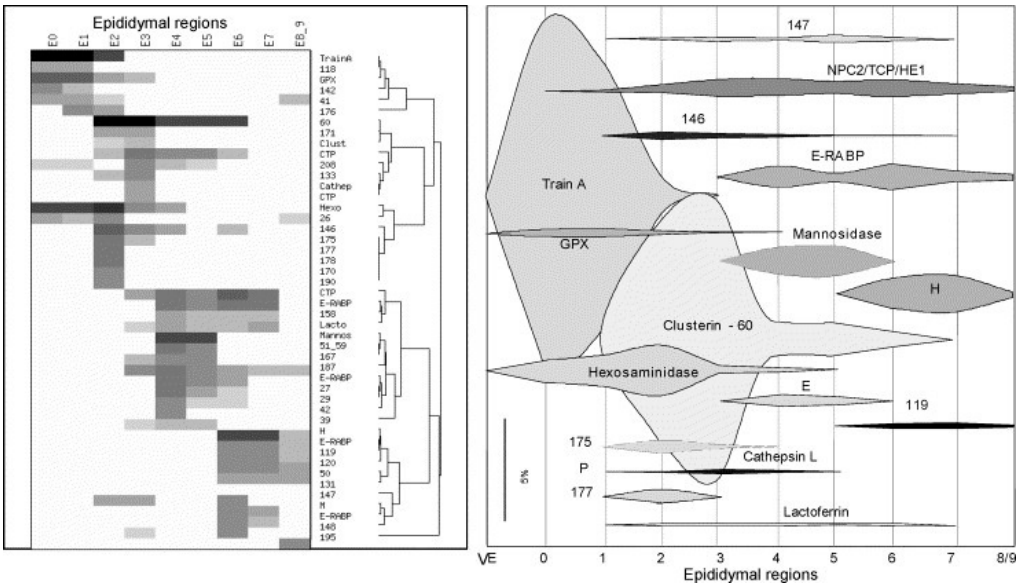

Figure 5: Diagrams of the proteins secreted (secretome) in the different regions of the boar epididymis. On the left panel, an expression profile by clustering of the proteins and isoforms (representing more than 0.15% of the total luminal secretion) is represented according to their secretion intensity and their regional expression. The cluster tree indicates at least six groups of secretion. For some proteins (i.e., E-RABP and CTP), the isoforms are different according to the epididymal regions. On the right panel, only the major secreted proteins are represented according to the epididymal regions. For each protein, the intensity of secretion is expressed as a percentage of the total labeled epididymal secretion (Dacheux et al. 2005).

In the study of epididymal investigation, the identification of the whole process of gene transcription and protein secretion should be achieved soon with the new technology for gene and protein identification by an overall study approach, such as cDNA arrays or proteomic analysis. For example, the proximal caput is characterized by the synthesis and secretion of glutathione peroxidase (GPX), hexosaminidase (HEX) and an RNase-Train A (Train A), and the middle caput by an intense secretion of clusterin, the presence of cathepsin L, lactoferrin and NCP2/CTP/HE1 protein. Secretion of α-mannosidase, retinoic acid binding protein (E-RABP) and an unidentified train E characterize the distal caput and proximal corpus. Only one protein is specific for the distal corpus, train H (36–40 kDa), while two minor proteins appear in the cauda

(Figure 5). The second requirement in the understanding of epididymal physiology is the study of gene regulation specificity. The perspectives for the understanding of sperm maturation and epididymal physiology in terms of sperm technology are promising, especially for the identification of new markers, both for genetic selection and for sperm evaluation for insemination and conservation (Dacheux et al. 2005).

2.5 Biological and physiological background of selected genes

2.5.1 Cluster-of-differentiation antigen 9

Cluster-of-differentiation antigen 9 (CD9) is a member of the tetraspanin or transmembrane 4 (TM4) superfamily of protein containing CD9, CD37, CD53, CD81 and CD82 (Horejsi and Vlcek 1991). As a member of tetraspanins CD9 expressed in many cell types and participates in many cellular processes (Boucheix et al. 2001). It plays important roles for cell migration and adhesion, malignant metastasis and reported to be a surface marker on mouse and rat male germline stem cells (Kanatsu-Shinohara et al. 2004). CD9 is also involved in prostate carcinoma (Wang et al. 2007). However, CD9 draws the highest attention for its role in sperm-egg fusion in mammal (Le Naour et al. 2000, Miyado et al. 2000). It is reported to be expressed in pig oocytes during early growth and meiotic maturation and participates in sperm-oocyte fusion while fertilization (Li et al. 2004). CD9 is mapped110 to SSC5q25 (Yubero 2003) and QTL influencing the testicular weight and seminiferous tubular diameter are reported on SSC5 in pig (Ren et al. 2009b). In spite of the vital roles of CD9 in sperm-oocyte interaction and in early growth and meiotic maturation of pig oocyte, no study was devoted to unravel its association with sperm quality and boar fertility through an association and expression study. Moreover, to the best of our knowledge, no studied is performed to evaluate the roles of *CD9* in boar reproductive tract through transcriptomic and proteomic expression.

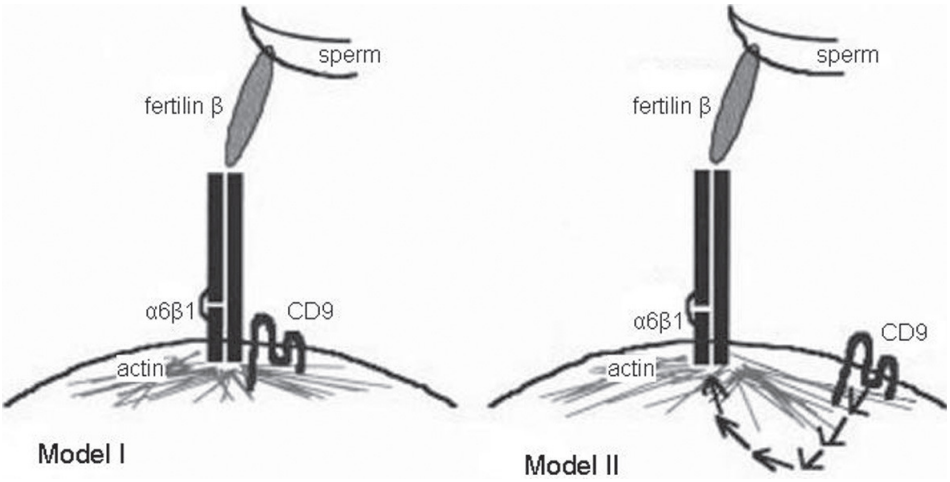

Figure 6: Models for the role of CD9 in binding of fertilin to α6β1. In Model I, fertilin β (ADAM 2) binds to α6β that is physically associated with CD9 and tethered to the actin cytoskeleton. In Model II, fertilin β binds to α6β1 that is tethered to the actin cytoskeleton; CD9 indirectly influences the association of α6β1 with the actin cytoskeleton and, therefore, its ability to bind fertilin β (Chen et al. 1999).

The experimental observations mentioned regarding the function of CD9, CD49f/CD29 and fertilin in reproduction processes have led to the construction of general hypothesis of fertilization comprising the interaction of the CD molecules in binding and fusion of sperm and egg. The adhesion of mouse gametes requires an interaction between fertilin and integrin α6β1 (Almeida et al. 1995) on the plasma membranes of the sperm and egg, respectively. According to Chen and Sampson (1999) fertilin β binds directly to the α6β1 integrin on the egg surface and this partnership mediates sperm-egg fusion. The tetraspan protein CD9 facilitates α6β1-mediated binding of fertilin β (ADAM2) to eggs. Perhaps CD9, or other integrin associated proteins, facilitate binding of other ADAMs to their respective integrin coreceptors (Nakamura et al. 1995). Two models for the role of CD9 in binding of fertilin to α6β1 have been considered by Chen et al (1999) (Figure 6). In model I, fertilin β binds to α6β1 that is physically associated with CD9 and tethered to the actin cytoskeleton. In model II, fertilin β binds to α6β1 that is tethered to the actin cytoskeleton; CD9 indirectly influences the association of α6β1 with the actin cytoskeleton and, therefore, its ability to bind fertilin β.

Several proteins playing a role in the fusion with the spermatozoa have been identified on mammalian oocyte membrane. Actually, two protein families appeared essential for this process: the transmembrane 4 superfamily proteins, called the tetraspanins, and the glycosyl-phosphatidylinositol-anchored family proteins (GPIAPs). Indeed, the fertility of CD9-deficient female mice is severely reduced and double cd9- and cd81 knockout mice are totally sterile (Rubinstein et al. 2006). Oocyte-specific GPI-AP-knockout female mice showed also severely reduced fertility (Alfieri et al. 2003), but cd55- and cd59-knockout mice did not (Holt et al. 2001, Sun et al. 1999). Thus, in absence of tetraspanin, in particular CD9 and CD81, or in absence of one or several GPI-APs, female mice are sterile. Gangliosides are involved in this organization since inhibition of the glycosphingolipid biosynthetic pathway reduces the interaction of CD82 with CD9 and CD151 in mammary epithelial cells (Odintsova et al. 2006). Furthermore, tetraspanins are one of the most abundant protein families found in exosomes. Several members of this family, including CD9, CD63, CD81, and CD82, are highly enriched in exosomes of virtually any cell type. Several tetraspanin partners, such as integrins, co-exist with tetraspanins in exosomes (Thery et al. 2002). CD9, has been shown to be essential for gamete fusion. Indeed, the fertility of CD9- deficient female mice is severely reduced because membrane fusion ability is lost in CD9-deficient eggs (Kaji et al. 2000, Le Naour et al. 2000, Miyado et al. 2000). Nevertheless, CD9 is not the only tetraspanin expressed by oocytes. Several hypotheses have been proposed to explain why CD9 is required for the gamete fusion process. (i) CD9 interacts in cis or laterally with molecules in the plasma membranes to form a tetraspanin web (Zhu et al. 2002). In addition, that CD9 controls the oocyte membrane reorganization that occurs during gamete fusion (Ziyyat et al. 2006). A recent report demonstrates the role of CD9 as an organizer of membrane functionality through CD9-associated proteins during fertilization (Glazar and Evans 2009). (ii) CD9 may also interact in trans, since it has been demonstrated to be the receptor for pregnancy-specific glycoprotein 17 (PSG17) in macrophages (Waterhouse et al. 2002). (iii) CD9 has the ability to maintain the proper structure and function of egg microvilli, as it has recently been demonstrated (Runge et al. 2007). (iv) The spermatozoon acquires CD9 from the oocyte membrane (Rubinstein et al. 2006) in a process similar to trogocytosis (Barraud-Lange et al. 2007). Indeed, CD9 is transferred from the oocyte membrane to the spermatozoon present in the

perivitelline space (PVS), and could induce a sperm membrane molecular reorganization.

2.5.2 Phospholipase C zeta

PLCz is mapped on SSC5q11-12 (ENSEMBL) where QTL for seminiferous tubule diameter and testicular weight (Ren et al. 2009b) and for number of still born (Cassady et al. 2001) are reported in pigs. It transcribes a key protein during sperm-egg fusion in mammals (Le Naour et al. 2000). PLCz mRNA transcripts are reported to be expressed in Landrace boars testis (Yoneda et al. 2006) as well as in other species like in testes of male mice (Yoshida et al. 2007), in mature epididymal sperm of mouse (Coward et al. 2006). Importantly, PLCz expression was suggested to be used as indicator for infertility in stallion since the concentration of PLCz was greatly reduced in infertile stallions (Gradil et al. 2006). The phospholipase-c zeta (PLCz) is in thirteen kinds of mammalian PLC which are classified into six models (Beta, Gamma, Delta, Epsilon, Zeta, Eta) according to structure. PLCz expression could be used as indicator for infertility as a study of fertility in stallion (Giesecke et al. 2010). Moreover, PLCz mRNA transcripts expressed in Landrace boars testis (Yoneda et al. 2006). Porcine sperm contain greater ability to induce Ca^{2+} oscillations than mouse sperm (Kurokawa et al. 2005). In the other species, PLCz expressed on testes of male mice (Yoshida et al. 2007), in mature epididymal sperm of mouse (Coward et al. 2006).

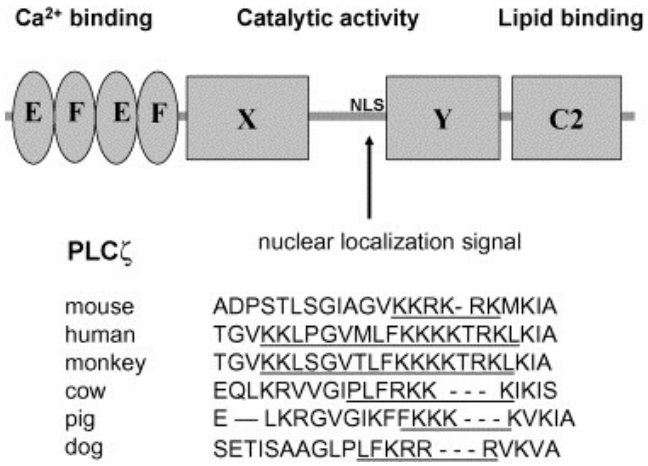

Figure 7: The predicted domain structure of PLCz. The putative nuclear localization sequence (NLS) of PLCz from different species. There is predicted to be 2 pairs of EF hand domains that appear to be involved in Ca^{2+} binding. The catalytic activity is associated with the X and Y domains and the C2 domain is considered to bind to phosphoinositde containing lipids. In mouse there is a nuclear localization sequence that appears to be functional in transporting PLCz into the pronuclei that form after fertilization (Larman et al. 2004). The predicted nuclear localization sequence is shown for the same region of primary sequence for some other mammalian species.

The domain structure of PLCz shares many similarities with PLCs of the δ class that have been described in detail previously (Figure 7) (Katan 1998, Rebecchi and Pentyala 2000). PLCz has X and Y catalytic domains that are common to all phosphoinositide-specific PLCs. In common with PLCδs there is a set of EF hand domains and a C2 domain. The one major difference between PLCz and PLCδ is the absence of a PH domain in all identified versions of PLCz. This means that PLCz is the smallest known mammalian PLC and has an overall size and domain structure that is most similar to plant PLCs (Rebecchi and Pentyala 2000).

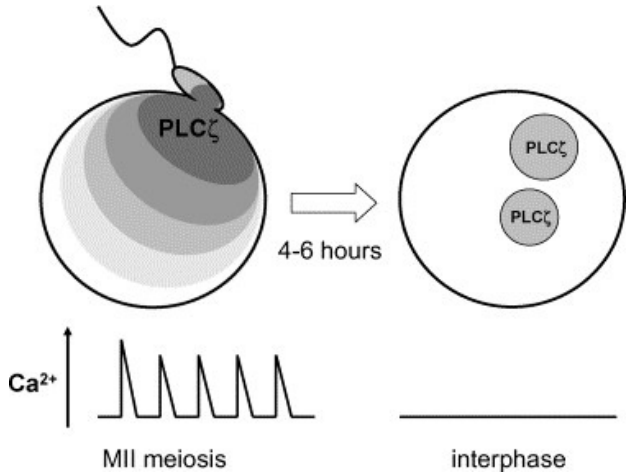

Figure 8: Schematic diagram of PLCz localization at fertilization. When the sperm fuses with the eggs the PLCz is released from the sperm head. PLCz is released from the sperm in a process that may take at least 15 min in the mouse (Knott et al. 2003). It then diffuses into the egg cytoplasm triggering a series of Ca^{2+} oscillations in the egg which is otherwise arrested at metaphase of the second meiotic division. The Ca^{2+} oscillations cease as the egg enters interphase and the zygote then forms two pronuclei into which PLCz is sequestered.

Following fertilization by sperm, the cytoplasmic Ca^{2+} oscillations in the egg cease around the time the two pronuclei form (Marangos et al. 2003). Some Ca^{2+} oscillations are subsequently observed during mitosis in mouse zygotes (Carroll 2001, Marangos et al. 2003). One proposal suggested to explain why Ca^{2+} oscillations stopped and started in such a cell cycle-dependent manner, was that the sperm factor was localized to pronuclei during interphase (Carroll 2001). This localization would result in the cessation of Ca^{2+} oscillations, and they would return when pronuclear envelope breakdown occurred during entry into mitosis. This mechanism is consistent with studies showing that there is a Ca^{2+}-releasing activity associated with fertilized, sperm-injected, or sperm extract-injected mouse embryos (Kono et al. 1995). The idea of a nuclear-associated sperm factor has now been strengthened by recent experimental observations with PLCz. When PLCz is tagged with Venus fluorescent protein or a Myc epitope, the expression of PLCz can be monitored in eggs and is associated with Ca^{2+} oscillations. When the Ca^{2+} oscillations stop the tagged PLCz can be seen to be

associated with the nascent pronuclei (Larman et al. 2004, Yoda et al. 2004). During the first mitosis, the tagged PLCz returns to the cytoplasm in temporal coincidence with the restart of Ca^{2+} oscillations (Larman et al. 2004). Although the Ca^{2+} oscillations have only been recorded to occur during the first mitosis in mouse zygotes, if exogenous PLCz is introduced into the embryos at later stages of development it continues to undergo nuclear sequestration (Sone et al. 2005). This suggests that the nuclear localization is a fundamental feature of mouse PLCz. The idea that PLCz is released from the sperm and then localized to pronuclei in a manner that is consistent with the termination of Ca^{2+} oscillations (Figure 8). There appear to be varying amounts and disparate solubilities of the PLCz in sperm from different species (Kurokawa et al. 2005). This was suggested by early studies which used cytosolic extracts from mechanically-disrupted hamster sperm that were extremely potent in causing Ca^{2+} oscillations in eggs of different species (Homa and Swann 1994, Swann 1990). In contrast, mechanical disruption of mouse sperm was unable to yield an active extract, and one of the first reports of a sperm factor activity found that egg activation could not be caused by mouse sperm extracts (Stice and Robl 1990). More recently, it has been shown that pig sperm contain significantly more PLCz than mouse sperm (Kurokawa et al. 2005). Moreover, whilst some of the PLCz in pig sperm is soluble and found in the cytosolic extract, a considerable proportion of PLC activity is retained in the sperm head and can only be extracted by high pH treatment (Kurokawa et al. 2005). Fractionation of pig sperm also suggests that the 70 kDa, full-length PLCz does not always correlate with PLC activity and the ability of sperm extracts to cause Ca^{2+} oscillations in eggs (Kurokawa et al. 2005). In this species, there is evidence that a proteolytically cleaved version of PLCz may be the active form. Not only are there likely to be differences in the amount and solubility of PLCz from different species but it seems likely that there are substantial differences in the relative potency of the PLCz from different species. We have found that it takes nearly a hundred times less human PLCz cRNA than mouse PCLz cRNA to cause Ca^{2+} oscillations in mouse eggs (Cox et al. 2002). We have also found that the mouse PLCz cRNA is less effective at generating Ca^{2+} oscillations in human eggs (Rogers et al. 2004). Whilst the precise expression levels of protein were not measured quantitatively in these experiments, the data suggests that human PLCz may be unusually effective in causing Ca^{2+} oscillations. When combined with the evidence that sperm from various mammals contain different

amounts of PLCz, it is plausible that the species-dependent variation enables a precise tuning of the effective 'dose' of PLCz delivered by a sperm. The species-specific sperm PLCz quantity and quality is thus adjusted to match the size and sensitivity of the recipient egg, that culminates in a series of robust Ca^{2+} oscillations lasting several hours in order to reliably effect egg activation.

The solubility of PLCz could also be related to the timing of oscillations. For example, at fertilization in hamster eggs, the sperm initiates Ca^{2+} oscillations within about 10 s of sperm-egg fusion, whereas in mouse there appears to be a delay of 1–3 min between sperm-egg fusion and the first Ca^{2+} increase (Lawrence et al. 1997, Miyazaki et al. 1993). The delay to the first Ca^{2+} increase is related to sperm because if zona-free hamster eggs are inseminated with mouse sperm there are also Ca^{2+} oscillations, but these start several minutes after sperm-egg fusion (Igusa et al. 1983). The rapid response of the hamster egg is therefore related to the sperm, and may be explained by the highly soluble nature of the 'sperm factor', and by implication PLCz, in hamster sperm extracts.

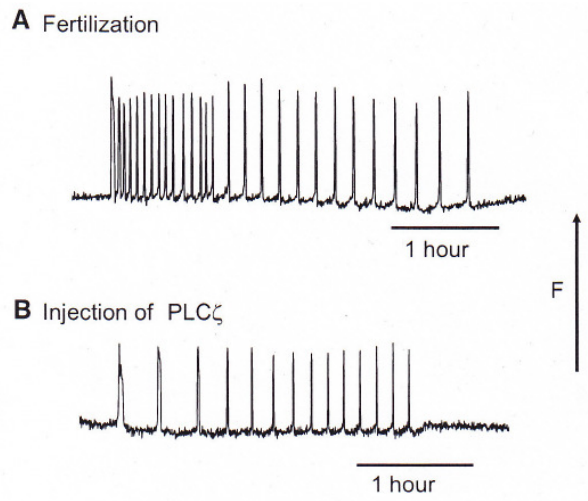

Figure 9: Intracellular Ca^{2+} oscillations in mouse eggs measured by the fluorescence of a Ca^{2+} sensitive dye (Oregon green BAPTA dextran). In (A), an egg is shown in response to fertilization, and in (B), the response of an egg is shown after microinjection of cRNA for mouse PLCz (Campbell and Swann 2006, Saunders et al. 2002). The fluorescence F on the y-axis is in arbitrary units, so the Ca^{2+} levels are not calibrated.

Ca^{2+} oscillations very similar to those seen at fertilization (Kouchi et al. 2004, Saunders et al. 2002). An example of a mouse egg undergoing Ca^{2+} oscillations after injection of PLCz cRNA. Immunodepletion of PLCz from sperm extracts abolishes their ability to cause Ca^{2+} oscillations in mouse eggs (Figure 9). This suggests that the previously described sperm factor is PLCz (Saunders et al. 2007, Swann et al. 2006). There is every indication that PLCz is involved in causing Ca^{2+} oscillations in fertilizing mammalian eggs. A PLCz isoform has now been identified in at eight mammalian species and in many cases it has been shown to trigger Ca^{2+} oscillations in eggs (Swann et al. 2006). The amount of PLCz that is required to trigger Ca2+ oscillations is within the range of PLCz in a single sperm (Saunders et al. 2002). The release of PLCz from sperm probably occurs well within the first hour after sperm-egg fusion and this correlates with the time over which the Ca^{2+} oscillations occur (Yoon and Fissore 2007). There also now evidence that PLCz is the factor responsible for causing Ca^{2+} oscillation afterintracytoplasmic sperm injection (Fujimotoa et al. 2004). Only one study to date has directly addressed the requirement of PLCz in normal fertilization. The knockdown of PLCz in sperm using a transgenic RNAi approach has been shown to reduce the number of Ca^{2+} oscillations and reduce the activation rates at fertilization (Knott et al. 2005). This transgenic approach generates mosaic expression in spermatogenic cells, and some sperm would still contain some PLCz that could account for why some sperm can still cause some Ca^{2+} oscillations and egg activation. One noteworthy feature, however, is that the transgene was never passed on from males to the next generation. This implies that sperm carrying the transgene, where PLCz would be most reduced, are not able to trigger an egg to activate and develop. This implies that PLCz is required for normal fertility in male mice (Knott et al. 2005). The actions of PLCz appears to be specific to egg since transgenic expression of PLCz in somatic cells appears to have little effect. In the ovary PLCz expression it leads to spontaneous egg activation and the subsequent development of ovarian teratocarcinomas (Yoshida et al. 2007). The mechanism of action and localization of PLCz in eggs is unclear. Studies using a Venus-tagged (hence highly fluorescent) version of PLCz have suggested that it is not specifically localized in the plasma membrane (Yoda et al. 2004). The apparent lack of specific plasma membrane localization could be due to the fact that PLCz lacks a PH domain which localizes the closely related PLCz 1 to the PIP2 in plasma membrane (Saunders et al. 2002). The parts of PLCz that may be involved in localization to a

source of PIP2 are the C2 domain and a region between the X and Y catalytic domains called the X-Y linker (Kouchi et al. 2005, Nomikos et al. 2005). The X-Y linker region has several basic residues that could help anchor it to PIP2 which is a very negatively charged phospholipid (Nomikos et al. 2007). However, it is still unclear whether the PIP2 that PLCz binds to is in the plasma membrane or an internal organelle. The only statement we can make about its localization is that PLCz enters the pronuclei as they form after mouse egg activation (Larman et al. 2004, Yoda et al. 2004, Yoon and Fissore 2007). This localization appears to involve a nuclear targeting region in the X-Y linker region of the protein that is the same as that proposed to bind to PIP2. The localization of PLCz in pronuclei is associated with the termination of Ca^{2+} oscillations and may act in conjunction with modifications to the InsP3 receptor to terminate Ca^{2+} signals at fertilization (Larman et al. 2004, Lee et al. 2006). More extensive reviews of PLCz can be found elsewhere (Saunders et al. 2007, Swann et al. 2006).

2.5.3 Cyclooxygenase isoenzyme type 2

COX-2 is mapped on the distal part of SSC9q (SSC9q24-26) (ENSEMBL) where QTL for semen pH and sperm abnormality rate (Xing et al. 2009) and for litter size (Bidanel et al. 2008) are reported in pigs. The prostaglandin is one of four families of eicosanoid (De Caterina and Basta 2001, Funk 2001, Soberman and Christmas 2003) which is synthesized from arachidonic acid by Cyclooxygenase isoenzyme type 2 (COX-2). The prostaglandin is required for spermatogenesis and function in male reproductive tracts of many species such as hamster Leydig cells (Frungieri et al. 2006, Matzkin et al. 2009), rat testis (Winnall et al. 2007, Yamaguchi et al. 2008), human Leydig cells (Sirianni et al. 2009), rat vas deferens (McKanna et al. 1998, Ruan et al. 2008), human prostate gland (Rubio et al. 2005) and responsible for the fertilization in female such as bovine embryo (El-Sayed et al. 2006), mice uterus (Lim et al. 1997), bovine endometrium (Liu et al. 2001), bovine uterus (Wehbrink et al. 2008).

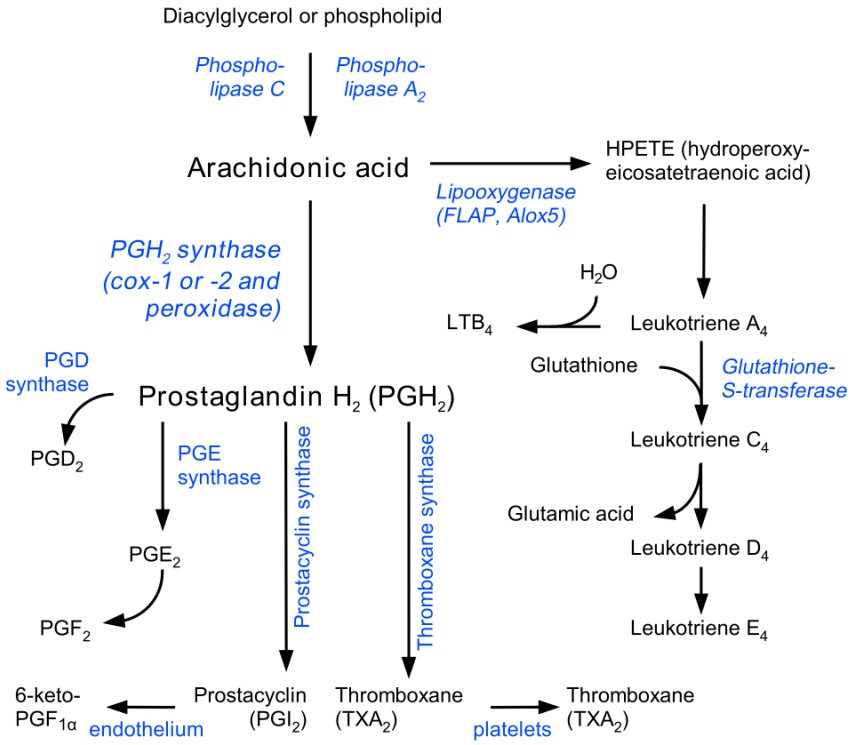

Figure 10: Overview of eicosanoid biosynthesis. Arachidonic acid is released via the actions of phospholipases and oxidized through the cyclooxygenase and peroxidase activities of 2 prostaglandin H synthase isozymes (commonly called COX-1 and COX-2). The intermediate PGG2 is reduced to PGH2. PG synthases convert PGH2 to prostaglandins and thromboxane A2. Lipoxygenases catalyze the regioisomeric introduction of molecular oxygen of arachidonic acid yielding the corresponding hydroperoxides (Helliwell et al. 2004, Simmons et al. 2004).

Two major metabolic routes, i.e., the COX and LOX pathways, control the biosynthesis of eicosanoids. COX-derived eicosanoids comprise prostaglandins (PG) and thromboxane A_2 (TXA_2), while hydroperoxyeicosatetraenic acids (HPETE) and leukotrienes (LT) are products of LOX-catalyzed arachidonic acid metabolism (Figure 10). Two isoforms of COX, i.e., COX-1 and COX-2, have been described: COX-1 is constitutively expressed in most tissues and appears to generate PG that control normal physiological functions such as platelet aggregation, regulation of renal blood flow and maintenance of the gastric mucosa. In contrast, COX-2 is transiently induced by

proinflammatory stimuli, growth factors, cytokines and tumor promoters resulting in increased rates of PG formation during tissue injury and repair. The production of PG is a tightly regulated process that requires free fatty acids as substrates in most cases, the expression and activity of the individual COX isozymes, and the cell type-selective expression and activity of different PG synthases (Helliwell et al. 2004, Simmons et al. 2004). The specificity of action of the different PG is likely to predominantly depend on the cell type-specific expression of the corresponding G protein-coupled seventransmembrane PG receptors (Hata and Breyer 2004). COX isozymes, PG synthases and PG receptors are major players of the COX pathway, which control both synthesis and function of PG. Increased COX-2 mRNA and protein that are observed in premalignant and malignant epithelial and nonepithelial tumors are consistently associated with the accumulation of COX-derived PG. In fact, PGE_2 has been found to be the major PG in human colorectal cancer (Rigas et al. 1993) and increased contents of PGE_2 and $PGF_{2\alpha}$ were observed in endometrial adenocarcinoma (Sales et al. 2004), and accumulation of PGE_2, $PGF_{2\alpha}$ and PGI_2 is a consistent feature of skin tumors (Furstenberger et al. 2003, Muller-Decker et al. 2002). In addition, PGE_2 was shown to prevent COX inhibitor-induced adenoma regression in APC^{Min} mice and $PGF_{2\alpha}$ reversed the indomethacin-induced inhibition of tumor development in mouse skin (Fischer et al. 1987, Hansen-Petrik et al. 2002). This data demonstrates that PG are critically involved in colorectal and skin carcinogenesis in these models. However, that COX-independent effects may also contribute to reduction of tumor formation is indicated by numerous studies that identified COX-2-independent antitumor targets, in particular, at high inhibitor doses (Baek and Eling 2006, Tegeder et al. 2001). The cell types that contribute to aberrant COX-2 expression and PGE_2 synthesis are less clear and may depend on the tissue type. In human colorectal cancer, increased COX-2 expression is present in the epithelial tumor parenchyma and in mononuclear cells, vascular endothelium, smooth muscle cells and fibroblasts. Moreover, COX-2 expression levels in the individual cell types may vary with tumor progression (Brown and DuBois 2005). In skin, COX-2 expression is restricted to basal keratinocytes and dendritic cells of the papilloma parenchyma and to endothelial cells and macrophages of the tumor stroma. In squamous cell carcinomas, a strong COX-2 expression is observed in keratinocytes and infiltrated inflammatory cells (Furstenberger et al. 2003).

3 Material and methods

3.1 Materials

In this section, materials used in this study such as animals, all other biological materials, chemicals, kits, reagents, media, soft wares, equipments and their sources are mentioned. The experimental protocol was carried out according to the rules and regulations of the German law of animal protection.

3.1.1 Animals

3.1.1.1 Boars for association analysis

Samples and phenotypes from 231 purebred Pietrain (PI) and 109 Pietrain × Hampshire crossbred (PIHA) boars born before 1996 up to 1999 were used in this study. Animals derived from an artificial insemination (AI) station and were mated to sows in commercial pig herds in North-Western Germany. They represent commercially used boars with high estimated breeding values in regards of production parameters. The AI station provided quality and quantity records of more than 46,000 ejaculates from these boars, including repeated measures of sperm concentration (SCON [$\times 10^8$ ml]), semen volume per ejaculate (VOL [ml]), sperm motility (MOT [%]), plasma droplets rate (PDR [%]) and abnormal spermatozoa rate (ASR [%]).Whole ejaculates were obtained from purebred Pietrain and crossbred Pietrain × Hampshire boars aged between two to five years. Semen was collected by the vinyl gloved hand method 2 times per week in 4 week intervals during a period of 2000 to 2001. These parameters were obtained from each ejaculate between January 2000 to December 2001 using light microscopy according to the guidelines of the World Health Organization (WHO). Additional fertility data including non return rate data at 42 days after insemination (NRR [%]) and number of piglet born alive (NBA)] per litter were available as the deviation from the population means within sow breed, parity of sow, farm and season classes.

3.1.1.2 Boars for mRNA and protein expression analysis

Boars from the AI station SuisAG (Sempach, Switzerland) were selected based on extreme phenotypes (high/low SCON and SMOT, and SVOL). The SCON (average sperm concentration) was highly negative (r^2 = -0.8) correlated with SVOL (average semen volume), whereas SCON was highly positive (r^2 = 0.7) correlated with SMOT (average sperm motility). Moreover, SVOL was highly negative (r^2 = -0.8) correlated with SMOT. Therefore, grouping was done on the basis of SCON, SVOL and SMOT (Table 2). A total of six animals were selected and equally divided into group I (G-I) with high SCON (> 262.32 $\times 10^6$ ml), high SMOT (> 76.59%) and low SVOL (< 215.24 ml/ejaculation) and group II (G-II) with low sperm concentration and motility, and high sperm volume. The significance difference between the two groups was calculated using proc t-test in SAS. There were differences for SCON ($p < 0.05$) and for SVOL ($p < 0.01$) between G-I and G-II, whereas for the SMOT the difference was not significant ($p = 0.12$). Reproductive tissues (testis, head of epididymis, body of epididymis, tail of epididymis, vas deferens, bulbourethral gland, vesicular glands and prostate gland), non reproductive tissues (brain, liver and skeletal muscle tissue) and semen samples (spermatozoa) of six boars (Duroc, Large White and Landrace) were collected for the mRNA and protein study.

3.1.2 Materials for laboratory analysis

3.1.2.1 Chemicals, kits, biological and other materials

Amersham Biosciences (Freiburg)	CyScribeTM GFXTM purification kit, CyScribe postlabelling kit
Applichem (Darmstadt, Germany)	50x Denhardt's solution
Applied Biosystems (Foster City)	SYBR® Green Universal PCR Master Mix
Beckman Coulter (Krefeld)	CEQ™ 8000 Genetic Analysis System, Dye TerminatorCycle Sequencing (DTCS), Glycogen
Biomol (Hamburg)	Phenol, Phenol/ Chlorophorm/Isoamyl alcohol (25:24:1), Lambda DNA Eco9lI

	(BstE II) and Lambda DNA HindIJJ
Biozym Diagnostic (Epicentre Technologies) (Hessisch-Oldendorf)	Sequagel XR Sequencing Gel (National Diagnostics), SequiTherm EXCELTM II DNA
Sequencing kit-LC, AmpliScribeTM T7 transcription kit Corning (Amsterdam)	GAPS II coated slides
Corning (Amsterdam)	GAPS II coated slides
DYNAL Biotech (Hamburg)	Dynabeads oligo (dT)25
Eppendorf (Hamburg)	2.5x RealMasterMix/ 20x SYBR Solution
Invitrogen Life Technologies (Karlsruhe)	DTT, SuperScriptTM II RNase H- Reverse Transcriptase, 5 X first strand buffer, Random Primers
MBI Fermentas (St. Leon-Rot)	Glycogen
MWG Biotech (Eberberg, Germany)	Oligonucleotide primers
Promega (Mannheim)	Random primer, BSA, pGEM®-T vector, RQ1 RNase-free DNase, RNasin (Ribonuclease inhibitor), 2X rapid ligation buffer, T4 DNA ligase, Pronto!TM Plus systems
Qiagen (Hilden)	RNeasy® Mini kit, QIAquick PCR Purification Kit, Mini EluteTM Reaction Cleanup Kit
Roche (Mannheim, Germany)	Horseradish peroxidase antidigoxigenin antibody
Roth (Karlsruhe)	Acetic acid, Agar, Ampicillin, Bromophenol blue, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Hydrochloric acid, Isopropyl -D-thiogalactoside (IPTG), Kohrso1in FF, Nitric acid, Peptone, Potassium dihydrogen phosphate, 2- Propanol, Silver nitrate, Sodium acetate, Sodium carbonate, Sodium

	chloride, Sodium hydroxide, Trichloromethane/chlorophorm, Tris, X-Gal (5-bromo-4-chloro-3- indolylbeta-D-galactopyranoside), Yeast extract
Sigma-Aldrich Chemie GmbH (Munich)	Acetic anhydride, Agarose, Ammonium acetate, Calcium chloride, Calcium chloride dihydrate, Calcium lactate, Dulbecco's phosphate buffered saline (D-PBS), Formaldehyde, GenEluteTM plasmid Miniprep kit, Heparin, Hepes, Hydroxylamin, Hypotaurin, Igepal, Isopropanol, Magnesium chloride, Magnesium chloride hexahydrate, Medium 199, 2-Mercaptoethanol, Mineral oil, Oligonucleotide primers, Penicillin, Phenol red solution, 10 X PCR reaction buffer, Potassium chloride, Sodium dodecyl sulfate (SDS), Sodium hydrogen carbonate, Sodium hydrogen phosphate, Sodium hydrogen sulfate, Sodium lactate solution (60%), Sodium pyruvate, Streptomycin sulfate, Taq DNA polymerase, yeast tRNA
STARLAB GmbH (Ahrensburg)	Rigid thin wall 96 X 0.2 ml skirted microplates for real-time PCR
Stratagene (Amsterdam)	5 α DH *Escherichia coli* competent cells
USB (Ohio)	ExoSAP-IT

3.1.2.2 Reagents and media

All solutions used in this investigation were prepared using deionized millipore water (ddH2O) and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

General reagents

DEPC-treated water:	DEPC	1.0
	Water added to	1,000.0
	Incubate overnight at 37°C and heat inactivate by autoclaving	
dd H2O:	Deionised millipore water	
LB-agar plate:	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH2O added to	800.0 ml
LB-broth:	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH2O added to	800.0 ml
TBE (10x) buffer:	Tris	108.0 g
	Boric acid	55.0 g
	EDTA (0.5 M)	40.0 ml
	ddH2O added to	1000.0 ml
TAE (50x) buffer, pH 8.0:	Tris	242.0 mg0
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml00
	ddH2O added to	1000.0 ml
TE (1x) buffer:	Tris (1 M)	10.0 ml
	EDTA (0.5 M)	2.0 ml
	ddH2O added to	1000.0 ml
X-gal:	X-gal	50.0 mg
	N, N'-dimethylformamide	1.0 ml

10x FA buffer, pH 7.0:	MOPS	41.8 g
	Sodium acetate	4.1 g
	EDTA (0.5M)	20.0 ml
	ddH2O added to	1000.0 ml
1.2% FA gel:	Agarose	1.2 g
	10 x FA buffer	10.0 ml
	RNase free H_2O	90.0 ml
	Ethidium bromide	2.0 µl
	Formaldehyde (37%)	1.8 ml
Agarose loading buffer:	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH2O added to	25 ml
Digestion buffer:	NaCl	100 mM
	Tris-HCl	50 mM
	EDTA pH 8.0	1mM
SDS solution:	Sodium dodecylsulfat in ddH2O	10% (w/v)
Proteinase K solution:	Protein K in 1 x TE buffer	2% (w/v)
dNTP solution:	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	ddH2O added to	400.0 µl
IPTG solution:	IPTG	1.2 g
	ddH2O added to	10.0 µl
3M Sodium Acetate, pH 5.2:	Sodium Acetate	123.1 g
	ddH2O added to	500 ml
1M EDTA, pH 8.0:	EDTA	37.3 g
	ddH2O added to	1000 ml
Phenol Chloroform:	Phenol : Chloroform	1 : 1 (v/v)

Reagents for Immunohistochemistery

0.2% Triton-X100:	Triton	2 ml
	10x PBS: added to	1,000.0 ml
0.3% BSA in PBS	PSA	3 g
	10x PBS: added to	1,000.0 ml
3% BSA in PBS	PSA	30 g
	10x PBS: added to	1,000.0 ml
4% PFA (pH 7.3):	Paraformaldehyde	10.00 g
	1 x PBS added to	250.00 ml
	Bring to 65°C under ventilation hood. Add 5 µl of 5 M NaOH for solution to become clear. Store protected from light and use within 2 weeks.	

Reagents for in western blotting

10x PBS:	NaCl	8.77 g
	NaH_2PO_4	1.50 g
	NaH_2PO_4	2.04 g
	Water added to	1,000.0 ml
1x PBS:	10x PBS	100.0 ml
	Water added to	1,000.0 ml
1x PBS-Tween (PBST):	1x PBS	999.50 ml
	Tween®20	0.50 ml
Tris-HCl (1M):	Tris	121.14 g
	Water added to	1,000.00 ml
	Adjust pH to the required pH.	
10% Tween 20:	DEPC-treated H2O	45.00 ml
	Tween®20	5.00 ml

3.1.3 Used softwares

Axio Vision	Carl Zeiss International
BLAST program	http://www.ncbi.nlm.nih.gov/BLAST
EndNote XI	Thomoson
Entrez Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene

Multiple Sequence Alignment	http://searchlauncher.bcm.tmc.edu/multialign/Options/clustalw.html
Primer Express® software	Applied Biosystems, Foster city, CA, USA
Primer 3 (version 4)	http://frodo.wi.mit.edu/primer3/
SAS (version 9.02)	SAS Institute Inc., NC, USA

3.1.4 Equipment

ABI PRISM® 7000 SDS	Applied Biosystems, Foster city, USA
Centrifuge Hermle, Wehingen	Applied Biosystems, Foster city, USA
CEQ™ 8000 Genetic Analysis System	Beckman Coulter GmbH, Krefeld
Electrophoresis (for agarose gels)	BioRad, Munich
Electrophoresis (vertical apparatus)	Consort, Turnhout
Fluorescence microscope (DM-IRB)	Leica, Bensheim, Germany
GFL 7601 hybridization chamber	Fisher scientific, Leicestershire, UK
GenePix 4000A scanner	Axon Instruments, Foster City, USA
HERA safe Bioflow safety hood	Heraeus Instruments, Meckenheim
Icycler	Bio-Rad Laboratories, München, Germany
Incubator	Heraeus, Hanau
Millipore apparatus	Millipore corporation, USA
Nanodrop 8000	Thermo scientific, Germany
PCR thermocycler (PTC100)	MJ Research, USA & BioRad, Germany
pH meter	Kohermann
Power supply	PAC 3000 Biorad, Munich
Savant SpeedVac®	TeleChem International, Sunnyvale
Spectrophotometer, Ultrospec™ 2100 *pro* UV/Visible	Amersham Bioscience, Munich
Thermalshake Gerhardt	John Morris scientific, Melbourne
Tuttnauer autoclave	Connections unlimited, Wettenberg
Ultra low freezer (-80°C)	Labotect GmbH, Gottingen
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany
Spectrophotometer (DU-62)	Beckman, Unterschleissheim-Lohhof

3.2 Methods

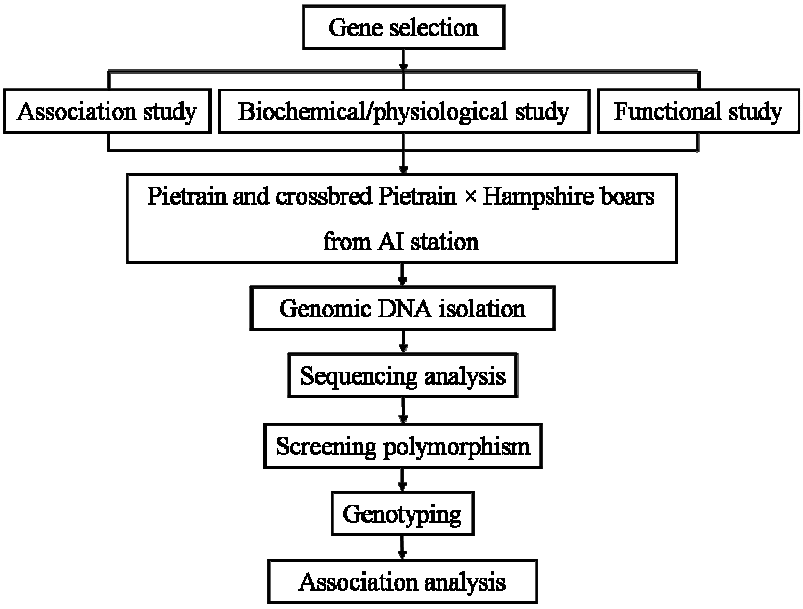

Figure 11: Overview of the association study

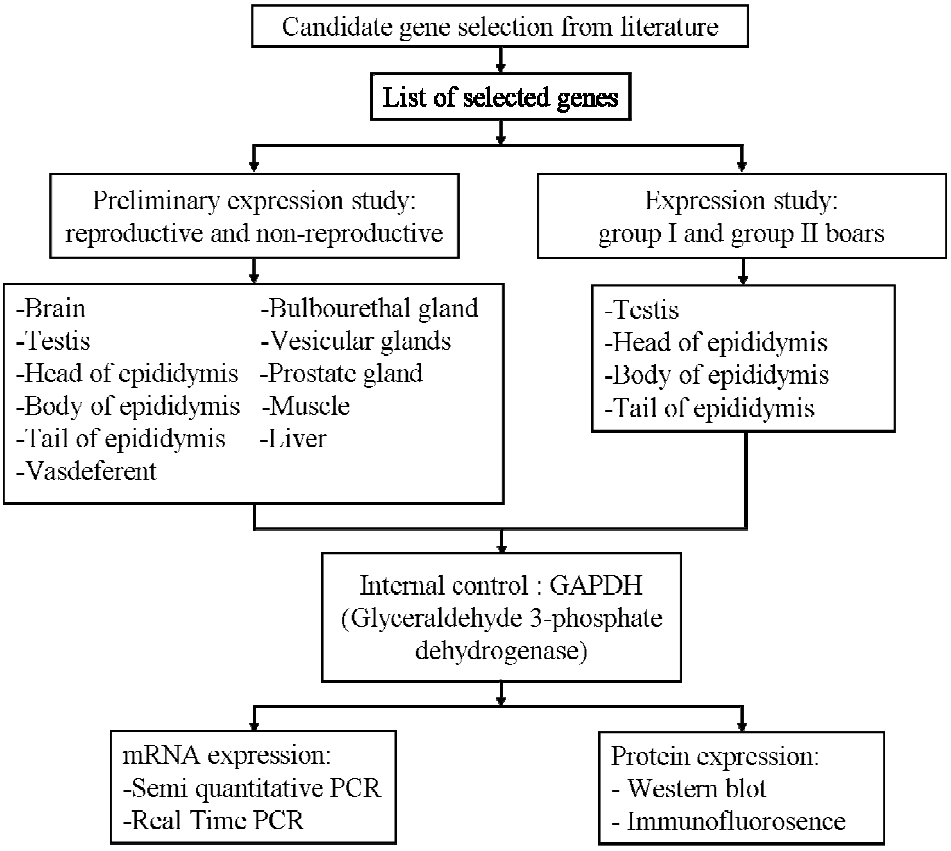

Figure 12: Overview of the expression profiling

3.2.1 DNA isolation

Genomic DNA was isolated from the sperm samples of 340 boars by standard protocol using mercaptoethanol treatment and proteinase K digestion followed by phenol/chloroform extraction and precipitation with isopropanol.

3.2.2 SNP screening

To screen for polymorphisms in the sequence of the porcine CD9, PLCz and COX-2 gene, samples from different pig breeds including Duroc × Pietrain (DUPI), Duroc × Berlin Miniature (DUMI), Pietrain (PI) and Pietrain × Hampshire (PIHA) were used. Genomic DNA was isolated from the sperm samples or skeletal muscle using standard phenol/chloroform extraction and precipitation with isopropanol. A working solution with the final concentration of 50 ng/µl was prepared and DNA samples were stored at 4°C. The porcine CD9 mRNA sequence (GenBank accession No. NM_214006), the porcine PLCz genomic DNA sequence (GenBank accession No. NC_010447) and the porcine COX-2 mRNA sequence (GenBank accession No. GU971651) were used for primer design using the software Primer3 (Rozen and Skaletsky 2000) (Table 2). Polymerase chain reactions (PCR) were performed in 20 µl volume containing 100 ng genomic DNA, 1×PCR buffer (with 1.5 mM $MgCl_2$), 0.25 mM of each dNTP, 5 pmol of each primer and 0.1 U Taq DNA polymerase (GeneCraft) using an initial denaturation at 95^{o}C for 5 min followed by 35 cycles of 30 s at 95^{o}C, 30 s at 58^{o}C, 45 s at 72^{o}C and final elongation of 10 min at 72^{o}C. The PCR reaction of PLCz primers was performed under the following conditions: initial denaturing at 95^{o}C for 5 min followed by 35 cycles of 30 s at 95^{o}C, 45 s at 58^{o}C, 45 s at 72^{o}C and final elongation of 10 min at 72^{o}C. The PCR reaction of COX-2 primers was performed under the following conditions: initial denaturing at 95^{o}C for 5 min followed by 40 cycles of 30 s at 95^{o}C, 45 s at 63^{o}C, 1 min at 72^{o}C and final elongation of 10 min at 72^{o}C. The PCR products were purified and comparatively sequenced by using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter). Individual sequences were compared to sequences of other species using the web-based program MULTIALIN (Corpet 1998) for alignment.

Table 2: Polymerase chain reaction primers used for SNPs screening

Gene	Primer pairs	Fragment (bp.)	SNP position
CD9	Fw: 5´-taatgggggaagtggaacaa-3´	523	Intron 6
	Rv: 5´-cgccaatgatgtggaact t-3´		g.358A>T
	Fw: 5´-tcttctcggggtcctgtttg-3´	383	3′UTR
	Rv: 5´-ttttcaggacgttgcatcatcttt-3´		g.1022C>T
PLCz	Fw: 5´-ggtgttcagaccgaaaggaa-3´	723	Intron 1
	Rv: 5´-aacagaaaggacttctgagtgga-3´		g.71T>C,
			g.158A>C
			Exon 2
			g.54T>G
	Fw: 5´-catgagatagactgccctctga-3´	511	Intron 3
	Rv: 5´-ctgaattcccagcagacattc-3´		g.10,444A>G,
			g.10,521T>G
			Exon 4
			g.99A>C,
			g.148T>C
COX-2	Fw: 5'-caacgcctctaccagtctgc-3'	372	Exon 2
	Rv: 5'-ttcgggtgcagtcacactta-3'		g.33C>T
	Fw: 5'-gtgcactacatacttacccacttc-3'	825	Intron 3
	Rv: 5'-aggcttcccagcttttata-3'		g.244C>G
	Fw: 5'-aaaagctgggaagccttttc-3'	596	Intron 4
	Rv: 5'-ggggatcagggatgaacttt-3'		g.56T>G
	Fw: 5'-ggctgcgggaacataataga-3'	474	Intron 7
	Rv: 5'-gcagctctgggtcaaacttc-3'		g.76A>G
	Fw: 5'-ttgatggccacgagtacaac-3'	561	Intron 8
	Rv: 5'-gatcgatagggcttcagcag-3'		g.55T>G
	Fw: 5'-gtcggctgacattttctggt-3'	442	3′UTR
	Rv: 5'-tgcccagaatgtctctccat-3'		g.68G>A,
			g.1708A>G

3.2.3 Genotyping

A total of 340 PI and PIHA boars were genotyped by restriction fragment length polymorphism (RFLP) analysis using the restriction enzymes HinfI and DraI (New England) for the CD9 fragments (Table 3) covering exon 6 to 7 (forward primer: 5´-taatgggggaagtggaacaa-3´; reverse primer: 5´-cgccaatgatgtggaactt-3´) and the 3´ UTR (forward primer: 5´-tcttctcggggtcctgtttg-3´, reverse primer: 5´-ttttcaggacgttgcatcatcttt-3´), respectively. After incubation at 37°C overnight fragments were visualized using electrophoresis in 3% (w/v) agarose gels. After checking the PCR products, genotyping primers (Table 3) of the exon 1 - exon 2 (forward: 5´-ggtgttcagaccgaaaggaa-3´ and reverse: 5´-aacagaaaggacttctgagtgga-3´) in PLCz sequence were used for restriction fragment length polymorphisms (RFLPs) analysis. The digestion of restriction enzyme Tsp509fI (New England) was carried out in 10 µl of reaction mixture of each sample and incubated for overnight at 65°C. Genotyping primers (Table 3) of the exon 9 (forward: 5´-tcgaccagagcagagagatgagat-3´ and reverse: 5´-accatagagcgcttctaactctgc-3´) in COX-2 sequence were also used for genotyping. The digestion of restriction enzyme BsrBI (New England) was carried out in 10 µl of reaction mixture of each sample and incubated for overnight at 37°C. Detection of RFLPs of 340 boars was carried out by electrophoresis in 3% (w/v) agarose gels.

Table 3: Polymerase chain reaction primers used for genotyping analysis

Gene	Primer sequence	Product size (bp)	Annealing temperature (◦C)
COX-2	Fw: 5'-tcgaccagagcagagagatgagat-3' Rv: 5'-accatagagcgcttctaactctgc-3'	278	58^{o}C
CD9	Fw: 5'-taatgggggaagtggaacaa-3' Rv: 5'-cgccaatgatgtggaactt-3'	523	58^{o}C
PLCz	Fw: 5'-ggtgttcagaccgaaaggaa-3' Rv: 5'-aacagaaaggacttctgagtgga-3'	723	58^{o}C

3.2.4 Statistical analysis for sperm quality traits

The association of CD9, PLCz and COX-2 with sperm quality and quantity traits was carried out by variance analysis (PROC MIXED) using the SAS software package (SAS Institute Inc., ver. 9.2). Statistical analyses were carried out using the following model

$$y_{ijklm} = \mu + breed_i + season_j + genotype_k + age_l + ejaculation_m + \varepsilon_{ijklm} \quad \text{[Model 1]}$$

where y_{ijklm} is the sperm quality traits (SCON, VOL, MOT, PDR, ASR); μ is the overall population mean; $breed_i$ is the fixed effect of the i-th breed (i = PI; PI×HA); $season_j$ is the fixed effect of the j-th season (j = 1 through 8; four seasons per year, in total eight seasons within 2 years from January 2000 to December 2001); $genotype_k$ is fixed effect of the k-th genotype (k= 1, 2 and 3); age_l is the effect of boar age (covariable); $ejaculation_m$ is the random effect of the m-th boar and ε_{ijklm} is the residual error.

3.2.5 Statistical analysis for fertility traits

The association analysis between CD9, PLCz and COX-2 and the fertility traits was carried out using the following generalized linear model (PROC GLM) in SAS.

$$y_{ijk} = \mu + breed_i + genotype_j + year_k + \varepsilon_{ijk} \quad \text{[Model 2]}$$

where y_{ijk} is the boar fertility trait (NRR and NBA); μ is the overall population mean; $breed_i$ is the fixed effect of the i-th breed (i = PI, PI×HA); $genotype_j$ is the fixed effect of the j-th genotype (j= 1, 2 and 3); $year_k$ is the fixed effect of the k-th boar year of birth (k = 1 through 3: boar born before 1996, in 1996–97 and in 1998–99), ε_{ijk} is the residual error.

The distribution of the genotypes was tested for Hardy–Weinberg equilibrium by chi-square (χ^2) test before using both polymorphisms for the association analysis. Least square means for CD9, PLCz and COX-2 genotypes were compared by t-test and P-values were adjusted by Tukey–Kramer correction.

3.3 Quantitative Real-Time PCR

Total RNA was isolated using TRI Reagent (Sigma) from different tissues of breeding boars (testis, head of epididymis or caput, body of epididymis or corpus, tail of epididymis or cauda, vas deferens, bulbourethral gland, vesicular glands, prostate gland, brain, liver, skeletal muscle tissue, spermatozoa). RNA was purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated using on-column RNase-Free DNase set (Promega) and quantified sphectrophotometrically (Nano Drop, ND8000). Furthermore, RNA integrity was checked by 2% agarose gel electrophoresis. First-strand cDNA were synthesized from individual RNA using Superscript II enzyme (Invitrogen). Primers for qRT-PCR were designed using the publicly available Primer3 tool and are listed in Table 2. Plasmids of 9-fold serial dilution were prepared and used as template for the generalization of the standard curve. In each run, the 96-well micro titer plate contained each cDNA sample, plasmid standards for the standard curves and no-template control. To ensure repeatability of the experiments, each sample was run in three replications. qRT-PCR was set up using 2 µl first-strand cDNA template, 7.6 µl deionized H_2O, 0.2 µM of upstream and downstream primers, and 10 µl 1x Power SYBR Green I master mix with ROX as reference dye (Applied Biosystems). The thermal cycling conditions were: 3 min at 94°C followed by 20 sec at 94°C (40 cycles) and 1 min at 60°C. Experiments were performed using ABI prism®7000 (Applied Biosystems) quantitative real-time PCR system. An amplification-based threshold and adaptive baseline were selected as algorithms. Common housekeeping gene GAPDH (GenBank: AF017079) was used for the data normalization. Final results were reported as the relative expression level compared after normalization of the transcript level using the housekeeping gene. Differences in CD9, PLCz and COX-2 genes expression were analyzed with the simple t-test in SAS software (SAS Institute Inc., ver. 9.2). Values of $p < 0.05$ were considered to indicate statistically significant differences.

Table 4: Primers used for real-time polymerase chain reaction

Gene	Primer sequence	Product size (bp)	Annealing temperature (◦C)
COX-2	Fw: 5'-caacgcctctaccagtctgc-3'	372	58°C
	Rv: 5'-ttcgggtgcagtcacactta-3'		
CD9	Fw: 5'-tcttctcggggtcctgtttg-3'	383	58°C
	Rv: 5'-ttttcaggacgttgcatcatcttt-3'		
PLCz	Fw: 5'-ggtgttcagaccgaaaggaa-3'	723	58°C
	Rv: 5'-aacagaaaggacttctgagtgga-3'		

3.4 Western Blotting

Total protein was isolated using TRI reagent (Sigma) from different tissues of breeding boars (testis, head of epididymis or caput, body of epididymis or corpus, tail of epididymis or cauda, vas deferens, bulbourethral gland, vesicular glands, prostate gland, brain, liver, skeletal muscle tissue, spermatozoa). The protein extracted from tissues was separated by SDS-PAGE (gradient 4-18%). Subsequently the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% Polyvinylpyrolidone) at room temperature for 1 h, the membrane was incubated with the anti-CD9 antibody purified from goat polyclonal antibody (Cat.nr. Sc7639; Santa Cruz). The membrane was incubated overnight at 4 °C with the anti-PLCz antibody purified from goat polyclonal antibody (Cat.No. Sc31748; Santa Cruz) and the anti-COX-2 antibody purified from goat polyclonal antibody (Cat.No. Sc1745; Santa Cruz) in the blocking medium (diluted 1:500). The horseradish peroxidase conjugated donkey anti-goat IgG secondary antibody (Cat.nr. Sc2020; Santa Cruz) was used as the secondary antibody. The chemiluminesce was detected by using the ECL plus western blotting detection system (Amersham Biosciences) and visualized by using Kodak BioMax XAR film (Kodak). GAPDH was used as a loading control and for normalizing. The membrane was stripped by incubation in 2% SDS, 100 mM Tris-HCl, 0.1% β-mercaptoethanol for 30 min at 60°C and re-probed with GAPDH antibody (Cat.nr. Sc20357; Santa Cruz).

3.5 Protein localization of CD9 by immunofluorescence

Due to the limitations of samples from G-I and G-II boars, we collected different fresh reproductive tissues from a healthy Pietrain boar after slaughtering for protein localization. Immunofluorescence staining was performed on 8 µm cryostat sections of snap frozen tissues. All sections were kept in -80ºC for further analysis. To block unspecific staining, sections were incubated for 30 min at room temperature with 5% bovine serum albumin in PBS (50 nM sodium phosphate, pH 7.4; 0.9% NaCl). The sections were incubated overnight at 4 ºC with the CD9 goat polyclonal primary antibody (Cat.No. Sc7639; Santa Cruz) diluted at 1:50 in PBST, the PLCz goat polyclonal primary antibody (Cat.No. Sc31748; Santa Cruz) and the COX-2 rabbit polyclonal primary antibody (Cat.No. Sc7951; Santa Cruz) diluted at 1:50 in PBST followed by six times (10 m per time) washing with PBS. Then, the sections of CD9 antibody and PLCz antibody were incubated 1 h at room temperature with the donkey anti-goat IgG-B conjugated with Rhodamine (TRITC) reactive water-soluble fluorescent dye (Cat.No. Sc2094; Santa Cruz) (dilution 1:200) followed by six times (10 m per time) washing with PBS. The detection of COX-2 secondary antibody sections were incubated in biotinylated donkey anti-rabbit IgG-B conjugated with Fluorescein Isothiocyanate (FITC) reactive water-soluble fluorescent dye (Cat.No. Sc2090; Santa Cruz) (dilution 1:200). Finally, the samples were counterstained with vectashield mounting medium (Vector Laboratories) containing 40,6-diamidino-2-phenyl indole (DAPI) and covered with a cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss). In case of negative controls, PBS was used instead of the primary antibody.

4. Results

4.1 Genotyping of candidate genes

4.1.1 Detection of polymorphisms in CD9, PLCz and COX-2 genes

The porcine CD9 mRNA, genbank accession No. NM_214006 were compared sequences from human, genbank accession No. NC_000012.

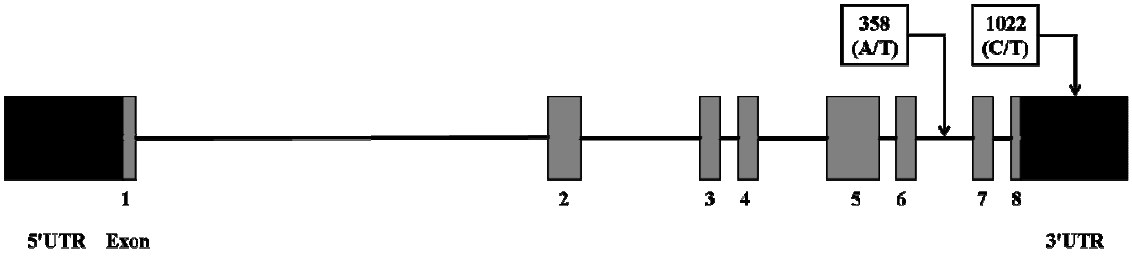

Figure 13: The genomic structure of porcine CD9 gene derived from comparative sequence of human genomic. Positions of SNPs, 5'UTR, exonic, intronic polymorphic and 3'UTR are displayed.

Two single nucleotide polymorphisms (SNPs) were revealed by sequencing of CD9 gene. An adenine (A)-to-thymine (T) transversion and a cytosine (C)-to-thymine (T) transition (g.358A>T in intron 6, genbank accession No. GU584195 and g.1022C>T in 3′UTR, genbank accession No. GU584195) were shown in two SNPs (Figure 13). The SNP g.358A>T was confirmed by PCR-RFLP with restriction enzyme HinfI (BioLabs).

The porcine PLCz DNA was 43857 bp, genbank accession No. NC_010447.

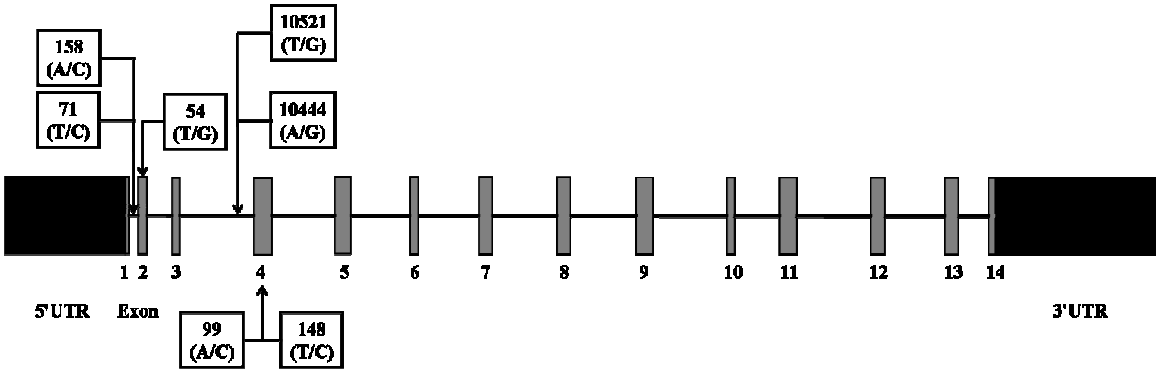

Figure 14: The genomic structure of porcine PLCz gene. Positions of SNPs, 5'UTR, exonic, intronic polymorphic and 3'UTR are displayed.

Sequencing result of PLCz gene revealed seven SNPs g.71T>C and g.158A>C in intron 1, g.54T>G in exon 2, g.10,444A>G and g.10,521T>G in intron 3, g.99A>C and g.148T>C in exon 4 (Figure 14). The SNP g.158A>C from intron 1 (genbank accession No. NC_010447) was confirmed by PCR-RFLP with restriction enzyme Tsp509I (BioLabs) and the SNP was a transition from Adenine (A)-to-Cytosine (C).

The porcine COX-2 mRNA, genbank accession No. NM_214321 were compared sequences from bovine, genbank accession No. NC_007314.

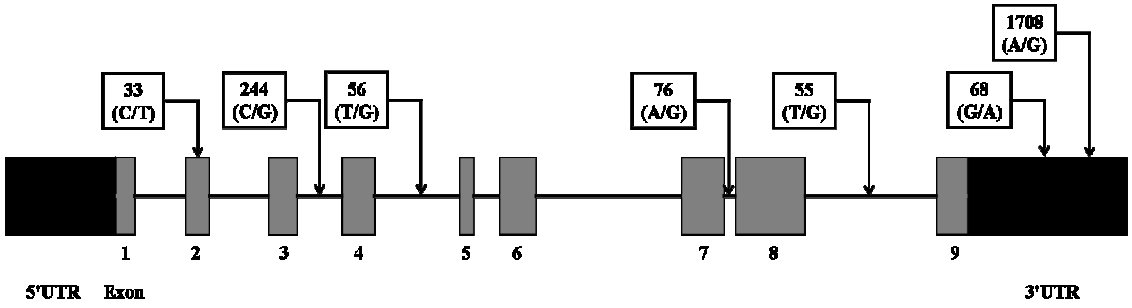

Figure 15: The genomic structure of porcine COX-2 gene derived from comparative sequence of bovine genomic. Positions of SNPs, 5'UTR, exonic, intronic polymorphic and 3'UTR are displayed.

Screening of SNP for COX-2 locus showed seven SNPs g.33C>T in exon 2, g.244C>G in intron 3, g.56T>G in intron 4, g.76A>G in intron 7, g.55T>G in intron 8, g.68G>A and g.1708A>G in 3'UTR (Figure 15). The SNP g.68G>A from intron 9 (genbank accession No. GU971651) was confirmed by PCR-RFLP with restriction enzyme BsrBI (BioLabs) and the SNP was a transition from Guanine (G)-to-Adenine (A).

4.1.2 Determination of genotypes

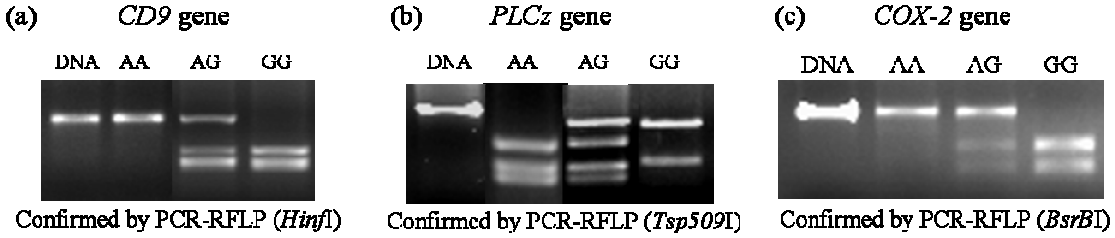

Figure 16: Description of polymorphisms in candidate genes CD9, PLCz and COX-2 tested by means of PCR-RFLP.

Polymorphisms in CD9, PLCz and COX-2 were detected in different pig breeds by comparative sequencing. PCR-RFLP technique was established in order to genotype animal samples (Figure 16). The PCR amplification fragment digestion of CD9 gene show HinfI cutting at nucleotide 523 bp (A) and 357, 166 bp (T) respectively. On the other hand, the polymorphism g.1022C>T was not segregated with the animals. The PCR amplification fragment digestion of PLCz gene show Tsp509I cutting at nucleotide 277, 235, 211 bp (A) and 488, 235 bp (C) respectively. The PCR amplification fragment digestion of COX-2 gene show BsrBI cutting at nucleotide 278 bp (A) and 165, 113 bp (G) respectively.

4.2 Analysis of boar reproductive traits

The boar reproductive traits were analysed with procedure MEANS of software SAS V9.2. The traits were compared among the different populations.

4.2.1 Comparison of sperm quality traits among boar populations

Sperm quality traits including sperm concentration [SCON (10^8/ml)], semen volume per ejaculate [VOL (ml)], sperm motility [MOT (%)], plasma droplets rate [PDR (%)] and abnormal spermatozoa rate [ASR (%)] were obtained from each ejaculate with the evaluation of semen quality. The general description of sperm quality traits are shown in Table 5.

Table 5: Means, standard deviation (SD), sample size, ranges of traits in the Pietrain (PI) population, the Pietrain × Hampshire (PIHA) population and the combined analysis of the PI and PIHA population (COMBINED)

Boars	Traits	Sample size	Means	SD	Minimum	Maximum
COMBINED	SCON (10^8/ml)[a]	49736	2.99	0.98	1	6
	VOL (ml)[a]	52609	250.52	69.25	23	549
	MOT (%)[a]	51497	85.22	4.18	65	95
	PDR (%)[a]	51672	5.33	3.36	0	15
	ASR (%)[a]	52205	5.99	3.99	0	20
PI	SCON (10^8/ml)[a]	38133	2.96	0.97	1	6
	VOL (ml)[a]	40242	244.36	64.98	23	549
	MOT (%)[a]	39444	85.30	4.16	65	95
	PDR (%)[a]	39608	5.21	3.38	0	15
	ASR (%)[a]	39997	6.02	3.88	0	20
PIHA	SCON (10^8/ml)[a]	11603	3.06	1.01	1	6
	VOL (ml)[a]	12367	270.58	78.32	41	549
	MOT (%)[a]	12053	84.98	4.24	70	95
	PDR (%)[a]	12064	5.72	3.22	0	15
	ASR (%)[a]	12208	5.82	4.31	0	20

[a]SCON: sperm concentration, VOL: semen volume per ejaculate, MOT: sperm motility, PDR: rate of sperm with proximal plasma droplets, ASR: abnormal spermatozoa rate

Means of SCON, VOL and PDR trait in the PIHA population were larger than these in the PI population, but their ranges were similar. Because sample size of the PI population was double as of the PIHA population. Means and standard deviation of SCON, MOT and PDR trait in the PI population were close to these in the COMBINED group. Means, standard deviation and ranges of MOT trait in all groups were very close.

4.2.2 Comparison of fertility traits among boar populations

Fertility data [non return rate (NRR42) and number of piglets born alive (NBA)] of each boar were given as the deviation from the population means within breed, parity of sow, farm and season classes. The general description of sperm quality traits are shown in Table 6.

Table 6: Sample size, means, standard deviation (SD) and ranges of fertility traits in the Pietrain (PI) population, the Pietrain × Hampshire (PIHA) population and the combined analysis of the PI and PIHA population (COMBINED)

Boars	Traits	Sample size	Means	SD	Minimum	Maximum
COMBINED	NRR42 (%)[b]	340	0.59	6.08	-24.07	18.62
	NBA (per litter)[b]	340	0.01	0.54	-2.97	1.37
PI	NRR42 (%)[b]	231	0.46	6.80	-24.07	18.62
	NBA (per litter)[b]	231	0.04	0.56	-1.69	1.37
PIHA	NRR42 (%)[b]	109	0.86	4.20	-12.23	13.79
	NBA (per litter)[b]	109	0.04	0.50	-2.97	1.10

[b] Fertility (NRR42, NBA) corrected with factors: parity, farm, season and breed

For NRR42 trait, the mean of the PIHA population was higher than of the PI population, but standard deviation turned out contrary to the means. The range values of the PIHA population were not as wide as those of the PI population. The means, standard deviation and ranges of the PI population were close to those of the COMBINED group due to bigger sample size than the PIHA population. For NBA trait, means and standard deviation in both populations were very close. The ranges values of the PIHA population were wider than those of the PI population.

4.3 Analysis of genotypes

Analysis of genotype was composed of calculation genotype of and allele frequencies and test of Hardy-Weinberg equilibrium for single locus within population.

4.3.1 Frequencies of genotypes and alleles

The genotype and allele frequencies of the diallelic loci within the PI and the PIHA boars were calculated. The results are shown in Table 7. The frequencies of homozygote AA and TT of the CD9 gene were close in the PI population and the PIHA population. Homozygote AA was most frequent and more than 2/3 in both populations, and homozygote TT in the PI population (0.16) and in the PIHA population (0.24) was rare. The heterozygote genotype in the PI population (0.08) and in the PIHA population (0.09) was similar. The allele frequency of CD9 gene in the PI population and the PIHA population was almost similar. Homozygote AA of the PLCz locus in the PI population (0.43) was more than in the PIHA population (0.39). Heterozygote AC was most frequent in both populations, and homozygote CC was rare frequent in both populations. The heterozygote AC in the PI population (0.51) and in the PIHA population (0.55) was similar. The homozygote CC in the PI population (0.06) and in the PIHA population (0.06) was the same. The frequency of allele A in the PI population (0.68) and in the PIHA population (0.66) was almost similar. The frequency of allele C in the PI population (0.32) and in the PIHA population (0.34) was similar. The frequency of homozygote AA of the COX-2 gene in the PI population (0.22) was more than in the PIHA population (0.09). Heterozygote AG was most frequent in both populations. Heterozygote genotype in the PI population (0.54) and in the PIHA population (0.57) was similar. Homozygote GG in the PI population (0.24) was less than in the PIHA population (0.34). The frequency of allele A of COX-2 locus in the PIHA (0.62) population was more than in the PI population (0.51). While, the frequency of allele G of COX-2 locus in the PI (0.49) population was more than in the PIHA population (0.38).

4.3.2 The test of Hardy-Weinberg equilibrium

The chi-square test revealed (Table 7) that the diallelic loci CD9, PLCz and COX-2 loci were in Hardy-Weinberg equilibrium the Pietrain (PI) population, the Pietrain × Hampshire (PIHA) population and the combined analysis of the PI and PIHA population (COMBINED).

Table 7: Genotype, allele frequencies and the chi-square test of porcine CD9, PLCz and COX-2 gene in different pig breeds

Locus	Breed	Number	Genotype frequency			Allele frequency		Chi-square test	
CD9			AA(n)	AT(n)	TT(n)	A	T	χ^2	p-value
	COMBINED	340	0.73(248)	0.08(28)	0.19(64)	0.77	0.23	0.60	0.74
	PI	231	0.76(175)	0.08(18)	0.16(38)	0.80	0.20	0.56	0.76
	PIHA	109	0.67(73)	0.09(10)	0.24(26)	0.72	0.28	0.61	0.74
PLCz			AA(n)	AC(n)	CC(n)	A	C	χ^2	p-value
	COMBINED	340	0.42(143)	0.52(177)	0.06(20)	0.68	0.32	0.04	0.98
	PI	231	0.43(99)	0.51(118)	0.06(14)	0.68	0.32	0.03	0.98
	PIHA	109	0.39(42)	0.55(60)	0.06(7)	0.66	0.34	0.05	0.98
COX-2			GG(n)	GA(n)	AA(n)	G	A	χ^2	p-value
	COMBINED	340	0.18(61)	0.55(187)	0.27(92)	0.54	0.46	0.01	0.99
	PI	231	0.22(51)	0.54(125)	0.24(55)	0.51	0.49	0.01	0.99
	PIHA	109	0.09(9)	0.57(62)	0.34(38)	0.62	0.38	0.05	0.98

PI: Pietrain population; PIHA: Pietrain × Hampshire population; COMBINED: the combined analysis of PI and PIHA; n: Number of boar by genotype

4.4 Association analysis between genotypes and phenotypes

4.4.1 Sperm quality traits

Analyses of variance were performed with the procedure “PROC MIXED” of the SAS software package (SAS Institute Inc., ver. 9.2). Statistical analyses were carried out using model 1 with the fixed effects of breed, sperm collection season (four seasons per year, in total eight seasons within 2 years from January 2000 to December 2001), age (covariable), genotype of boars and random permanent effect of the boar (repeated measurement). The sperm quality data were also analysed for PI and PIHA separately as well as for both together named COMBINED. Results of the statistical analysis are presented as least square means (LSM) and standard errors (SE) of least square means. Rare genotypes of candidate genes were excluded from the statistical analysis.

4.4.1.1 Association between CD9 genotypes with sperm quality traits in the COMBINED group

The statistical results are given as least square means and their standard errors. Only CD9 loci was significantly associated with MOT ($p < 0.001$), PDR ($p < 0.001$) and ASR ($p < 0.01$) in the COMBINED group. In case of MOT and PDR, genotype AA were significantly ($p < 0.05$) different from genotype AT. Genotype AA were significantly ($p < 0.01$) different from genotype TT in case of MOT and ASR. Moreover, genotype AA and TT were significantly ($p < 0.001$) different in case of PDR (Table 8). For no significant effect relevant loci, the results are shown in appendix.

4.4.1.2 Additive and dominance effects on sperm quality traits in the COMBINED group

At this locus, the additive effect seemed to be highly significant in MOT ($p < 0.01$), PDR ($p < 0.001$) and ASR ($p < 0.01$). The statistical analyses revealed that allele A of the CD9 locus had a positive effect on MOT but had a negative effect on PDR and ASR (Table 8).

Table 8: Association between CD9 genotypes with sperm quality traits in the COMBINED group

Trait		CD9 genotype (μ±SE)			Effect (μ±SE)	
		AA	AT	TT	Additive	Dominance
	Nr. of observations[#]	34420	3899	8157		
SCON (10^8/ml)		3.03±0.04	3.17±0.12	2.89±0.08	0.07±0.05	-0.20±0.13
VOL (ml)		257.73±3.41	262.64±9.25	263.64±6.40	-2.95±3.55	-2.10±9.83
MOT (%)		85.18±0.24[ac]	83.51±0.64[b]	83.62±0.44[d]	0.78±0.25**	0.89±0.68
PDR (%)		5.33±0.18[ae]	6.40±0.49[b]	6.86±0.34[f]	-0.77±0.19***	-0.30±0.52
ASR (%)		6.12±0.20[c]	6.86±0.55	7.44±0.38[d]	-0.66±0.21**	-0.08±0.58

Least square mean significantly for genotypes with different superscripts (a, b: $p < 0.05$; c, d: $p < 0.01$; e, f: $p < 0.001$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). [#]repeated measurement

4.4.1.3 Association between CD9 genotypes with sperm quality traits in the PI population

CD9 polymorphism was associated with MOT ($p < 0.05$) and PDR ($p < 0.05$) in the PI population. In case of MOT, genotype AA were significantly ($p < 0.05$) different from genotype AT. For PDR, genotype AA were significantly ($p < 0.05$) different from genotype AT and TT (Table 9).

4.4.1.4 Additive and dominance effects on sperm quality traits in the PI population

The additive effect significant in MOT ($p < 0.05$) and PDR ($p < 0.05$). The statistical analyses revealed that allele A of the CD9 locus had a positive effect on MOT but had a negative effect on PDR (Table 9).

4.4.1.5 Association between CD9 genotypes with sperm quality traits in the PIHA population

CD9 loci was significantly associated with MOT ($p < 0.001$), PDR ($p < 0.001$) and ASR ($p < 0.01$) in PIHA population. Genotype AA were significantly ($p < 0.01$) different from genotype TT in MOT, PDR and ASR. Genotype AT were significantly ($p < 0.05$) different from genotype TT only in PDR (Table 10).

4.4.1.6 Additive and dominance effects on sperm quality traits in the PIHA population

At CD9 locus, the additive effect significant in MOT ($p < 0.05$), PDR ($p < 0.05$) and ASR ($p < 0.05$). The statistical analyses revealed that allele A of the CD9 locus had a positive effect on MOT but had a negative effect on PDR and ASR (Table 10).

Table 9: Association between CD9 genotypes with sperm quality traits in the PI population

Trait	CD9 genotype (μ±SE)			Effect (μ±SE)	
	AA	AT	TT	Additive	Dominance
Nr. of observations[#]	22105	2158	5083		
SCON (10^8/ml)	2.98±0.05	3.11±0.14	2.88±0.11	0.049±0.058	-0.18±0.16
VOL (ml)	246.24±3.36	245.03±10.29	245.25±7.54	0.50±4.13	0.72±11.09
MOT (%)	85.47±0.24[a]	83.75±0.73[b]	84.32±0.54	0.58±0.29*	1.14±0.79
PDR (%)	4.99±0.19[a]	6.50±0.57[b]	6.17±0.42[b]	-0.59±0.23*	-0.92±0.62
ASR (%)	6.12±0.21	6.57±0.63	6.86±0.46	-0.37±0.25	-0.08±0.68

Least square mean significantly for genotypes with different superscripts (a, b: $p < 0.05$; * $p < 0.05$); [#]repeated measurement

Table 10: Association between CD9 genotypes with sperm quality traits in the PIHA population

Trait	CD9 genotype (μ±SE)			Effect (μ±SE)	
	AA	AT	TT	Additive	Dominance
Nr. of observations[#]	6439	1079	2497		
SCON (10^8/ml)	3.13±0.08	3.25±0.21	2.92±0.13	0.11±0.08	-0.22±0.22
VOL (ml)	264.78±6.96	287.10±19.21	283.60±11.89	-9.41±6.89	-12.92±20.42
MOT (%)	85.19±0.48[c]	83.95±1.34	82.65±0.83[d]	1.27±0.48*	-0.03±1.42
PDR (%)	5.45±0.34[c]	5.43±0.93[a]	7.62±0.58[db]	-1.09±0.34*	1.10±0.99
ASR (%)	5.80±0.40[c]	6.95±1.11	8.41±0.69[d]	-1.31±0.40*	0.15±1.18

Least square mean significantly for genotypes with different superscripts (a, b: $p < 0.05$; c, d: $p < 0.01$; * $p < 0.05$); [#]repeated measurement

4.4.1.7 Association between PLCz genotypes with sperm quality traits in the PIHA population

PLCz loci was significantly associated with SCON ($p < 0.001$) in the PIHA population. Genotype AC were significantly ($p < 0.05$) different from genotype CC in SCON (Table 11).

4.4.1.8 Additive and dominance effects on sperm quality traits in the PIHA population

At PLCz locus, the dominance effect significant in SCON ($p < 0.05$). The statistical analyses revealed that allele C of the PLCz locus had a positive effect on SCON (Table 11).

4.4.2 The gene-gene interaction on the sperm quality and fertility trait

Finally, we examined whether genetic interaction exists between CD9, PLCz and COX-2 SNPs, because molecular interaction is known between the protein products of these genes. Statistically significant gene-gene interaction was detected (Table 12). Significant gene-gene interaction between CD9 and PLCz was detected in MOT in the COMBINED group ($p < 0.01$) and the PIHA population ($p < 0.05$), PDR in the COMBINED group ($p < 0.001$), the PI population ($p < 0.05$) and the PIHA population ($p < 0.05$) and ASR in the PIHA population ($p < 0.05$). The interaction between CD9 and COX-2 polymorphisms was confirmed by the association with MOT in the COMBINED group ($p < 0.05$), PDR in the COMBINED group ($p < 0.01$) and the PIHA population ($p < 0.05$) and PDR in the PIHA population ($p < 0.01$). The interaction between PLCz and COX-2 with respect to NRR42 in the PI population ($p < 0.05$) showed an associated when the PLCz genotype was combined with the COX-2 genotype. CD9, PLCz and COX-2 showed an associated for PDR in the COMBINED group ($p < 0.05$) when all alleles were present.

Table 11: Association between PLCz genotypes with sperm quality traits in the PIHA population

Trait		PLCz genotype (μ±SE)			Effect (μ±SE)	
		AA	AC	CC	Additive	Dominance
	Nr. of observations[#]	4485	4954	576		
SCON (10^8/ml)		3.12±0.09	2.95±0.09[a]	3.62±0.25[b]	-0.25±0.14	0.42±0.16*
VOL (ml)		268.08±8.97	272.82±8.63	280.39±24.47	-6.15±13.03	1.41±15.63
MOT (%)		85.16±0.67	84.07±0.65	86.18±1.84	-0.51±0.98	1.60±1.17
PDR (%)		5.78±0.48	6.23±0.46	4.69±1.30	0.55±0.69	-0.99±0.83
ASR (%)		6.22±0.56	6.62±0.53	6.26±1.52	-0.38±0.97	-0.02±0.81

Least square mean significantly for genotypes with different superscripts (a, b: $p < 0.05$; * $p < 0.05$); [#]repeated measurement

Table 12: The association between CD9, COX-2 and PLCz genotypes with sperm quality traits and fertility traits in the Pietrain (PI) population, the Pietrain × Hampshire (PIHA) population and the combined analysis of the PI and PIHA population (COMBINED)

Locus	Trait	COMBINED	PI	PIHA
CD9*PLCz	SCON (10^8/ml)	0.21	0.39	0.16
	VOL (ml)	0.81	0.96	0.64
	MOT (%)	$p < 0.01$	0.06	$p < 0.05$
	PDR (%)	$p < 0.001$	$p < 0.05$	$p < 0.05$
	ASR (%)	0.17	0.76	$p < 0.05$
	NRR42 (%)	0.98	0.84	0.28
	NBA (per litter)	0.59	0.72	0.79
CD9*COX-2	SCON (10^8/ml)	0.51	0.81	0.51
	VOL (ml)	0.62	0.84	0.59
	MOT (%)	$p < 0.05$	0.16	0.23
	PDR (%)	$p < 0.01$	0.07	$p < 0.05$
	ASR (%)	0.16	0.74	$p < 0.01$
	NRR42 (%)	0.65	0.86	0.44
	NBA (per litter)	0.74	0.66	0.94
PLCz*COX-2	SCON (10^8/ml)	0.73	0.63	0.10
	VOL (ml)	0.88	0.95	0.67
	MOT (%)	0.86	0.85	0.45
	PDR (%)	0.66	0.44	0.51
	ASR (%)	0.77	0.92	0.53
	NRR42 (%)	0.07	$p < 0.05$	0.35
	NBA (per litter)	0.75	0.56	0.98
CD9* PLCz*COX-2	SCON (10^8/ml)	0.33	0.33	0.57
	VOL (ml)	0.69	0.85	0.70
	MOT (%)	0.09	0.24	0.16
	PDR (%)	$p < 0.05$	0.10	0.18
	ASR (%)	0.68	0.98	0.09
	NRR42 (%)	0.42	0.17	0.38
	NBA (per litter)	0.82	0.82	0.83

4.5 Expression of CD9, PLCz and COX-2 mRNA in reproductive and non-reproductive tissues of breeding boars

4.5.1 Expression of CD9 mRNA in reproductive and non-reproductive tissues of boars

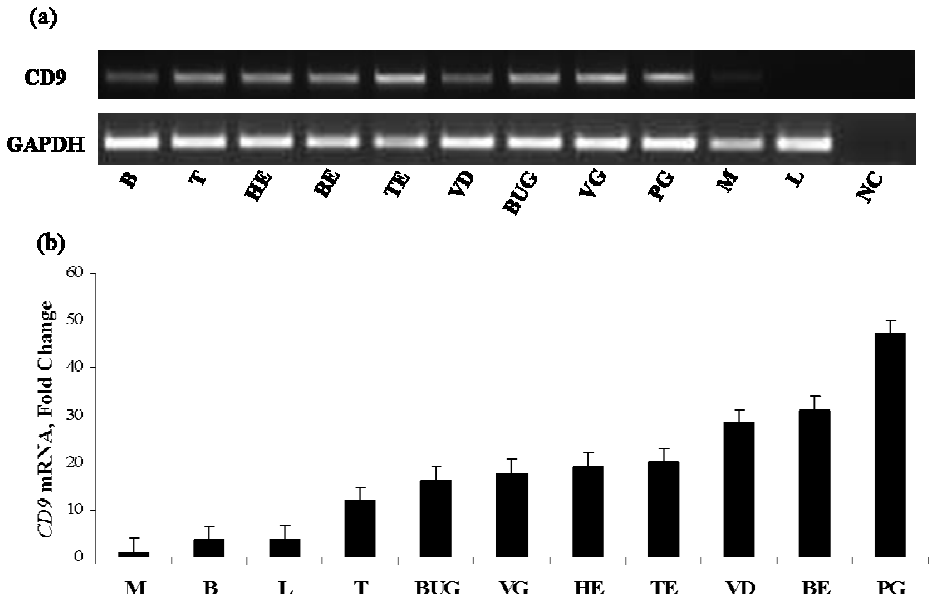

Figure 17: mRNA expression of CD9 in different reproductive and non-reproductive tissues. (a) Expression of CD9 and GAPDH in different tissues by semi-quantitative RT PCR. (b) CD9 mRNA expression in the different tissues using qRT-PCR (data was normalized by GAPDH). (B: Brain, T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, BUG: Bulbourethral gland, VG: Vesicular glands, PG: Prostate gland, M: Muscle, L: Liver).

The semi-quantitative reverse transcription PCR result of GAPDH showed no remarkable differences among tissues (Figure 17a). CD9 mRNA expression was high in reproductive tissues such as in prostate gland, then in body of the epididymis, vas deferens, tail of epididymis, head of epididymis (Figure 17b) and lower expression was found in non-reproductive tissue (muscle, brain and liver).

4.5.2 Expression of PLCz mRNA in reproductive and non-reproductive tissues of boars

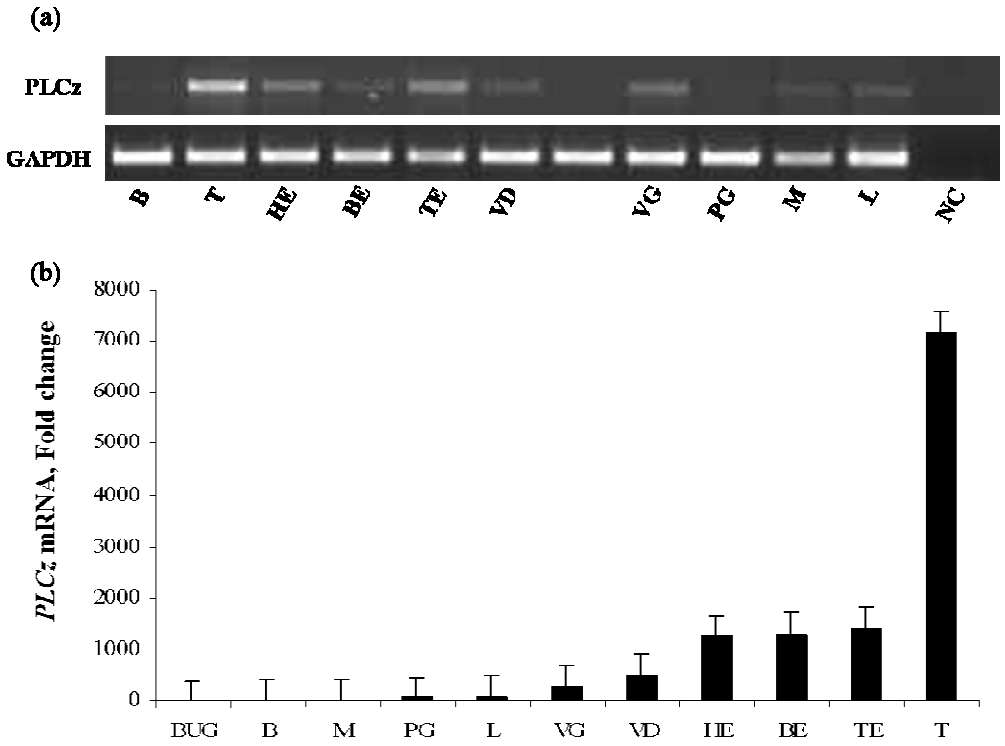

Figure 18: mRNA expression of PLCz in different reproductive and non-reproductive tissues. (a) Expression of PLCz and GAPDH in different tissues by semi-quantitative RT PCR. (b) PLCz mRNA expression in the different tissues using qRT-PCR (data was normalized by GAPDH). (B: Brain, T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, BUG: Bulbourethral gland, VG: Vesicular glands, PG: Prostate gland, M: Muscle, L: Liver).

The semi-quantitative reverse transcription PCR result of GAPDH showed no remarkable differences among tissues (Figure 18a). PLCz mRNA showed higher level of expression in testis, then in tail of epididymis, body of epididymis, head of epididymis, and lower PLCz mRNA expressed in vas deferens, vesicular glands, liver, prostate gland, muscle and bulbourethral gland (Figure 18b).

4.5.3 Expression of COX-2 mRNA in reproductive and non-reproductive tissues of boars

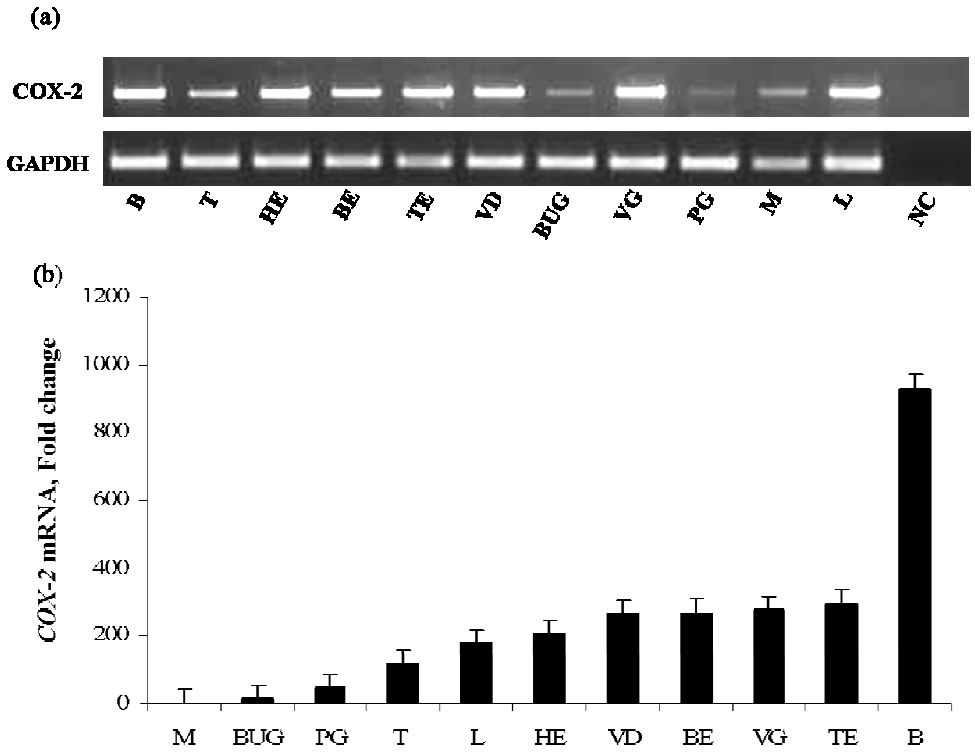

Figure 19: mRNA expression of COX-2 in different reproductive and non-reproductive tissues. (a) Expression of COX-2 and GAPDH in different tissues by semi-quantitative RT PCR. (b) COX-2 mRNA expression in the different tissues using qRT-PCR (data was normalized by GAPDH). (B: Brain, T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, BUG: Bulbourethral gland, VG: Vesicular glands, PG: Prostate gland, M: Muscle, L: Liver).

The detection any variation in COX-2 expression levels under different experimental conditions by semi-quantitative reverse transcription PCR. The semi-quantitative reverse transcription PCR result of GAPDH showed no remarkable differences among tissues (Figure 19a). Higher levels of COX-2 was expressed in reproductive tissues such as in tail of epididymis, vesicular glands, body of epididymis, vas deferens, head of epididymis, then in testis, and lowest expression was in prostate gland, bulbourethral

gland. The expression of COX-2 mRNA in non-reproductive tissues was very high in brain, then in liver, and lowest expression was in muscle (Figure 19b).

4.6 Protein expression of CD9, COX-2 and PLCz in reproductive and non-reproductive tissues of boars

4.6.1 Expression of CD9 protein in reproductive and non-reproductive tissues of boars

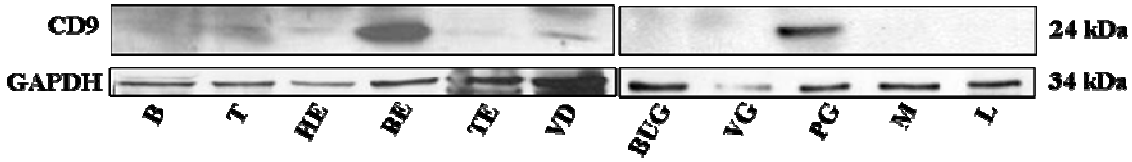

Figure 20: Protein expression of CD9 in different reproductive and non-reproductive tissues by western blotting. The antibody recognized a protein at 24 kDa of CD9. (B: Brain, T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, BUG: Bulbourethral gland, VG: Vesicular glands, PG: Prostate gland, M: Muscle, L: Liver).

The proteins were detected in reproductive and non-reproductive tissues to confirm the translation level of those mRNA into protein by western blot analysis (Figure 20). The higher detection of CD9 protein with 24 kDa molecular weigh was found in body of epididymis and in prostate gland of boars.

4.6.2 Expression of PLCz protein in reproductive and non-reproductive tissues of boars

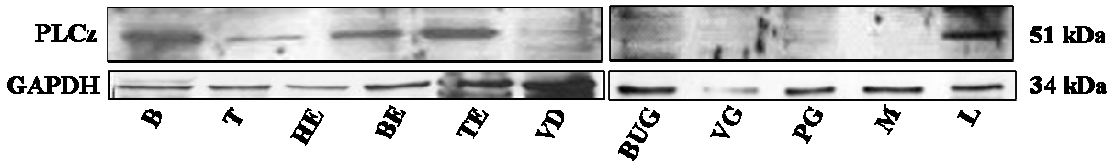

Figure 21: Protein expression of PLCz in different reproductive and non-reproductive tissues by western blotting. The antibody recognized a protein at 51 kDa of PLCz. (B: Brain, T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, BUG: Bulbourethral gland, VG: Vesicular glands, PG: Prostate gland, M: Muscle, L: Liver).

The proteins were detected in reproductive tissues to confirm the translation level of PLCz mRNA into protein by western blot analysis (Figure 21). PLCz protein with 51 kDa molecular weigh was expressed in testis, head of epididymis, body of epididymis, tail of epididymis, and liver.

4.6.3 Expression of COX-2 protein in reproductive and non-reproductive tissues of boars

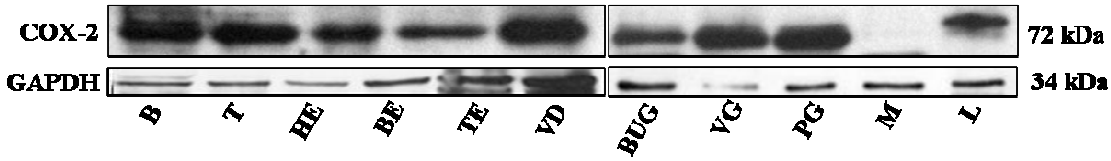

Figure 22: Protein expression of COX-2 in different reproductive and non-reproductive tissues by western blotting. The antibody recognized a protein at 72 kDa of COX-2. (B: Brain, T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, BUG: Bulbourethral gland, VG: Vesicular glands, PG: Prostate gland, M: Muscle, L: Liver).

The proteins were detected in reproductive tissues to confirm the translation level of COX-2 mRNA into protein by western blot analysis (Figure 22). The higher detection of COX-2 protein with 72 kDa molecular weigh was showed higher levels in reproductive tissues and in liver.

4.7 Localization of CD9, COX-2 and PLCz protein in boar reproductive tissues by immunofluorescence

4.7.1 Histological section of reproductive tissues of breeding boar

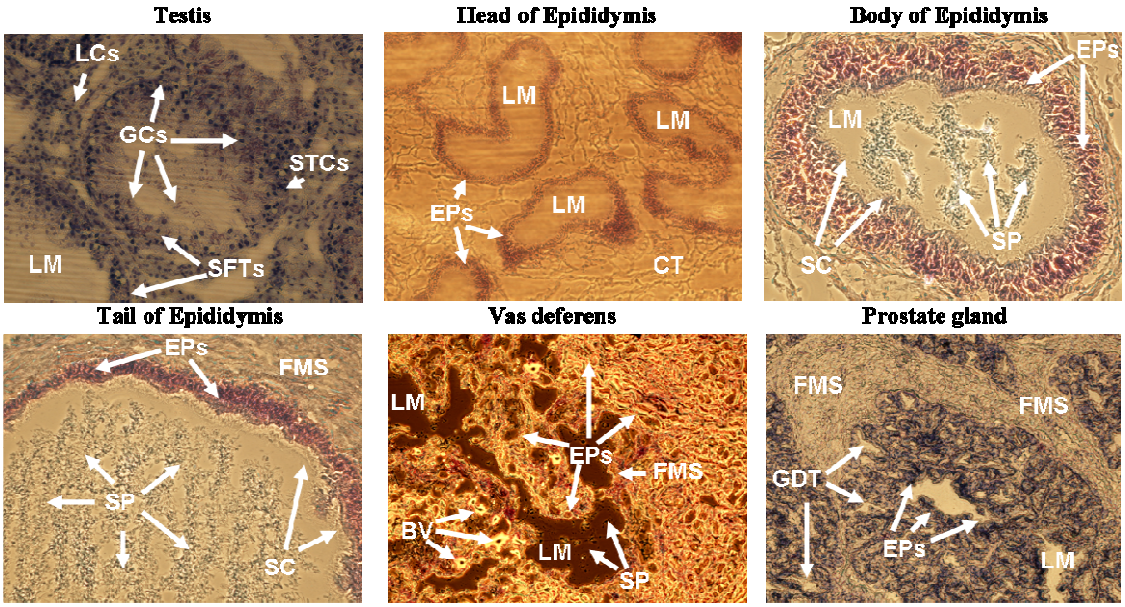

Figure 23: Histological section of reproductive tissues (LCs = Leydig cells; EPs = Epithelial cells; BV = Blood vessel; SFTs = Seminiferous tubules; CT = Connective tissue; FMS = Fibromuscular stroma cell; LM = Lumen ; SP = Spermatozoa; GDT = Glandular tissue; GCs = Germ cells; STCs = Sertoli; SC = Stereocilia).

Control animals showed a compact and regular arrangement of cells in the testis, head of epididymis, body of epididymis, tail of epididymis, vas deferens and prostate gland (Figure 23). In testes of the animals degenerating the Leydig cells, Sertoli cells, Germ cells, Lumen and tubules with disorganized seminiferous epithelium were visible. Epithelial cells, connective tissue, stereocilia, fibromuscular stroma cell and spermatozoa within the lumen were found in head, body and tail of epididymis. Cross-sections show different staining properties of vas deferens and prostate gland tissues, the histologic specimen of epithelial cells, blood vessel, fibromuscular stroma cell, lumen, glandular tissue were stained with haematoxylin.

4.7.2 Localization of CD9 protein in boar reproductive tissues by immunofluorescence

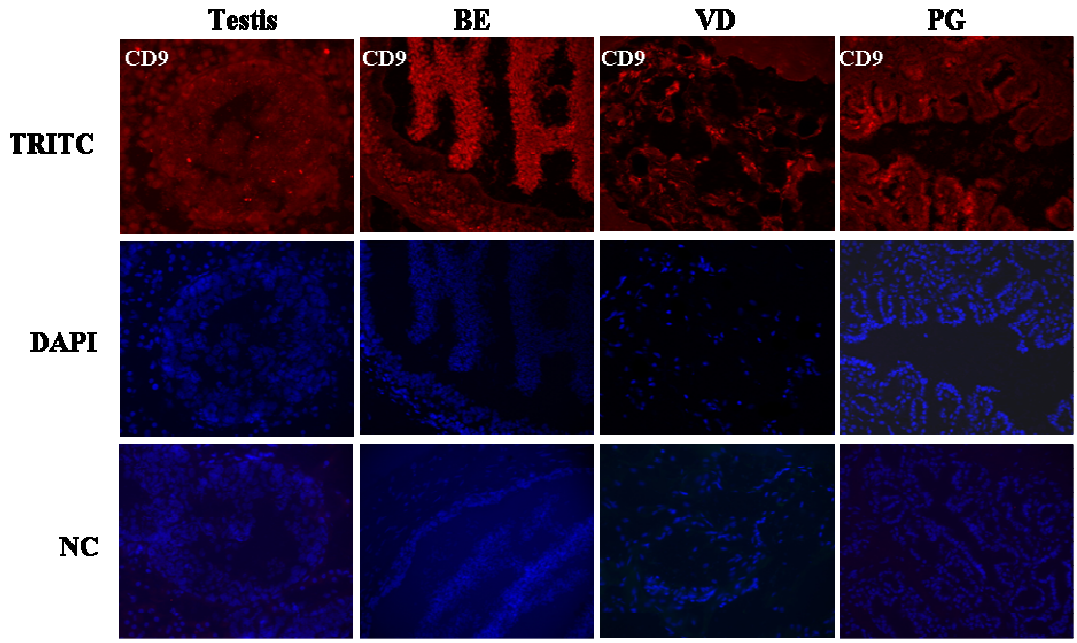

Figure 24: Localization of CD9 protein in different parts of reproductive tract. The cell nuclei were counterstained with DAPI. Magnification 40X. (BE: Body of epididymis, VD: Vas deferens, PG: Prostate gland).

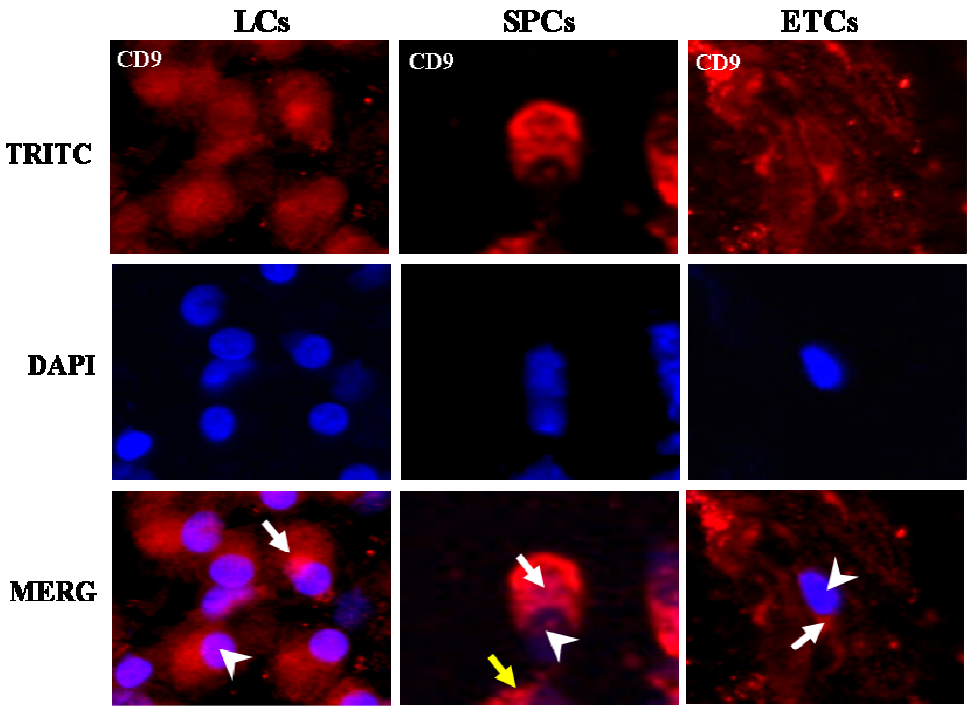

Figure 25: Immunofluorescence detection of CD9 in Leydig cells, Sertoli cells and germ cells of testis. Cytoplasm of Leydig cells were stained of CD9 (arrows) and the Leydig cell nuclei were counterstained with DAPI (arrow head). The immunofluorescence of CD9 protein localized in acrosomal region (arrow) and acrosomal membrane (arrow head) of spermatozoa in epididymis. CD9 protein in epithelial cells, fibro muscular cells, stereo cilia of body of epididymis and spermatozoa within lumen of epididymis. Epithelial cells of vas deferens and prostate gland were stained by CD9. The cell nuclei were counterstained with DAPI. Magnification 40X. (LCs: Leydig cells, SPCs: Spermatozoa cells, ETCs: Epithelial cells).

Testis, body of epididymis, vas deferens and prostate gland sections were stained through the same optical panel for the cell surface CD9 protein expression (Figure 24). The Leydig cells and Sertoli cells in testis, epithelial cells in epididymis, vas deferens and prostate gland gave signal for CD9 immunoreactivity (Figure 25). In case of spermatogenesis the CD9 protein expressed in spermatogonium, spermatocytes and spermatids. CD9 protein expressed highly in Leydig cells (arrow). Cytoplasmic CD9 staining was observed in the stratified epithelial cells of body of epididymis. The CD9 protein expressed in the spermatozoa located within the different regions of

reproductive tract. CD9 protein was expressed in acrosomal region (arrow) and acrosomal membrane (arrow head).

4.7.3 Localization of PLCz protein in boar reproductive tissues by immunofluorescence

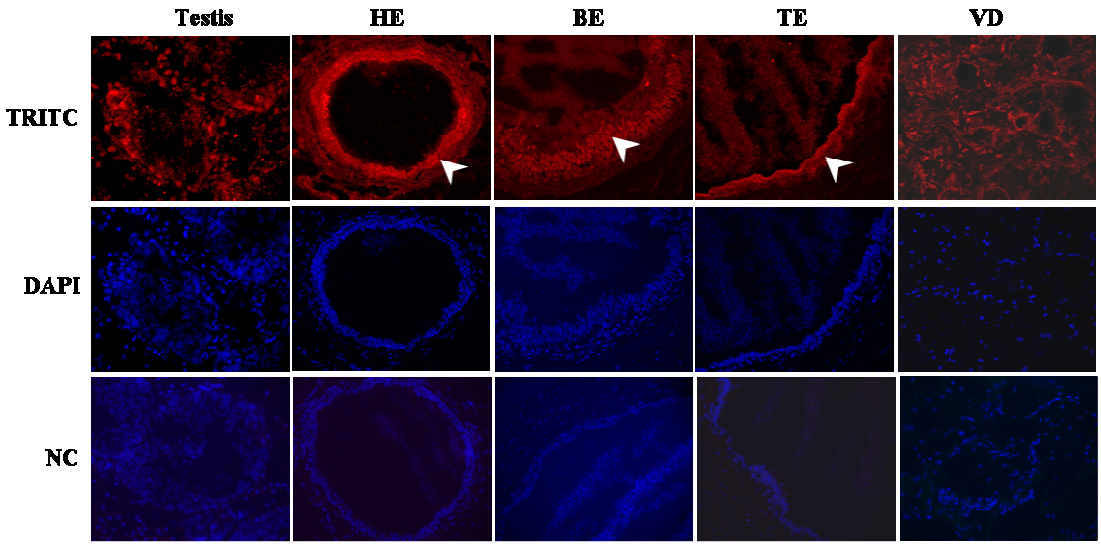

Figure 26: Localization of PLCz protein in different parts of reproductive tract. PLCz protein expression in testis, head of epididymis, body of epididymis, tail of epididymis, vas deferens. The cell nuclei were counterstained with DAPI. Magnification 40X. (HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens).

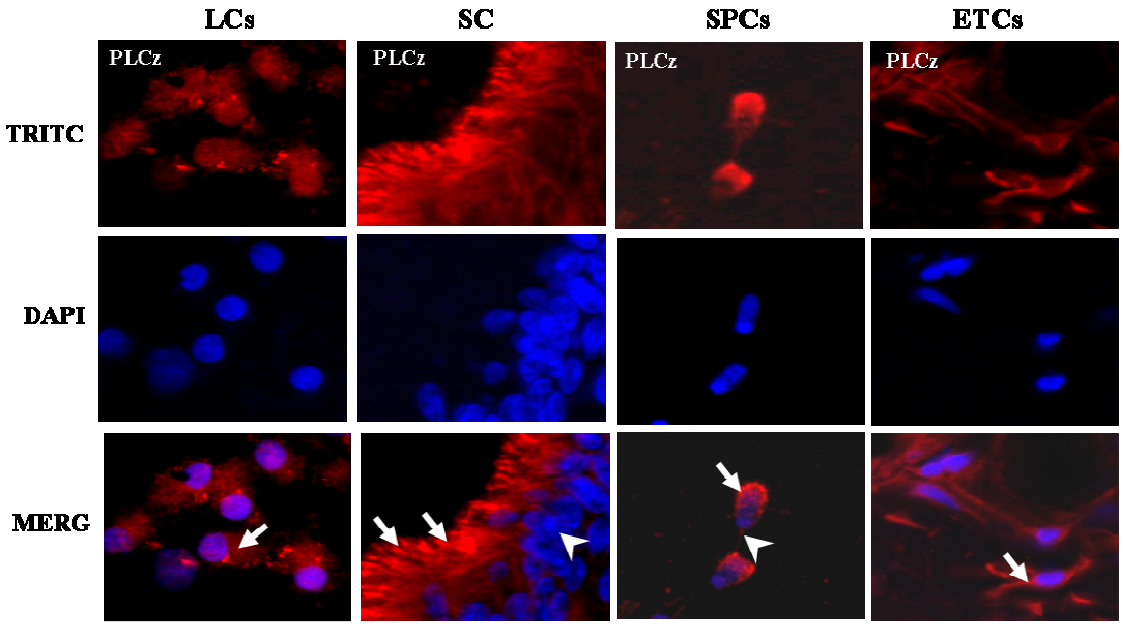

Figure 27: PLCz protein expression in epithelial cells (arrows) of head of epididymis, body of epididymis and tail of epididymis, respectively. PLCz protein expression in vas deferens. PLCz protein expression in cytoplasm (arrow) of Leydig cells in testis. PLCz protein expression in stereo cilia (arrows) in epididymis. PLCz protein expression in acrosomal region (arrow) and post acrosomal region (arrow head) of spermatozoa in epididymis. PLCz protein expression in cytoplasm of epithelial cells (arrow) in vas deferens. The cell nuclei were counterstained with DAPI. Magnification 40X. (LCs: Leydig cells, SC: Sertoli cells, SPCs: Spermatozoa cells, ETCs: Epithelial cells).

We observed a clearly cytoplasm labeled staining for PLCz expression in Leydig cells (arrow) of testis (Figure 27). PLCz protein expressed in stereo cilia cells (arrow) and in epithelial cells of head of epididymis, body of epididymis and tail of epididymis (arrow head). In addition, expression of PLCz at the vas deferens was also confirmed in cytoplasm of epithelial cells. In case of spermatogenesis process, PLCz protein expressed in acrosomal region (arrow), and mitochondria sheet (arrow head) of spermatozoa within epididymis.

4.7.4 Localization of COX-2 protein in boar reproductive tissues by immunofluorescence

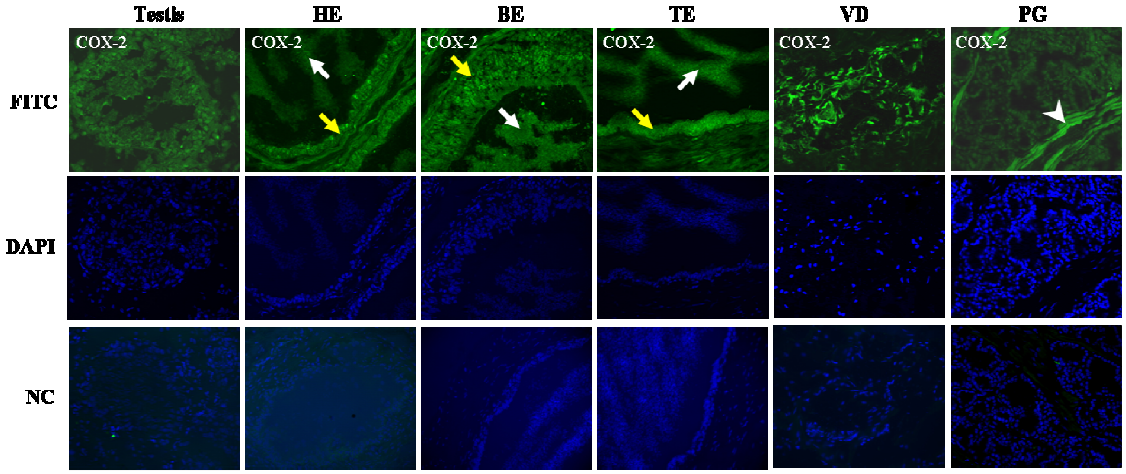

Figure 28: Localization of COX-2 protein in different parts of reproductive tract. COX-2 protein expression in testis, head of epididymis, body of epididymis, tail of epididymis, vas deferens and prostate gland. The cell nuclei were counterstained with DAPI. Magnification 40X. (HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis, VD: Vas deferens, PG: Prostate gland).

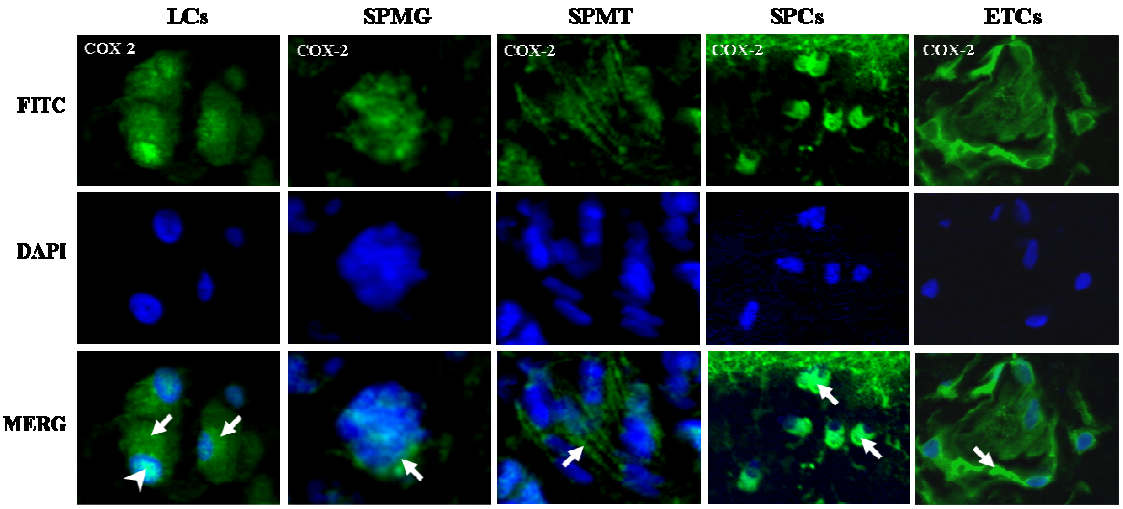

Figure 29: COX-2 protein expression in epithelial cells (yellow arrows) and spermatozoa (arrows) within head of epididymis, body of epididymis and tail of epididymis, respectively. COX-2 protein expression in fibromuscular stroma (arrow head) of prostate gland. COX-2 protein expression in cytoplasm (arrows) and perinuclei (arrow head) of Leydig cells in testis. COX-2 protein expression in cytoplasm of spermatogonium (arrow) in seminiferous tubule. COX-2 protein expression in tail of spermatids (arrow) in seminiferous tubule. COX-2 protein expression in acrosomal region (arrows) of spermatozoa in epididymis. COX-2 protein expression in cytoplasm of epithelial cells (arrow) in vas deferens. The cell nuclei were counterstained with DAPI. Magnification 40X. (LCs: Leydig cells, SPMG: Spermatogonium, SPMT: Spermatid, SPCs: Spermatozoa cells, ETCs: Epithelial cells).

COX-2 immunofluorescence was observed in Leydig cells (arrow), spermatogonium (arrow), tail of spermatids (arrow) of testis (Figure 29). The most intense COX-2 immunofluorescence remained cytoplasm, but the staining extended into the perinuclear of Leydig cells in testis (arrow head). COX-2 immunofluorescence was more distinct in spermatozoa within lumen of epididymis (arrow) and appeared to have a consistent with localization to the acrosomal region of spermatozoa (arrow). COX-2 protein was expressed in epithelial cells of epididymis (yellow arrow), vas deferens (arrow) and in fibro muscular cells of prostate gland (arrow head).

4.8 Expression of CD9, COX-2 and PLCz mRNA in different reproductive tissues and spermatozoa from G-I and G-II boars

4.8.1 Expression of CD9 mRNA in different reproductive tissues and spermatozoa from G-I and G-II boars

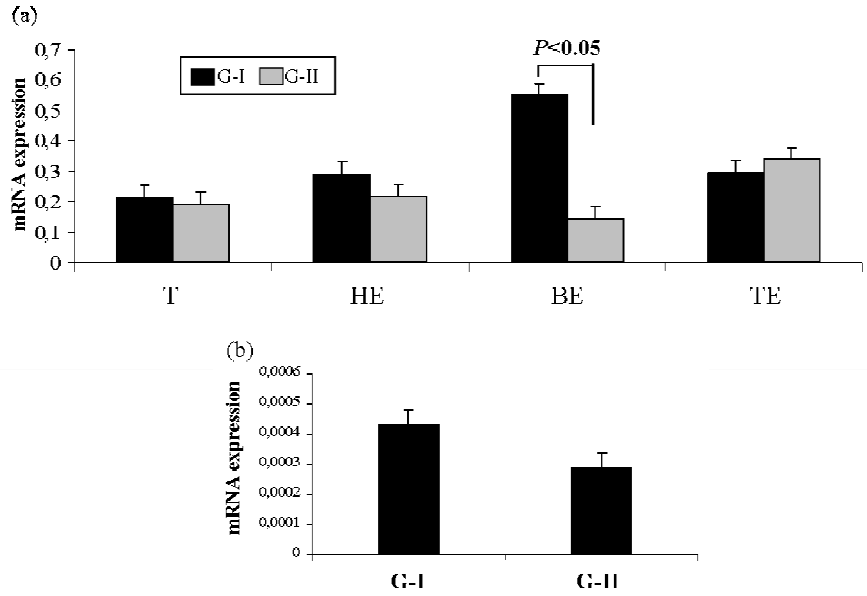

Figure 30: mRNA expression of CD9 in different reproductive tissues including spermatozoa. (a) CD9 mRNA expression in different reproductive tissues from G-I and G-II boars by qRT-PCR. (b) The differential CD9 mRNA expression in spermatozoa from G-I and G-II boars by qRT-PCR. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis).

The CD9 mRNA was higher in all reproductive tissues from G-I boars except tail of epididymis (Figure 30a). However, this expression was significantly higher in the body of epididymis ($p<0.05$). The mRNA expression of CD9 was higher in spermatozoa of G-I boars than that of G-II boars (Figure 30b).

4.8.2 Expression of PLCz mRNA in different reproductive tissues and spermatozoa from G-I and G-II boars

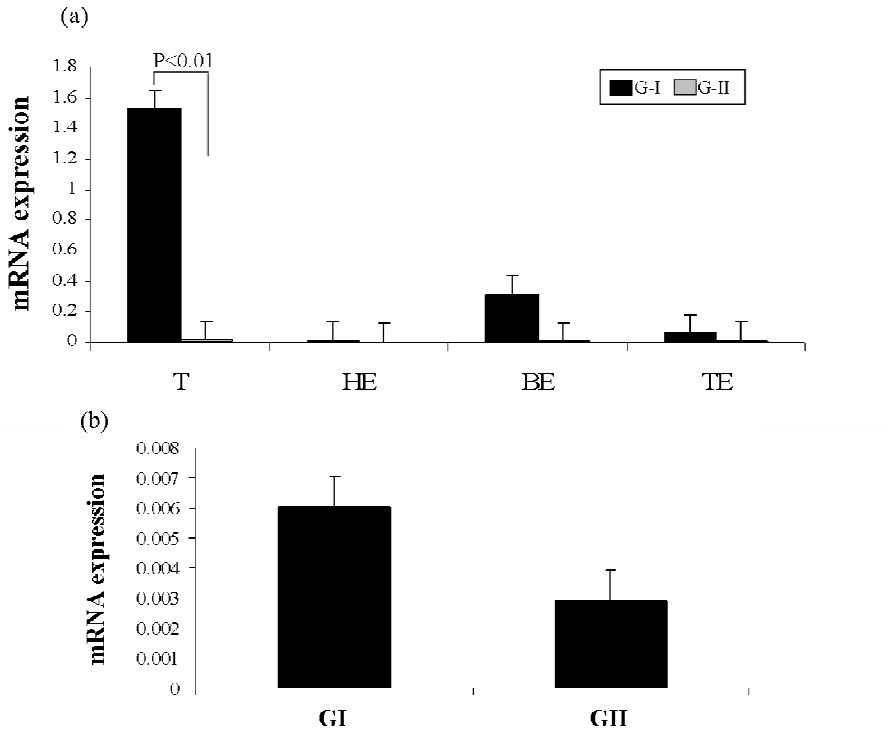

Figure 31: mRNA expression of PLCz in different reproductive tissues including spermatozoa. (a) PLCz mRNA expression in different reproductive tissues from G-I and G-II boars by qRT-PCR. (b) The differential PLCz mRNA expression in spermatozoa from G-I and G-II boars by qRT-PCR. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis).

PLCz mRNA was higher in all reproductive tissues from G-I boars (Figure 31a). However, this expression was significantly higher in the testis ($p<0.01$). The mRNA expression of PLCz was higher in spermatozoa of G-I boars than that of G-II boars (Figure 31b).

4.8.3 Expression of COX-2 mRNA in different reproductive tissues and spermatozoa from G-I and G-II boars

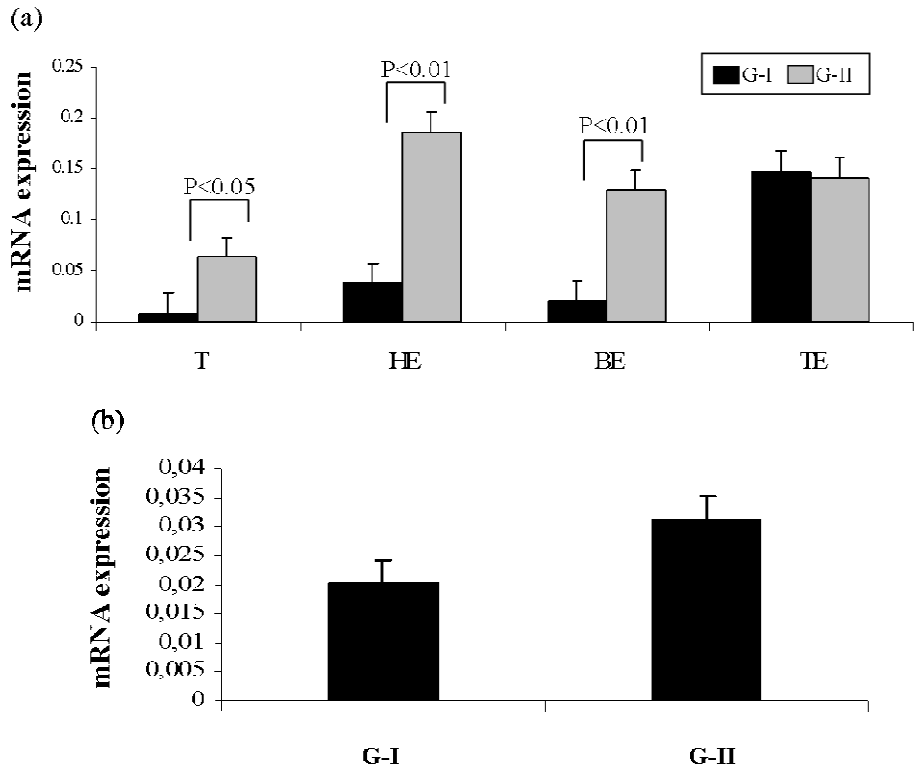

Figure 32: mRNA expression of COX-2 in different reproductive tissues including spermatozoa. (a) COX-2 mRNA expression in different reproductive tissues from boars with G-I boars and G-II boars by qRT-PCR. (b) The differential COX-2 mRNA expression in spermatozoa from G-I boars and G-II boars by qRT-PCR. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis).

The COX-2 mRNA was significantly higher in testis ($p<0.05$), head of epididymis ($p<0.01$) and body of epididymis ($p<0.01$) from G-I boars except tail of epididymis (Figure 32a). However, this expression was significantly higher in the testis ($p<0.01$). The mRNA expression of COX-2 was higher in spermatozoa of G-II boars than that of G-I boars (Figure 32b) but this difference was not significant.

4.9 Protein expression of CD9, COX-2 and PLCz in different reproductive tissues and spermatozoa from G-I and G-II boars

4.9.1 Expression of CD9 protein in different reproductive tissues and spermatozoa from G-I and G-II boars

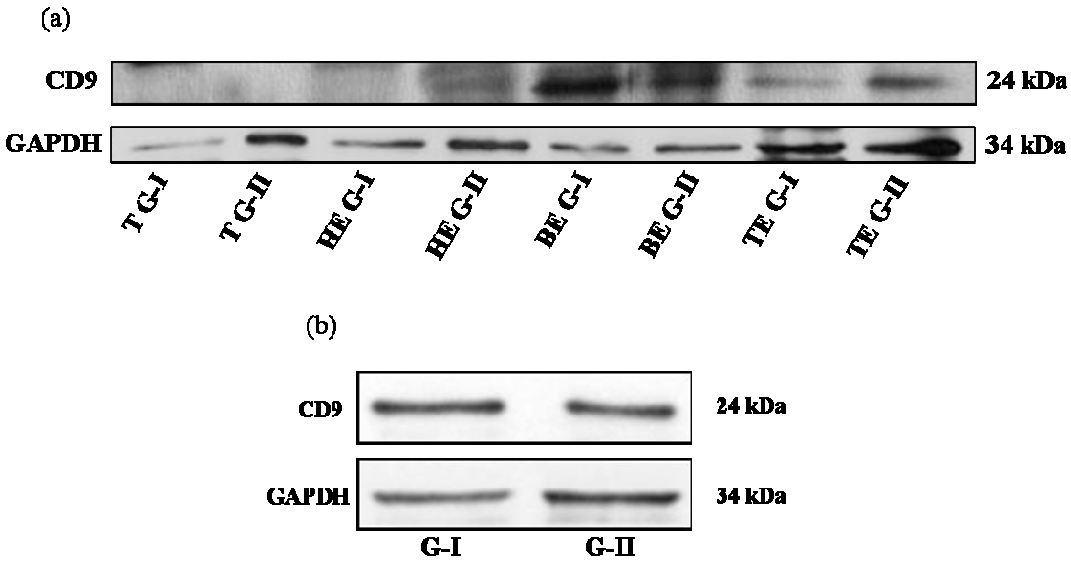

Figure 33: Protein expression of CD9 in different reproductive tissues including spermatozoa. (a) The protein expression in different reproductive tissues from G-I and G-II boars by western blotting. (b) The expression of 24 kDa CD9 protein in spermatozoa from G-I and G-II boars by western blotting. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis).

The CD9 protein expression was higher in tissues from G-I boars than those of collected from G-II boars (Figure 33a). The difference was not significant which are coincided with the proteomic study of western blot result in spermatozoa (Figure 33b).

4.9.2 Expression of PLCz protein in different reproductive tissues and spermatozoa from G-I and G-II boars

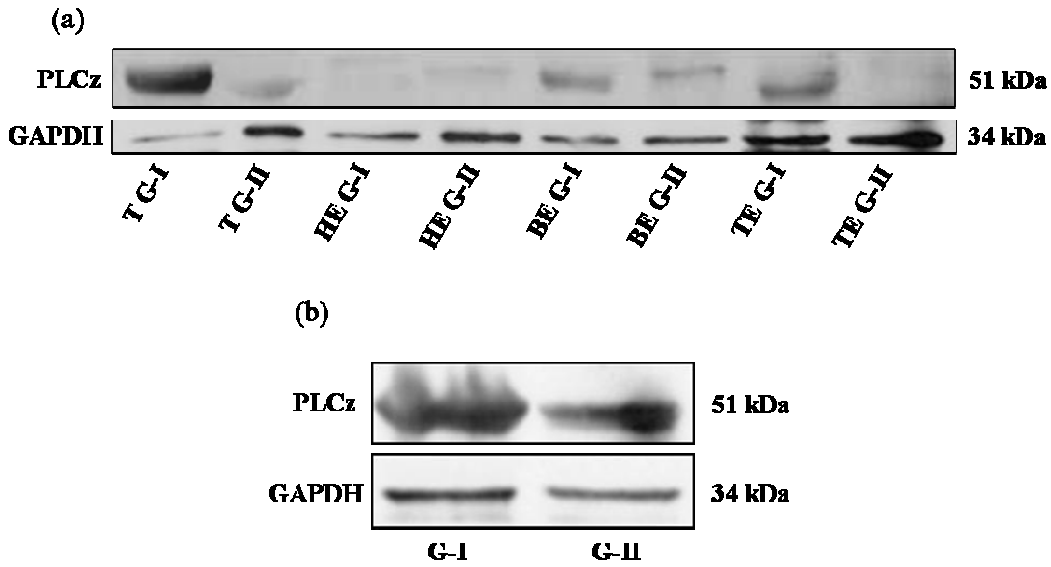

Figure 34: Protein expression of PLCz in different reproductive tissues including spermatozoa. (a) The protein expression in different reproductive tissues from G-I and G-II boars by western blotting. (b) The expression of 51 kDa PLCz protein in spermatozoa from G-I and G-II boars by western blotting. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis).

PLCz proteins expression was higher in testis, body of epididymis and tail of epididymis from G-I boars than those of collected from G-II boars (Figure 34a). However, this PLCz mRNA was translated into protein in spermatozoa of boars with both G-I boars and G-II boars (Figure 34b).

4.9.3 Expression of COX-2 protein in different reproductive tissues and spermatozoa from G-I and G-II boars

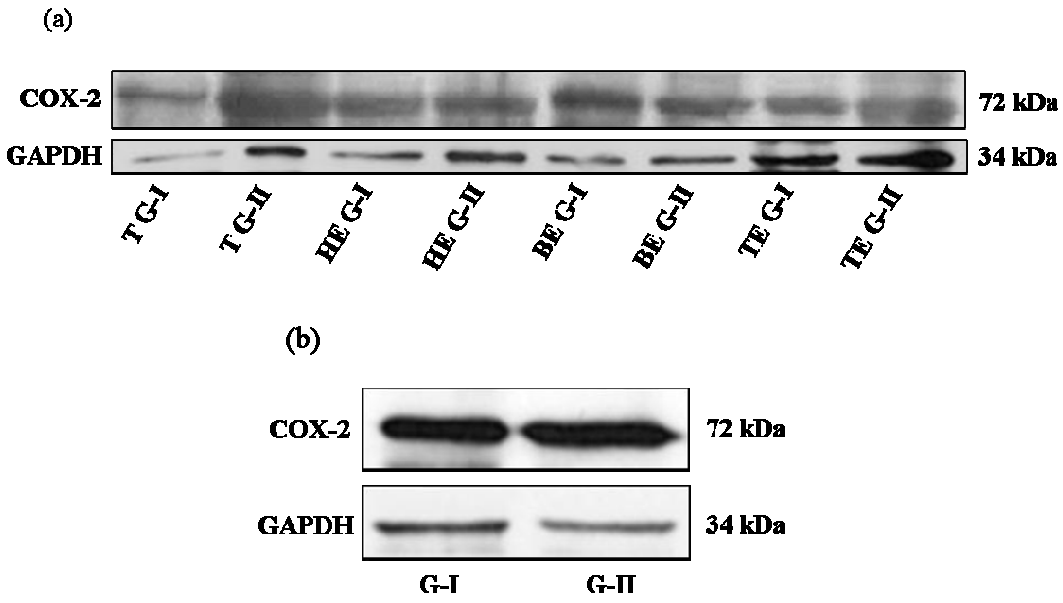

Figure 35: Protein expression of COX-2 in different reproductive tissues including spermatozoa. (a) The protein expression in different reproductive tissues from G-I and G-II boars by western blotting. (b) The expression of 72 kDa COX-2 protein in spermatozoa from G-I and G-II boars by western blotting. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis).

The proteins expression of COX-2 was higher in tissues from boars with both G-I boars and G-II boars (Figure 35a). The protein expression of COX-2 was seemed to be higher in spermatozoa of G-II boars than that of G-I boars (Figure 35b) but this difference was not significant which are coincided with the proteomic study of western blot result.

5. Discussion

In previous study, the identification individual genes especially promising for reproductive traits such as litter size, due to the low heritability and the existence of appropriate genetic markers was found (Spotter et al. 2005). Traits related to fertility of boar are of low heritability (h^2 = 0.01–0.06) and are strongly affected by environmental and genetic effects of the boar itself, the dam and the offspring (See 2000). Therefore, effects of single loci are expected to be low and require a higher number of animals to be identified. In contrast, sperm quality traits have moderate to medium heritability (h^2 = 0.19–0.37) (See 2000). In this thesis, CD9, PLCz and COX-2 were in Hardy-Weinberg equilibrium in all tested boar populations, implying that these population with constant allele and genotype frequencies (Falconer and Mackay 1996).

5.1 Identification of CD9 gene as a candidate gene for boar sperm quality and fertility

An association of the CD9 gene with sperm motility, plasma droplets rate and abnormal spermatozoa rate in boars has been detected for the first time. In case of MOT, semen from animals having genotype AA had significantly higher motility than that of animals having AT or TT genotype. Animals carrying genotype AA had significantly lower PDR than that of animals carrying AT or TT genotype. Animals with genotype AA showed significantly lower ASR than that of animals with genotype TT. Moreover, a significant additive effect was observed in MOT, PDR and ASR. No polymorphism in CD9 was reported in pig yet. However, polymorphism screening and association of CD9 with male fertility is reported in bulls by Daghigh-Kia (2007), where SNP g.95T>C in exon 9 showed a positive effect on sperm concentration and sperm motility ($p < 0.05$) in bulls. This SNP in this thesis was obtained in intron 6 which is subjected to less functional constraint and may change the primary structure of CD9. Though silent, it could affect CD9 function by altering the mRNA stability (Capon et al. 2004). Moreover, it may affect mRNA splicing, or is linked to variation elsewhere in the coding sequence that subsequently affects the amino acid sequence (Hickford et al. 2009). The association of such SNPs with observed traits may be explained by the influence of the intron on mRNA metabolism including initial transcription, editing and polyadenylation of the pre-mRNA, translation and decay of the mRNA product.

Moreover, there is increasing number of reports regarding the role of introns in regulating the expression level of a gene or tissue specific expression pattern (Jiang et al. 2000, Pagani and Baralle 2004, Virts and Raschke 2001). However, no significant association could found for boar fertility traits in this study. Lin et al (2006a). also could not find association with boar fertility traits in the same population. Therefore, the identification of the association of this CD9 gene with fertility traits by the gene expression study is might be important and can be used to support the function of this gene in boar reproduction.

In this thesis, mRNA expression analysis by qRT-PCR demonstrated that the porcine CD9 gene was expressed higher in reproductive tissues than in non-reproductive tissues. CD9 is reported to be expressed as a surface marker in the male germline stem cells of both mouse and rat (Kanatsu-Shinohara et al. 2004). As a surface protein, CD9 was expressed in Leydig cells, Sertoli cells and germ cells within testis, in the epithelial cells of epididymis, vas deferens and prostate gland and in spermatozoa within the lumen of epididymis, suggesting that CD9 might have an important role in spermatogenesis and in sperm cell development in seminiferous tubules and epididymis. CD9 protein is reported to be expressed in the cytoplasm and nucleus of spermatogonia, spermatocyte, spermatid and spermatozoa located in the human testis (Jae Ho et al. 2006). Recently, by immunochemistry study, it has been found that CD9 is distributed in a similar pattern in porcine tissues, being present on testis (Yubero et al. 2010). Furthermore, CD9 immunoreactive sites are reported in prespermatogonia of rat and a similar expression pattern was also observed for CD9 protein in the meiotic and spermiogenic phase of spermatogenesis in the fetal rat testis (Laura and Abraham 2005). We detected CD9 on the acrosomal membrane of spermatozoa. Therefore, our results make it plausible that the CD9 might play an important role in the spermatogenesis in pig. After spermatozoa are produced in the seminiferous tubules additional maturation processes are necessary before they can participate in fertilization which occurs in the epididymis gaining the ability to be motile, to be fertile, and losing the cytoplasmic droplet. This study demonstrates that CD9 mRNA transcripts are present in pig testis and epididymis and also are expressed in the others parts of reproductive tract and accessory glands. However, no study concerning to CD9 mRNA expression in boar reproductive tissue is reported yet. CD9 was localized previously at pig ovarian tissues, oocytes, and liquid nitrogen frozen spermatozoa (Li et al. 2004). We localized CD9 protein in spermatozoa

located within the different parts (seminiferous tubules of testis and epididymis) of boar reproductive tract, but Li et al (2004) could not stain CD9 in sperm membrane. The explanation might be that Li et al (2004) performed immunostaining of CD9 protein in spermatozoa frozen in liquid nitrogen. We used freshly collected testicle and other parts of boar reproductive tract. CD9 protein is reported to be expressed on spermatogonial stem cells (Klassen et al. 2001, Mattioli et al. 2009, Oka et al. 2002, Oritani et al. 1996) differentiate boar spermatozoa from other somatic cell types based on integrin alpha 6 and CD9 molecules on the cell plasma membrane.

In mouse more than 2300 different genes are reported to be involved at the different stages of spermatogenesis and sperm development (Schultz et al. 2003). Tetraspanins are abundantly expressed transmembrane proteins, with at least 28 distinct family members in mammals (Hemler 2001). Consequently, members of this family are involved in the coordination of intracellular and intercellular processes, including signal transduction, cell proliferation, adhesion, and migration; cell fusion and host-parasite interactions (Levy and Shoham 2005). Sperm-oocyte fusion is one of the most impressive events in sexual reproduction and recent research emphasized greatly on the various molecules that are involved in sperm-oocyte binding and fusion. The factors involved in this important membrane fusion event, fertilization, have been sought for a long time (Stein et al. 2004) and the search for sperm surface proteins that function in sperm-egg interaction process are limited (Kaji et al. 2000, Miyado et al. 2008). The most attention has recently been given to the sperm members of the ADAM family specifically fertilinβ (ADAM2) and cyritestin (ADAM3) (Primakoff and Myles 2002) and Izumo (Inoue et al. 2005). On the other hand, several egg surface proteins are reported (Primakoff and Myles 2002). Several studies have focused on CD9 proteins as candidate molecules involved in sperm-oocyte fusion and reported that lack of or interference with the function of CD9 protein disrupted the sperm-oocyte fusion in mouse and hamster (Kaji et al. 2000, Kaji and Kudo 2004, Miyado et al. 2008). CD9 is reported to be observed at the epithelial cells and is regulated by androgens in human prostate (Chuan et al. 2005). This gene was expressed in epithelial cells of prostate gland in this study. CD9 expression was reported to be reduced significantly in prostate cancer progression in human (Wang et al. 2007). In conclusion, the CD9 might playing role in spermatogenesis validated through association study and by profiling of mRNA and protein expression in non-reproductive and reproductive tissues including

spermatozoa. This thesis revealed association and functional evidence that CD9 might be used as a potential candidate gene in selection programs aimed to improve fertility performance in commercial male pigs. However, this study has to be validated in other animal population in order to evaluate its potentiality for selection programme in pig breeding.

5.2 Identification of PLCz gene as a candidate gene for boar sperm quality and fertility

The population in this thesis based difference of the genotype frequencies was observed at the PLCz locus and the allele frequencies were similar. The homozygous CC was rare and heterozygous AC was more frequent. This study revealed an important influence of PLCz variant on sperm concentration. Although association study was rare for PLCz in boars, but Daghigh-Kia (2007) reported a polymorphism of PLCz at 2749 (G>A) in intron 6 in bulls. However, our identified polymorphism in PLCz was at g.158A>C in intron 1 and it is difficult to conclude how this genetic variation affects sperm quality traits. Though 'silent', it could affect PLCz function by altering the mRNA stability (Capon et al. 2004). Moreover, it may affect mRNA splicing, or is linked to variation elsewhere in the coding sequence that subsequently affects the amino acid sequence (Hickford et al. 2009). Moreover, there is increasing number of reports regarding the role of introns in regulating the expression level of a gene or tissue specific expression pattern (Jiang et al. 2000, Pagani and Baralle 2004, Virts and Raschke 2001). The DNA variations of PLCz are identified in both intron and exon and are reported to have important effect on the infertility of men (Heytens et al. 2009). Because of the lack of SNP analysis in PLCz genomic, the mRNA expression analysis is neccessery to identify the function of coding region in PLCz porcine associated with their sperm quality as well as with fertility traits.

The gene expression analysis of this thesis demonstrated that the porcine PLCz gene was expressed more in the reproductive tissues than the non-reproductive tissues. PLCz mRNA expression is also reported in the testis of Landrace boars (Yoneda et al. 2006), in testicular cells as well as in mature epididymal sperm (Coward et al. 2006) and in testis, brain, heart and skeletal muscle (Yoshida et al. 2007) in mice. PLCz is important for mature sperm cell at the final phase of spermatogenesis, it is absent from primary and secondary spermatocyte but present in round spermatids (Sousa et al. 1996).

Moreover, PLCz is reported to influence the Ca^{2+} oscillations process which is very important for successful fertilization and porcine sperm is reported to have greater ability to induce Ca^{2+} oscillations than mouse sperm (Kurokawa et al. 2005). The concentration of this PLCz protein appears to be high in the acrosomal region of head and post-acrosomal regions of spermatozoa. The post-acrosomal regions also contains the mitochondria, which are essential for spermatozoa motility and the head contains the proximal centriole, which plays a critical role in fertilization (Sathananthan et al. 1996). PLCz was also localized in the post-acrosomal region in mouse, bull and human sperm (Yoon and Fissore 2007). The localization of PLCz in post-acrosomal region at head of sperm is important because this part rapidly exposed to the ooplasm following gamete fusion and PLCz is reported to have effect in oocyte-penetrating by the sperm acrosome for successful fertilization (Fujimotoa et al. 2004). When the study of gene expression comparison between two groups of boar was analysed, the expression of PLCz gene in reproductive tissues and spermatozoa of G-I boars were higher than G-II boars. The protein expression was coincided with mRNA expression for PLCz. There was a significant PLCz mRNA expression in testis and epididymis of G-I boars higher than the expression in testis of G-II boars. PLCz mRNA and protein expression was tended to be higher in G-I that that of G-II in this study. It has been shown in human that the sperm cell with defective expression of PLCz have deficiency in their ability to activate the oocyte (Heytens et al. 2009). Furthermore, PLCz is involved in the triggering of Ca^{2}+ oscillations in eggs for successful fertilization (Nomikos et al. 2005). However, the protein expression analysis of this gene might predict their functions in reproductive process of male pig.

5.3 Identification of COX-2 gene as a candidate gene for boar sperm quality and fertility

In this thesis showed that the genotype and allele frequencies of the COX-2 gene differed between the PI and PIHA population. Whereas, the homozygous AA was rare and heterozygous AG was more frequent in all populations. The association between polymorphism in COX-2 and sperm quality and fertility traits failed to reach the significance level. We could not find any significant association between the DNA variant of COX-2 with any traits in this thesis. This is coincided with a previous study were observed among different Chinese pig breeds as one possible reason the lack of

differences between the phenotype was mentioned (Ding et al. 2006). *COX-2* is highly inducible at sites of inflammation and tumor and overexpression of COX-2 has been shown to be involved in invasion, angiogenesis and apoptosis through production of prostaglandin E_2 (Campa et al. 2004). Prostaglandin is responsible for inflammation and pain, and orchitis is one of the most important causes for the abnormal spermatozoa production and infertility in mammals. Importantly, there were the previous studies could explain about COX-2 mRNA function in reproductive traits. Winnall et al (2007) reported the abundance of COX-2 in testis, liver, lung, kidney, prostate, adrenal glands, brain cortex, epididymis, and vas deferens in adult rat by quantitative real-time PCR. However, due to the limitation of COX-2 expression report in pig, it is difficult to compare our results with other study. COX-2 protein is reported to release from the epithelium in vas deferens and inhibit smooth muscle contraction (Ruan et al. 2008). COX-2 protein was also detected in low-grade inflammation generated by sperm debris in the lumen of rat vas deferens (McKanna et al. 1998). COX-2 protein was expressed in prostate gland in this study which is supported by the previous study detected COX-2 protein in human prostate glands (Rubio et al. 2005). Moreover, the constitutive expression of COX-2 mRNA is observed in spermatogonia, spermatocytes, and sertoli cells of rat (Yamaguchi et al. 2008). However, the protein expression was analysed to support the function of this gene in reproductive traits. In this study, the localization of COX-2 protein investigated in leydig cells, spermatogonium and spermatids within the testis and suggests that COX-2 may effect in boar spermatogenesis process. COX-2 silencing is reported to cause a significant decreased in phosphorylation, aromatase expression, and cell proliferation in rat and human Leydig cell (Sirianni et al. 2009). COX-2 protein and mRNA abundance was reported in the hamster Leydig cells (Frungieri et al. 2006). The higher staining of COX-2 is evidenced in tail of spermatids and in acrosomal region of mature spermatozoa in this study which may indicate that the COX-2 protein is shifted from tail of the spermatid to the acrosomal region of mature spermatozoa during the spermatogenesis process. This finding might support the general model of the sperm development since protein and enzymes are propelling from tail and accumulated in the acrosome of be released while fertilization (Abraham 2001).

The COX-2 was tended to be higher in G-II indicated that higher COX-2 might have negative effect on boar sperm quality and fertility. COX-2 is reported to be over

expressed in men with impaired fertility (Frungieri et al. 2002). Moreover, several researches detected COX-2 in the testicular biopsies with abnormal spermatogenesis and male with infertility but could not detected in the testicular biopsies with normal morphology in human (Dinchuk et al. 1995, Frungieri et al. 2002, Robert et al. 1999, Schell et al. 2007). Likewise, COX-2 was not detectable in the normal human testis, but there was over expression in human testicular cancer (Hase et al. 2003). Additionally, the COX-2 inhibitor (NSAIDS) is reported to have a positive effects on sperm quality associated with increased ejaculation volume in human (Gambera et al. 2007). It could be suggested that the higher expression of COX-2 might indicate the presence of low grade inflammation by producing higher prostaglandin and could contribute to produce poor quality sperm and to infertility. Therefore, our results provide evidence that the observed induced protein was indeed COX-2 in spermatogenesis process of male pig.

On the other hand, the phospholipase C zeta (PLCz) is a mammalian phospholipase C (PLC), an essential fatty acid for arachidonic acid synthesis which is the precursor for inflammatory prostaglandins (Walter 2003). Moreover, PLCz contributes to Ca^{2+} oscillations in pigs which is important for the successful fertilization (Kurokawa et al. 2005). Prostaglandin is synthesized from arachidonic acid by cyclooxygenase isoenzyme type 2 (COX-2) or prostaglandin G/H synthase-2 (PTGH2). This implies that both PLCz and COX-2 are involved in prostaglandin synthesis. In conclusion, it might suggest that the higher expression of COX-2 might indicate the presence of low grade inflammation by producing higher prostaglandin that could contribute to produce poor quality sperm and to infertility. Comparatively high expression of PLCz might be beneficial for sperm quality since its variant has effect on sperm concentration and it expressed higher in boar with good sperm quality. But, over expression of PLCz might be not be good for sperm quality and fertility since PLCz could promote the arachidonic acid synthesis which is the precursor for prostaglandin. Therefore, the results of this study might be valuable to shed light on the relations between PLCz and COX-2 in spermatogenesis process in male pigs.

6. Summary

The present study has been carried out to investigate the associations of three selected genes with boars sperm quality and fertility traits that reflect the success rate of artificial inseminations. Cluster-of-differentiation antigen 9 (CD9), phospholipase-c zeta (PLCz) and cyclooxygenase isoenzyme type 2 (COX-2) were selected based on their biological correlations and physiological functions in the male reproduction.

An association of the CD9 gene with sperm motility, plasma droplets rate and abnormal spermatozoa rate in boars has been detected for the first time in pigs. CD9 loci were significantly associated with MOT, PDR and ASR in the COMBINED group. CD9 polymorphism was associated with MOT and PDR in the PI population. CD9 locus was significantly associated with MOT, PDR and ASR in the PIHA population. The results suggest an important influence of PLCz, because its genetic variation shows association with sperm concentration. PLCz loci was significantly associated with SCON in the PIHA population.

In this study, mRNA expression analysis by qRT PCR demonstrated that the porcine CD9, PLCz and COX-2 genes were specifically expressed in reproductive tissues. CD9 mRNA expression was high in reproductive tissues such as in prostate gland, then in body of the epididymis, vas deferens, tail of epididymis, head of epididymis and lower expression was found in non-reproductive tissue (muscle, brain and liver). PLCz mRNA showed higher level of expression in testis, then in tail of epididymis, body of epididymis, head of epididymis, and lower PLCz mRNA expressed in vas deferens, vesicular glands, liver, prostate gland, muscle and bulbourethral gland. Higher levels of COX-2 was expressed in reproductive tissues such as in tail of epididymis, vesicular glands, body of epididymis, vas deferens, head of epididymis, then in testis, and lowest expression was in prostate gland, bulbourethral gland. The expression of COX-2 mRNA among the non-reproductive tissues was very high in brain, then in liver, and lowest expression was in muscle.

The deduced protein porcine CD9, PLCz and COX-2 may be of relevance in most male fertility and could play a key role in the regulation of reproductive tissues function. The proteins were detected in reproductive and non-reproductive tissues to confirm the translation level of those mRNA into protein by western blot analysis. The higher detection of CD9 protein with 24 kDa molecular weigh was found in body of

epididymis and in prostate gland of boars. The proteins were detected in reproductive tissues to confirm the translation level of PLCz mRNA into protein by western blot analysis. PLCz protein with 51 kDa molecular weigh was expressed in testis, head of epididymis, body of epididymis, tail of epididymis, and liver. The proteins were detected in reproductive tissues to confirm the translation level of COX-2 mRNA into protein by western blot analysis. The higher detection of COX-2 protein with 72 kDa molecular weigh was showed higher levels in reproductive tissues and in liver.

This results make it plausible that the CD9, PLCz and COX-2 might play an important role in the spermatogenesis in pig. Testis, body of epididymis, vas deferens and prostate gland sections were stained through the same optical panel for the cell surface CD9 protein expression. The Leydig cells and Sertoli cells in testis, epithelial cells in epididymis, vas deferens and prostate gland gave signal for CD9 immunoreactivity. In case of spermatogenesis the CD9 protein expressed in spermatogonium, spermatocytes and spermatids. CD9 protein expressed highly in Leydig cells. Cytoplasmic CD9 staining was observed in the stratified epithelial cells of body of epididymis. The CD9 protein expressed in the spermatozoa located within the different regions of reproductive tract. CD9 protein was expressed in acrosomal region and acrosomal membrane. We observed a clearly cytoplasm labeled staining for PLCz expression in Leydig cells of testis. PLCz protein expressed in stereo cilia cells and in epithelial cells of head of epididymis, body of epididymis and tail of epididymis. In addition, expression of PLCz at the vas deferens was also confirmed in cytoplasm of epithelial cells. In case of spermatogenesis process, PLCz protein expressed in acrosomal region, and mitochondrial sheet of spermatozoa within epididymis. COX-2 immunofluorescence was observed in Leydig cells, spermatogonium, and tail of spermatids of testis. The most intense COX-2 immunofluorescence remained in cytoplasm, but the staining extended into the perinuclear of Leydig cells in testis. COX-2 immunofluorescence was more distinct in spermatozoa within the lumen of epididymis and appeared to have a consistent with localization to the acrosomal region of spermatozoa. COX-2 protein was expressed in the epithelial cells of epididymis, vas deferens and in fibro muscular cells of prostate gland.

Our study revealed association and functional evidence that CD9 could be used as a potential candidate gene in selection programs aimed to improve fertility performance in commercial male pigs. The CD9 mRNA was higher in all reproductive tissues from G-I

boars except tail of epididymis. However, this expression was significantly higher in the body of epididymis. The mRNA expression of CD9 was higher in spermatozoa of G-I boars than that of G-II boars. The CD9 protein expression was higher in tissues from G-I boars than those of collected from G-II boars. The difference was not significant which are coincided with the proteomic study of western blot result in spermatozoa. In this study, it was found that PLCz influence the synthesis of arachidonic acid or promotes the activity of cyclooxygenase isoenzyme type 2 in spermatogenesis process. PLCz mRNA was higher in all reproductive tissues from G-I boars. However, this expression was significantly higher in the testis. The mRNA expression level of PLCz was not showed in spermatozoa of boars with both of G-I boars and G-II boars. The COX-2 mRNA was significantly higher in testis, head of epididymis and body of epididymis from G-I boars except tail of epididymis. However, this expression was significantly higher in the testis. The mRNA expression of COX-2 was higher in spermatozoa of G-II boars than that of G-I boars but this difference was not significant. PLCz proteins expression was higher in testis, body of epididymis and tail of epididymis from G-I boars than those of collected from G-II boars. However, this PLCz mRNA was translated into protein in spermatozoa of boars with both G-I boars and G-II boars. The proteins expression of COX-2 was higher in tissues from boars with both G-I boars and G-II boars. The protein expression of COX-2 was seemed to be higher in spermatozoa of G-II boars than that of G-I boars but this difference was not significant which were coincided with the proteomic study of western blot result.

7 Zusammenfassung

Mit der vorliegenden Studie wurde die Assoziation von drei ausgewählten Genen mit der Spermaqualität und Fruchtbarkeitsmerkmalen von Ebern, die die Erfolgsrate der künstlichen Besamung reflektieren, untersucht. Cluster-of-differentiation Antigen 9 (CD9), Phospholipase-C-zeta (PLCz) und Cyclooxygenase-Isoenzym Typ 2 (COX-2) wurden aufgrund ihrer biologischen Korrelation und ihrer physiologischen Funktionen mit männlichen Reproduktionsmerkmalen ausgewählt. Zum ersten Mal konnte bei Schweinen eine Verbindung des CD9 Gens mit der Spermienmotilität, Plasmatropfrate und abnormale Spermienrate nachgewiesen werden. Signifikante Assoziationen konnten zwischen dem CD9 Loci mit MOT, PDR und ASR in einer gemischten Population ermittelt werden. Der bei CD9 entdeckte Polymorphismus steht mit MOT und PDR in einer PI Population in Verbindung. Ebenso zeigt der CD9 Loci signifikante Assoziationen mit MOT, PDR und ASR in einer PIHA Population. Die Ergebnisse weisen des weiteren auf einen wichtigen Einfluss von PLCz, aufgrund der Assoziation seiner genetischen Variation, mit der Spermienkonzentration hin. Der PLCz Loci zeigt eine signifikante Verbindung mit SCON in einer PIHA Population.

Die mRNA-Expressionsanalyse erfolgte in dieser Studie mittels der qRT-PCR. Die Expressionsanalyse erbrachte, dass die Gene CD9, PLCz und COX-2 speziell in reproduktiven Geweben exprimiert werden. Die Expressionsergebnisse zeigten, dass die CD9 mRNA Expression im reproduktiven Gewebe, wie in der Prostata, im Nebenhoden, Samenleiter, Nebenhodenschwanz, sowie im Nebenhodenleiter höher war und niedriger im nicht-reproduktiven Gewebe (Muskel, Gehirn und Leber).

PLCz mRNA zeigte eine höhere Expression im Hoden, als im Nebenhodenschwanz, Nebenhoden und im Nebenhodenleiter. Im Samenleiter, in der vesikuläre Drüsen-, Leber-, Prostata-, Muskel- und bulbourethrales Drüse war die PLCz mRNA niedriger exprimiert. Das COX-2 Gen war im reproduktiven Gewebe wie im Nebenhodenschwanz, in der vesikuläre Drüsen, in den Nebenhoden, im Samenleiter und im Nebenhodenleiter höher exprimiert, als im Hoden und zeigte eine geringere Expression in der Prostata und der bulbourethrales Drüse. Im nicht-reproduktiven Gewebe war die COX-2 mRNA Expression höher im Gehirn als in der Leber und eine geringere Expression konnte in der Muskulatur beobachtet werden. Die von den CD9, PLCz und COX-2 Genen translatierten Proteine können eine große Bedeutung in der

männlichen Fruchtbarkeit und bei der Regulierung der Funktion von reproduktivem Gewebe spielen. Die Proteine wurden im reproduktiven als auch im nicht-reproduktiven Geweben nachgewiesen, um das mRNA Translationsniveau in Proteine mittels Western-Blot-Analysen zu bestätigten. Ein höheres CD9 Proteinlevel konnte in den Nebenhoden und der Prostata von Ebern ermittelt werden. Dieses Protein hat ein Molekulargewicht von 24 kDa und wurde im Nebenhoden und der Prostatadrüse nachgewiesen. Die Proteine wurden in reproduktiven Geweben ermittelt und mittels des Western-Blot-Verfahrens konnte die PLCz mRNA Translation nachgewiesen werden. Das PLCz Protein wiegt 51 kDa und wurde in den Hoden, Nebenhodenleiter, Nebenhoden sowie Nebenhodenschwanz und in der Leber exprimiert. Die Proteine wurden im reproduktiven Geweben nachgewiesen und das Translationsniveau der COX-2 mRNA in Protein konnte durch die Western-Blot-Methode bestätigt werden. Das COX-2 Protein hat ein Molekulargewicht von 72 kDa und zeigte ein höheres Expressionsniveau im reproduktiven Gewebe und in der Leber. Diese Ergebnisse machen es wahrscheinlicher, dass CD9, PLCz und COX-2 eine wichtige Rolle in der Spermatogenese bei Schweinen spielen könnten. Für die CD9 Proteinexpression in der Zelloberfläche wurden Abschnitte von Hoden, Nebenhoden, Samenleiter und Prostata angefärbt. Es kam sowohl in den Leydig-Zellen und Sertoli-Zellen in den Hoden, Epithelzellen der Nebenhoden, Samenleiter und in der Prostata zu einem CD9 Immunreaktivität. Das CD9 Protein exprimiert bei der Spermatogenese in den Spermatogonium, Spermatozyten und den Spermatiden und es weist eine höhere Expression in den Leydig-Zellen auf. Eine zytoplasmatische CD9 Anfärbung konnte in geschichteten Epithelzellen der Nebenhoden beobachtet werden. Das CD9 Protein exprimiert in den Spermien die innerhalb verschiedenen Regionen der Fortpflanzungsorgane lokalisiert sind. Das CD9 Protein war sowohl in der akrosomalen Region als auch in der akrosomalen Membran exprimiert. Ebenso konnte bei der PLCz Untersuchung ein deutliches Zytoplasma in den Leydig-Zellen des Hodens beobachtet werden. Das PLCz Protein exprimiert in stereo Zilien-Zellen und in den Epithelzellen der Nebenhodenleiter sowie im Körper und Schwanz des Nebenhodens. Zusätzlich konnte die Expression von PLCz am Samenleiter als auch im Zytoplasma von Epithelzellen bestätigt werden. Beim Prozess der Spermatogenese konnten PLCz Proteinexpressionen in der akrosomalen Region, und im mitochondrialen Blatt der Spermien innerhalb der Nebenhoden ermittelt werden. COX-2 Immunfluoreszenz

wurde in Leydig-Zellen, Spermatogonium und im Schwanz der Spermatiden im Hoden beobachtet. Die intensivste COX-2 Immunfluoreszenz erwies sich im Zytoplasma, aber die Anfärbung zeigte eine Erweiterung im perinukleären Raum der Leydig-Zellen im Hoden. COX-2 Immunfluoreszenz war deutlich ausgeprägter in den Spermien innerhalb der Lumen des Nebenhodens und schien eine Übereinstimmung mit der Lokalisierung der akrosomalen Region von Spermien zu haben. Das COX-2 Protein war in den Epithelzellen der Nebenhoden, Samenleiter und in den fibro Muskelzellen der Prostata exprimiert.

Durch Assoziation und funktionelle Analysen konnte in dieser Studie CD9 als ein mögliches Kandidatengen in Zuchtprogrammen ermittelt werden, das gezielt auf eine verbesserte Fruchtbarkeitsleistung bei kommerziellen männlichen Schweinen wirkt. Die CD9 mRNA war bei allen reproduktiven Gewebe der G-I Eber höher exprimiert, mit einer Ausnahme im Nebenhodenschwanz. Allerdings war die Expression im Nebenhoden signifikant höher. Die CD9 mRNA Expression war in Spermien der G-I Eber höher als die der G-II Eber. Im Vergleich zum Gewebe der G-II Eber war die CD9 Proteinexpression im Gewebe der G-I Eber höher. Der Unterschied war nicht signifikant und konnte durch die Proteom-Studie mit Hilfe der Western-Blot-Analysen bestätigt werden. In dieser Studie konnte festgestellt werden, dass PLCz die Synthese von Arachidonsäure beeinflussen oder die Aktivität der Cyclooxygenase-Isoenzym Typ 2 im Spermatogeneseprozess fördern könnte. In allen reproduktiven Gewebe der G-I Eber war die PLCz mRNA höher exprimiert. Allerdings konnte eine signifikant höhere Expression in den Hoden beobachtet werden. Es konnte sowohl bei den G-I Ebern als auch bei den G-II Ebern in den Spermien kein PLCz Expressionsniveau ermittelt werden. COX-2 zeigte ein signifikant höheres Expressionsnieveau in den Hoden, im Nebenhodenkopf und im Körper des Nebenhodens bei den G-I Ebern, mit der Ausnahme des Nebenhodenschwanzes. Allerdings war die Expression in den Hoden signifikant höher. Die mRNA Expression von COX-2 war in Spermien von G-II Eber höher als bei G-I Eber, jedoch war dieser Unterschied nicht signifikant höher. Die PLCz Proteinexpression war im Hoden, Nebenhoden und im Nebenhodenschwanz bei G-I Ebern höher als die Expression bei den G-II Ebern. Dennoch konnte die Translation von PLCz mRNA in Proteine in den Spermienen bei beiden G-I und G-II Ebern beobachtet werden. Die Proteinexpression von COX-2 war in beiden Geweben der GI Eber und G-II Eber höher. Ebenso konnte eine höhere Proteinexpression von COX-2 in Spermien

der G-II Eber ermittelt werden, allerdings war dieser Unterschied nicht signifikant. Dieses Ergebnis konnte durch der Proteom-Studie mittels Western-Blot bestätig werden.

8 References

Abraham LK (2001): Spermatid manchette: plugging proteins to zero into the sperm tail. Mol Reprod Dev 59(4), 347-349

Akinci MAM, Turner H, Taveras M, Wolosin JM (2009): Differential gene expression in the pig limbal side population: implications for stem cell cycling, replication, and survival. Invest Ophthalmol Vis Sci 50(12), 5630-5638

Alfieri J, Martin A, Takeda Jea (2003): Infertility in female mice with an oocyte-specific knockout of GPI-anchored proteins. J Cell Sci 116, 2149-2155

Alm K, Peltoniemi OAT, Koskinen E, Andersson M (2006): Porcine field fertility with two different insemination doses and the effect of sperm morphology. Reprod Domestic Anim 41(3), 210-213

Almeida EA, Huovila AP, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, et al. (1995): Mouse egg integrin α6β1 functions as a sperm receptor. Cell 81, 1095-1104

Althouse GC (1997): Evaluating porcine semen for artificial insemination. Part I. standard tests. The compendium. Swine Medicine S 30 Food Animal

Aron TC, Alexandre B, Nicolas P, Jacques GL, David WS (2007): Presumptive pre-sertoli cells express genes involved in cell proliferation and cell signalling during a critical window in early testis differentiation. Mol Reprod Dev 74(12), 1491-1504

At-Taras EE, Berger T, McCarthy MJ, Conley AJ, Nitta-Oda BJ, Roser JF (2006): Reducing estrogen synthesis in developing boars increases testis size and total sperm production. J Androl 27, 552-559

Baek S, Eling T (2006): Changes in gene expression contribute to cancer prevention by *COX* inhibitors. Prog Lipid Res 45, 1-16

Barraud-Lange V, Naud-Barriant N, Bomsel M, Wolf J-P, Ziyyat A (2007): Transfer of oocyte membrane fragments to fertilizing spermatozoa. FASEB J 21, 3446-3449

Bidanel J, Rosendo A, Iannuccelli N, Riquet J, Gilbert H, Caritez J, Billon Y, Amigues Y, Prunier A, Milan D (2008): Detection of quantitative trait loci for teat number and female reproductive traits in Meishan x Large White F2 pigs. Animal 2, 813-820

Bidanel JP, Prunier A, Iannuccelli N, Milan D (2001): Detection of quantitative trait loci for male and female reproductive traits in Meishan x Large White F2 pigs. in Book of Abstracts of the 51st Annual Meeting of the European Association for Animal Production. Wageningen Pers, Wageningen, the Netherlands. 54

Bissonnette N, Levesque-Sergerie J-P, Thibault C, Boissonneault G (2009): Spermatozoal transcriptome profiling for bull sperm motility: a potential tool to evaluate semen quality. Reproduction 138(1), 65-80

Booth WD (1980): A study of some major testicular steroids in the pig in relation to their effect on the development of male characteristics in the prepubertally castrated boar. J Reprod Fertil 59(1), 155-162

Borg CL, Wolski KM, Gibbs GM, O'Bryan MK (2010): Phenotyping male infertility in the mouse: how to get the most out of a 'non-performer'. Hum Reprod Update 16(2), 205-224

Borg KE, Lunstra DD, Christenson RK (1993): Semen characteristics, testicular size, and reproductive hormone concentrations in mature duroc, meishan, fengjing, and minzhu boars. Biol Reprod 49, 515-521

Boucheix C, Duc GHT, Jasmin C, Rubinstein E (2001): Tetraspanins and malignancy. Expet Rev Mol Med 3(4), 1-17

Brown J, DuBois R (2005): *COX-2*: a molecular target for colorectal cancer prevention. J Clin Oncol 23, 2840-2855

Caballero I, Vazquez JM, García EM, Parrilla I, Roca J, Calvete JJ, Sanz L, Martínez EA (2008): Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa. Theriogenology 70(8), 1352-1355

Campa D, Zienolddiny S, Maggini V, Skaug V, Haugen A, Canzian F (2004): Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer. Carcinogenesis 25 (2), 229-235

Campbell K, Swann K (2006): Ca^{2+} oscillations stimulate an ATP increase during fertilization of mouse eggs. Dev Biol 298, 225-233

Capon F, Allen M, Ameen M, Burden A, Tillman D, Barker J (2004): A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups. Hum Mol Genet 13(20), 2361-2368

Carroll J (2001): The initiation and regulation of Ca^{2+} signalling at fertilization in mammals. Semin Cell Dev Biol 12, 37-43

Cassady J, ohnson R, Pomp D, Rohrer G, Van VL, Spiegel E, Gilson K (2001): Identification of quantitative trait loci affecting reproduction in pigs. J Anim Sci 79(3), 623-633

Chen H, Sampson NS (1999): Mediation of sperm-egg fusion: evidence that mouse egg *α6β1* integrin is the receptor for sperm fertilinbeta. Chem Biol 6, 1-10

Chen MS, Tung KS, Coonrod SA, Takahashi Y, Bigler D, Chang A, Yamashita Y, Kincade PW, Herr JC, White JM (1999): Role of integrin-associated protein *CD9* in binding between sperm *ADAM 2* and the egg integrin α6β1: implications for murine fertilization. Proc Natl Acad Sci U S A 96, 11830-11835

Chuan Y, Pang S, Bergh A, Norstedt G, Pousette A (2005): Androgens induce CD-9 in human prostate tissue. Int J Androl 28(5), 291-296

Cocquet J, Ellis PJI, Yamauchi Y, Mahadevaiah SK, Affara NA, Ward MA, Burgoyne PS (2009): The multicopy gene Sly represses the sex chromosomes in the male mouse germline after meiosis. PLoS Biol 7(11):e1000244

Colenbrander B, Feitsma H, Grooten HJ (1993): Optimizing semen production for artificial insemination in swine. J Reprod Fert Suppl 48, 207-215

Cooper T, Yeung C-H (2003): Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. Microsc Res Techn 61, 28-38

Corpet F (1998): Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 16, 10881-10890

Correa JR, Heersche G, Zavos PM (1997): Sperm membrane functional integrity and response of frozen-thawed bovine spermatozoa during the hypoosmotic swelling test incubation at varying temperatures. Theriogenology 47(3), 715-721

Coward K, Kubota H, Hibbitt O, McIlhinney J, Kohri K, Parrington J (2006): Expression of a fluorescent recombinant form of sperm protein phospholipase C zeta in mouse epididymal sperm by in vivo gene transfer into the testis. Fertil Steril 85, 1281-1289

Cox LJ, Larman MG, Saunders CM, Hashimoto K, Swann K, Lai FA (2002): Sperm phospholipase Cζ from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. Reproduction 124, 611-623

Dacheux L, Castella S, Gatti JL, Dacheux F (2005): Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology 63, 319-341

Daghigh-Kia H (2007): Identification and SNP detection for preimplantation active genes and their association with embryo development and male fertility in cattle. Dissertation, der Rheinischen Friedrich - Wilhelms - Universität Bonn p. 135

Daniel SJ, Scott A J, Yu ZHI, Joshua N F, Gregory S K, William W W (2007): Identification of testis-specific male contraceptive targets. Ann NY Acad Sci 1120, 36-46

Dawson KA (2006): Nutrigenomics: feeding the genes for improved fertility. Anim Reprod Sci 96(3-4), 312-322

De Caterina R, Basta G (2001): n-3 fatty acids and the inflammatory response biological background. Eur Heart J Suppl 3, 42-49

Dekkers J (2003): Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. J Anim Sci 81(1), 5

Demars J, Riquet J, Feve K, Gautier M, Morisson M, Demeure O, Renard C, Chardon P, Milan D (2006): High resolution physical map of porcine chromosome 7 QTL region and comparative mapping of this region among vertebrate genomes. BMC Genomics 7, 13

Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, et al. (1995): Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 378, 406-409

Ding NS, Ren DR, Guo YM, Ren J, Yan Y, Ma JW, Chen KF, Huang LS (2006): Genetic variation of porcine prostaglandin-endoperoxide synthase 2 (PTGS2) gene and its association with reproductive traits in an Erhualian × Duroc F2 population. Acta Genetica Sin 33(3), 213-219

Ducos A, Berland HM, Pinton A, Guillemot E, Seguela A, Blanc MF, Darre A, Darre R (1998): Nine new cases of reciprocal translocation in the domestic pig (Sus scrofa domestica L.). J Hered 89, 136-142

Eckardstein SV, Cooper TG, Rutscha K, Meschede D, Horst J, Nieschlag E (2000): Seminal plasma characteristics as indicators of cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations in men with obstructive azoospermia. Fertil Steril 73, 1226-1231

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard M-A, Schellander K, Tesfaye D (2006): Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. Physiol Genomics 28(1), 84-96

Evans ACO, Forde N, Gorman GMO, Zielak AE, Lonergan P, Fair T (2008): Use of microarray technology to profile gene expression patterns important for reproduction in cattle. Reprod Domestic Anim 43(2), 359-367

Falconer D, Mackay T (1996): Introduction to quantitative genetics (fourth edition). 480

Fischer S, Furstenberger G, Marks F, Slaga T (1987): Events associated with mouse skin tumor promotion with respect to arachidonic acid metabolism: a comparison between SENCAR and NMRI mice. Cancer Res 47, 3174-3179

Flowers WL (1998): Artificial insemination in animals. In: E. Knobil and J.D. Neill, Editors, Encyclopedia of reproduction, Academic Press, San Diego, CA, USA. 296

Flowers WL (2008): Genetic and phenotypic variation in reproductive traits of AI boars. Theriogenology 70(8), 1297-1303

Flowers WL (2002): Increasing fertilization rate of boars: influence of number and quality of spermatozoa inseminated. J Anim Sci 80(1), 47-53

Flowers WL (1997): Management of boars for efficient semen production. J Reprod Fert Suppl 52, 67-78

Foote RH (1968): Standards for sperm concentrations: poystyrene latex particles as an aid in quality control. Proc. Tech Conf on AI and Reprod 95-97

Foote RH (2002): Within-herd use of boar semen at 5°C, with a note on electronic monitoring of oestrus. Reprod Domestic Anim 37(1), 61-63

Ford JJ, Wise TH, Lunstra DD, Rohrer GA (2001): Interrelationships of porcine X and Y chromosomes with pituitary gonadotropins and testicular size. Biol Reprod 65, 906-912

FranÃ§a LR, Silva VA, Chiarini-Garcia Hl, Garcia SK, Debeljuk L (2000): Cell proliferation and hormonal changes during postnatal development of the testis in the pig. Biol Reprod 63(6), 1629-1636

Frungieri MB, Gonzalez-Calvar SI, Parborell F, Albrecht M, Mayerhofer A, Calandra RS (2006): Cyclooxygenase-2 and prostaglandin F2[alpha] in syrian hamster Leydig cells: inhibitory role on luteinizing hormone/human chorionic gonadotropin-stimulated testosterone production. Endocrinology 147(9), 4476-4485

Frungieri MB, Weidinger S, Meineke V, Köhn FM, Mayerhofer A (2002): Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPARγ: possible relevance to human fibrotic disorders. Proc Natl Acad Sci U S A 99(23), 15072-15077

Fujimotoa S, Yoshidaa N, Fukuia T, Amanaia M, Isobeb T, Itagakib C, Izumib T, Perry A (2004): Mammalian phospholipase Cζ induces oocyte activation from the sperm perinuclear matrix Dev Biol 274, 370-383

Funk CD (2001): Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871 – 1875

Furstenberger G, Marks F, Muller-Decker K (2003): Cyclooxygenase-2 and skin carcinogenesis. Prog Exp Tumor Res 37, 72-89

Gadea J (2005): Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology 63(2), 431-444

Gadea J, Matás C, Lucas X (1998): Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Anim Reprod Sci 54(2), 95-108

Gambera L, Serafini F, Morgante G, Focarelli R, De Leo V, Piomboni P (2007): Sperm quality and pregnancy rate after COX-2 inhibitor therapy of infertile males with abacterial leukocytospermia. Hum Reprod 22(4), 1047-1051

García-Herrero S, Garrido N, Martínez-Conejero J, Remohí J, Pellicer A, Meseguer M (2010): Ontological evaluation of transcriptional differences between sperm of infertile males and fertile donors using microarray analysis. J Assist Reprod Gen 27(2-3), 67-68

Garniera J, Klont R, Plastow G (2003): The potential impact of current animal research on the meat industry and consumer attitudes towards meat. Meat Sci 63, 79-88

Georges M (2007): Mapping, fine mapping, and molecular dissection of quantitative trait loci in domestic animals. Annu Rev Genomics Hum Genet 8, 131-162

Giesecke K, Sieme H, Distl O (2010): Infertility and candidate gene markers for fertility in stallions: a review. Vet J 185(3), 265-271

Gillan L, Evans G, Maxwell WMC (2005): Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63(2), 445-457

Glazar A, Evans J (2009): Immunoglobulin superfamily member IgSF8 (*EWI-2*) and *CD9* in fertilisation: evidence of distinct functions for *CD9* and a *CD9*-associated protein in mammalian sperm-egg interaction. Reprod Fertil Dev 21, 293-303

Gradil C, Yoon S, Brown J, He C, Visconti P, Fissore R (2006): PLCζ: a marker of fertility for stallions. Anim Reprod Sci 94 23-25

Griffin DK, Finch KA (2005): The genetic and cytogenetic basis of male infertility. Hum Fertil 8, 19-26

Griffin J, Seal MC, Flowers WL (2006): Effect of neonatal environment on adult reproductive function in boars. J Anim Sci 84 (1), 205

Guarducci E, Nuti F, Becherini L, Rotondi M, Balercia G, Forti G, Krausz C (2006): Estrogen receptor alpha promoter polymorphism: stronger estrogen action is coupled with lower sperm count. Hum Reprod 21, 994-1001

Guyonnet B, Marot G, Dacheux JL, Mercat MJ, Schwob S, Jaffrezic F, Gatti JL (2009): The adult boar testicular and epididymal transcriptomes. BMC Genomics 10(1), 369

Hammond AC, Olson TA, Jr CCC, Bowers EJ, Randel RD, Murphy CN, Vogt DW, Tewolde A (1996): Heat tolerance in two tropically adapted Bos taurus breeds, Senepol and Romosinuano, compared with Brahman, Angus, and Hereford Cattle in Florida. J Anim Sci 74, 295-303

Hansen-Petrik M, McEntee M, Jull B, Shi H, Zemel M, Whelan J (2002): Prostaglandin E(2) protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apc(Min/+) mice. Cancer Res 62, 403-408

Harder RR, Lunstra DD, Johnson RK (1995): Growth of testes and testicular morphology after eight generations of selection for increased predicted weight of testes at 150 days of age in boars. J Anim Sci 73(8), 2186-2192

Hase T, Yoshimura R, Matsuyama M, Kawahito Y, Wada S, Tsuchida K, Sano H, Nakatani T (2003): Cyclooxygenase-1 and -2 in human testicular tumours. Eur J Cancer 39(14), 2043-2049

Hata A, Breyer R (2004): Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther 103, 147-166

He Z, Chan WY, Dym M (2006): Microarray technology offers a novel tool for the diagnosis and identification of therapeutic targets for male infertility. Reproduction 132(1), 11-19

Helliwell R, Adams L, Mitchell M (2004): Prostaglandin synthases: recent developments and a novel hypothesis. Prostaglandins Leukot Essent Fatty Acids 70, 101-113

Hemler M (2001): Specific tetraspanin functions. J Cell Biol 155, 1103-1108

Heytens E, Parrington J, Coward K, Young C, Lambrecht S, Yoon SY, Fissore RA, Hamer R, Deane CM, Ruas M, et al. (2009): Reduced amounts and abnormal forms of phospholipase C zeta (PLC[zeta]) in spermatozoa from infertile men. Hum Reprod 24(10), 2417-2428

Hickford J, Forrest R, Zhou H, Fang Q, Han J, Frampton C (2009): Polymorphisms in the ovine myostatin gene (MSTN) and their association with growth and carcass traits in New Zealand Romney sheep Anim Genet 41(1), 64-72

Hillbrand FW, Glodek P (1984): Halothane sensitivity and semen quality of boars in 3 lines of pigs. In 35th Annual Meeting of the EAAP. 2 (5), 11-12

Hoei-Hansen CE (2008): Application of stem cell markers in search for neoplastic germ cells in dysgenetic gonads, extragonadal tumours, and in semen of infertile men. Cancer Treat Rev 34(4), 348-367

Holt D, Botto M, Bygrave A, Hanna S, Walport M, Morgan B (2001): Targeted deletion of the *CD59* gene causes spontaneous intravascular hemolysis and hemoglobinuria. Blood 98, 442-449

Holt WV, Van Look KJW (2004): Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction 127(5), 527-535

Homa ST, Swann K (1994): A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. Human Reprod 9, 2356-2361

Horejsi V, Vlcek C (1991): Novel structurally distinct family of leucocyte surface glycoproteins including CD9, CD37, CD53 and CD63. FEBS Letters 288(1-2), 1-4

Houde AA, Murphy BD, Mathieu O, Bordignon V, Palin MF (2008): Characterization of swine adiponectin and adiponectin receptor polymorphisms and their association with reproductive traits. Anim Genet 39(3), 249-257

Huang Y-T, Johnson RK (1996): Effect of selection for size of testes in boars on semen and testis traits. J Anim Sci 74, 750-760

Humblot P, Ducrocq V, Csilla N (1993): Relationship between semen characteristics of young and adult bulls in the Normande breed. Lives Prod Sci 35(3), 265-281

Igusa Y, Miyazaki S, Yamashita N (1983): Periodic hyperpolarizing responses in hamster and mouse eggs fertilized with mouse sperm. J Physiol 340, 633-647

Inoue N, Ikawa M, Isotani A, Okabe M (2005): The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434, 234-238

Jae Ho L, Wolfgang E, Karim N (2006): Stem cell protein Piwil2 modulates expression of murine spermatogonial stem cell expressed genes. Mol Reprod Dev 73(2), 173-179

Jean-Louis D, Jean Luc G, Françoise D (2003): Contribution of epididymal secretory proteins for spermatozoa maturation. Microsc Res Tech 61(1), 7-17

Jean-Louis D, Sandrine C, Jean Luc G, FranÃ§oise D (2005): Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology 63(2), 319-341

Jiang Z, Cote J, Kwon J, Goate A, Wu J (2000): Aberrant splicing of tau pre-mRNA caused by intronic mutations associated with the inherited dementia frontotemporal dementia with parkinsonism linked to chromosome 17 Mol Cell Biol 20, 4036-4048

Johnson LA, Maxwell WMC, Dobrinsky JR, Welch GR (1995): Staining sperm for viability assessment. Reprod Domestic Anim 31(1), 37-47

Johnson LA, Weitze KF, Fiser P, Maxwell WMC (2000): Storage of boar semen. Anim Reprod Sci 62(1-3), 143-172

Kaji K, Kudo A (2004): The mechanism of sperm-oocyte fusion in mammals Reproduction 127, 423-429

Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, Sakagami J, Tada N, Miyazaki S, Kudo A (2000): The gamete fusion process is defective in eggs of *Cd9*-deficient mice. Nat Genet 24, 279-282

Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004): *CD9* Is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70(1), 70-75

Katan M (1998): Families of phosphoinositide-specific phospholipase C: structure and function. Biochim Biophys Acta 1436, 5-17

Kato S, Yasui T, Nano I, Kanda S (1984): Migration of cytoplasmic droplet in boar spermatozoa by centrifugation. Jap J Animal AI Res 6, 15-18

Kennedy B, Wilkins J (1984): Boar, breed and environmental factors influencing semen characteristics of boars used in artificial insemination. Can J Anim Sci 64, 833-843

Kim H, Kang T-y, Joo H-g, Shin T (2006): Immunohistochemical localization of galectin-3 in boar testis and epididymis. Acta Histochem 108(6), 481-485

Klassen H, Schwartz M, Bailey A, Young M (2001): Surface markers expressed by multipotent human and mouse neural progenitor cells include tetraspanins and non-protein epitopes. Neurosci Lett 312, 180-182

Knott J, Kurokawa M, Fissore R, Schultz R, Williams C (2005): Transgenic rnai reveals role for mouse sperm phospholipase C z in triggering ca^{2+} oscillations during fertilization. Biol Reprod 72, 992-996

Knott JG, Kurakawa M, Fissore RA (2003): Release of Ca^{2+} oscillation-inducing sperm factor during mouse fertilization. Dev Biol 260, 536-547

Kono T, Carroll J, Swann K, Whittingham DG (1995): Nuclei from fertilized mouse embryos have calcium-releasing activity. Development 121, 1123-1128

Kouchi Z, Fukami K, Shikano t, Oda S, Nakamura Y, Takenawa T, Miyazaki S (2004): Recombinant phospholipase C zeta has high Ca^{2+} sensitivity and induces Ca^{2+} oscillations in mouse eggs. J Biol Chem 279, 10408-10412

Kouchi Z, Nakamuray S, Shirakawa H, Fukami K, Miyazaki S (2005): The role of ef-hand domains and C2 domain in regulation of enzymatic activity of phospholipase C zeta. J Biol Chem 280, 21015-21021

Kurokawa M, Sato K-i, Wu H, He C, Malcuit C, Black SJ, Fukami K, Fissore RA (2005): Functional, biochemical, and chromatographic characterization of the complete [Ca2+]i oscillation-inducing activity of porcine sperm. Dev Biol 285(2), 376-392

Larman M, Saunders C, Carroll J, Lai F, Swann K (2004): Cell cycle-dependent ca2+ oscillations in mouse embryos are regulated by nuclear targeting of plcζ. J Cell Sci 117, 2513-2521

Laura LT, Abraham LK (2005): The ADAM-integrin-tetraspanin complex in fetal and postnatal testicular cords. Birth Defects Research Part C: Embryo Today: Reviews 75(2), 130-141

Lawrence Y, Whitaker M, Swann K (1997): Sperm-oocyte fusion is the prelude to the initial Ca^{2+} increase at fertilization in the mouse. Development 124, 223-241

Le Naour F, ccedil, ois, Rubinstein E, Jasmin C, Prenant M, Boucheix C (2000): Severely reduced female fertility in CD9-deficient mice. Science 287(5451), 319-321

Lee B, Yoon S, Fissore R (2006): Regulation of fertilization-initiated [ca*2*+]i oscillations in mammalian eggs: a multi-pronged approach. Semin Cell Dev Biol 17, 274-284

Levy S, Shoham T (2005): Protein-protein interactions in the tetraspanin web. Physiology 20, 218-224

Lhote D, Serres C, Laissue P, Oulmouden A, Rogel-Gaillard C, Montagutelli X, Vaiman D (2007): Centimorgan-range one-step mapping of fertility traits using interspecific recombinant congenic mice. Genetics 176, 1907-1921

Li YH, Hou Y, Ma W, Yuan JX, Zhang D, Sun QY, Wang WH (2004): Localization of CD9 in pig oocytes and its effects on sperm-egg interaction. Reproduction 127(2), 151-157

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK (1997): Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91(2), 197-208

Lin CL, Jennen DGJ, Ponsuksili S, Tholen E, Tesfaye D, Schellander K, Wimmers K (2006a): Haplotype analysis of beta-actin gene for its association with sperm quality and boar fertility. J Anim Breed Genet 123(6), 384-388

Lin CL, Ponsuksili S, Tholen E, Jennen DGJ, Schellander K, Wimmers K (2006b): Candidate gene markers for sperm quality and fertility of boar. Anim Reprod Sci 92(3-4), 349-363

Liu J, Antaya M, Goff AK, Boerboom D, Silversides DW, Lussier JG, Sirois J (2001): Molecular characterization of bovine prostaglandin G/H synthase-2 and regulation in uterine stromal cells. Biol Reprod 64(3), 983-991

Long CR, Dobrinsky JR, Johnson LA (1999): In vitro production of pig embryos: comparisons of culture media and boars. Theriogenology 51(7), 1375-1390

Lunstra DD, Ford JJ, Christenson RK, Allrich RD (1986): Changes in Leydig cell ultrastructure and function during pubertal development in the boar. Biol Reprod 34(1), 145-158

Majumdar SS, Usmani A, Bhattacharya I, Sarda K, Gautam M, Sharma D, Basu S, Dhup S (2009): A method for rapid generation of transgenic animals to evaluate testis genes during sexual maturation. J Reprod Immunol 83(1-2), 36-39

Makinen A, Andersson M, Hakkinen A, Kuosmanen S (1999): A reciprocal translocation between autosomes 8 and 10 in a boar used for artificial insemination service and its effects on litter size. Anim Reprod Sci 56, 237-243

Malmgren L, Larsson K (1984): Semen quality and fertility after heat stress in boars. Acta Vet Scand 25, 425-435

Marangos P, FitzHarris G, Carroll J (2003): Ca^{2+} oscillations at fertilization in mammals are regulated by the formation of pronuclei. Development 130, 1461-1472

Mathevon M, Buhr MM, Dekkers JCM (1998a): Environmental, management, and genetic factors affecting semen production in Holstein bulls. J Dairy Sci 81, 3321-3330

Mathevon M, Dekkers JCM, Buhr MM (1998b): Environmental, management, and genetic factors affecting semen production in French Montbéliard bulls. Livestock Prod Sci 55, 65-77

Mattioli M, Gloria A, Mauro A, Gioia L, Barboni B (2009): Fusion as the result of sperm-somatic cell interaction. Reproduction 138, 679-687

Matzkin ME, Gonzalez-Calvar SI, Mayerhofer A, Calandra RS, Frungieri MB (2009): Testosterone induction of cyclooxygenase 2 (COX2) expression and prostaglandin F2[alpha] (PGF2[alpha]) production in hamster Leydig cells. Reproduction 138, 163

McCoard SA, Lunstra DD, Wise TH, Ford JJ (2001): Specific staining of Sertoli cell nuclei and evaluation of Sertoli cell number and proliferative activity in meishan and white composite boars during the neonatal period. Biol Reprod 64, 689-695

McKanna JA, Zhang MZ, Wang JL, Cheng HF, Harris RC (1998): Constitutive expression of cyclooxygenase-2 in rat vas deferens. Am J Physiol Regul Integr Comp Physiol 275(1), 227-233

Merks JWM, Ducro-Steverink DWB, Feitsma H (2000): Management and genetic factors affecting fertility in sows. Reprod Domestic Anim 35, 261-266

Milan D, Bidanel JP, Iannuccelli N, Riquet J, Amigues Y, Gruand J, Roy PL, Renard C, Chevalet C (2002): Detection of quantitative trait loci for carcass composition traits in pigs. Genet Sel Evol 34, 705-728

Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A, et al. (2000): Requirement of CD9 on the egg plasma membrane for fertilization. Science 287(5451), 321-324

Miyado K, Yoshida K, Yamagata K, Sakakibara K, Okabe M, Wang X, Miyamoto K, Akutsu H, Kondo T, Takahashi Y, et al. (2008): The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. Proc Natl Acad Sci U S A 105, 12921-12926

Miyazaki S, Shirakawa H, Nakada K, Honda Y (1993): Essential role of the inositol 1,4,5-trisphosphate/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. Dev Biol 58, 62-78

Moe M, Lien S, Aasmundstad T, Meuwissen T, Hansen M, Bendixen C, Grindflek E (2009): Association between SNPs within candidate genes and compounds related to boar taint and reproduction. BMC Genetics 10(1), 32

Muller-Decker K, Neufang G, Berger I, Neumann M, Marks F, Furstenberger G (2002): Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. Proc Natl Acad Sci USA 99, 12483-11248

Muller CH (2000): Rationale, interpretation, validation, and uses of sperm function tests. J Androl 21(1), 10-30

Nakamura K, Iwamoto R, Mekada E (1995): Membrane-anchored heparin-binding EGF-like growth factor (*HB-EGF*) and diphtheria toxin receptor-associated protein (*DRAP27)*/ *CD9* form a complex with integrin α3β1 at cell-cell contact sites. J Cell Biol 129, 1691-1705

Nomikos M, Blayney L, Larman M, Campbell K, Rossbach A, Saunders C, Swann K, Lai F (2005): Role of phospholipase C-ζ domains in Ca2+-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca2+ oscillations. J Biol Chem 280, 31011-31018

Nomikos M, Mulgrew-Nesbitt A, Pallavi P, Mihalyne G, Zaitseva I, Swann K, Lai F, Murray D, Mclaughlin S (2007): Binding of phosphoinositid-specific phospholipase czeta (plcz) to phospholipid membranes. J Biol Chem 282, 16644-16653

Odintsova E, Butters T, Monti E, Sprong H, van Meer G, Berditchevsk iF (2006): Gangliosides play an important role in the organization of *CD82*-enriched microdomains. Biochem J 400, 315-325

Oka M, Tagoku K, Russell T, Nakano Y, Hamazaki T, Meyer E, Yokota T, Terada N (2002): CD9 is associated with leukemia inhibitory factor-mediated maintenance of embryonic stem cells. Mol Biol Cell 13, 1274-1281

Okwun OE, Igboeli G, Ford JJ, Lunstra DD, Johsnson L (1996): Number and function of Sertoli cells, number and yield of spermatogonia, and daily sperm production in three breeds of boar. J Reprod Fert 107, 137-149

Oritani K, Wu X, Medina K, Hudson J, Miyake K, Gimble J, Burstein S, Kincade P (1996): Antibody ligation of CD9 modifies production of myeloid cells in long- term cultures. Blood 87, 2252-2261

Ørstavik KH (2008): Genetic causes of male infertility. Tidsskr Nor Laegeforen 128, 324-326

Pagani F, Baralle F (2004): Genomic variants in exons and introns: identifying the splicing spoilers. Nat rev Genet 5, 389-396

Pérez-Llano B, Lorenzo JL, Yenes P, Trejo A, García-Casado P (2001): A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology 56(3), 387-398

Petrunkina AM, Waberski D, Gunzel-Apel AR, Topfer-Petersen E (2007): Determinants of sperm quality and fertility in domestic species. Reproduction 134(1), 3-17

Ponsuksili S, Murani E, Schellander K, Schwerin M, Wimmers K (2005): Identification of functional candidate genes for body composition by expression analyses and evidencing impact by association analysis and mapping. Biochim Biophys Acta 1730(1), 31-40

Primakoff P, Myles DG (2002): Penetration, adhesion, and fusion in mammalian sperm-egg Interaction. Science 296(5576), 2183-2185

Rathje TA, Johnson RK, Lunstra DD (1995): Sperm production in boars after nine generations of selection for increased weight of testis. J Anim Sci 73, 2177-2185

Rebecchi M, Pentyala S (2000): Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 80, 291-335

Rempel LA, Nonneman DJ, Wise TH, Erkens T, Peelman LJ, Rohrer GA (2010): Association analyses of candidate single nucleotide polymorphisms on reproductive traits in swine. J Anim Sci 88(1), 1-15

Ren D, Xing Y, Lin M, Wu Y, Li K, Li W, Yang S, Guo T, Ren J, Ma J, et al. (2009a): Evaluations of boar gonad development, spermatogenesis with regard to semen characteristics, libido and serum testosterone levels based on large white Duroc x Chinese Erhualian crossbred boars. Reprod Domest Anim 44(6), 913-919

Ren DR, Ren J, Xing YY, Guo YM, Wu YB, Yang GC, Mao HR, Huang LS (2009b): A genome scan for quantitative trait loci affecting male reproductive traits in a large white Duroc x Chinese Erhualian resource population. J Anim Sci 87(1), 17-23

Rigas B, Goldman I, Levine L (1993): Altered eicosanoid levels in human colon cancer. J Lab Clin Med 122, 518-523

Robert L, Charles D L, Christopher LEE, Howard T (1999): Cyclooxygenase-deficient mice: A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. Ann N Y Acad Sci 889, 52-61

Robinson J, Quinton V (2002): Genetic parameters of early neo-natal piglet survival and number of piglets born, Proceeding of the Seventh World Congress Genetics Applied Livestock Production Montpellier, France

Robinson JAB, Buhr MM (2005): Impact of genetic selection on management of boar replacement. Theriogenology 63(2), 668-678

RodrÃguez-MartÃnez H (2006): State of the art in farm animal sperm evaluation. Reprod Fertil Dev 19(1), 91-101

Rogers NT, Hobson E, Pickering S, Lai FA, Braude P, Swann K (2004): PLCζ causes Ca^{2+} oscillations and parthenogenetic activation of human oocytes. Reproduction 128, 697-702

Rohrer GA, Ford JJ, Wise TH, Vallet JL, Christenson RK (1999): Identification of quantitative trait loci affecting female reproductive traits in a multigeneration Meishan-White composite swine population. J Anim Sci 77, 1385-1391

Rohrer GA, Wise TH, Lunstra DD, Ford JJ (2001): Identification of genomic regions controlling plasma FSH concentrations in Meishan-White Composite boars. Physiol Genomics 6, 145-151

Rolf C, Behre H, Nieschlag E (1996): Reproductive parameters of older compared to younger men of infertile couples. Int J Androl 19, 135-142

Rothschild M, Jacobson C, Vaske D, Tuggle C, Wang L, Short T, Eckardt G, Sasaki S, Vincent A, McLaren D, et al. (1996): The estrogen receptor locus is associated with a major gene influencing litter size in pigs. Proc Natl Acad Sci U S A 93, 201-205

Rozeboom KJ (2000): Extension swine husbandry: evaluating boar semen quality Anim Sci Facts ANS 00-812S, 7

Rozeboom KJ, Troedsson MH, Hodson HH, Shurson GC, Crabo BG (2000): The importance of seminal plasma on the fertility of subsequent artificial inseminations in swine. J Anim Sci 78(2), 443-448

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365 – 386

Ruan YC, Wang Z, Du JY, Zuo WL, Guo JH, Zhang J, Wu ZL, Wong HY, Chung YW, Chan HC, et al. (2008): Regulation of smooth muscle contractility by the epithelium in rat vas deferens: role of ATP-induced release of PGE2. J Physiol 586(20), 4843-4857

Rubinstein E, Ziyyat A, Prenant M, Wrobel E, Wolf J, Levy S, Le Naour F, Boucheix C (2006): Reduced fertility of female mice lacking *CD81*. Dev Biol 290, 351-358

Rubio J, Ramos D, López-Guerrero JA, Iborra I, Collado A, Solsona E, Almenar S, Llombart-Bosch A (2005): Immunohistochemical expression of Ki-67 antigen, Cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. Eur Urol 48(5), 745-751

Runge K, Evans J, He Z, Gupta S, McDonald K, Stahlberg H, Primakoff P, Myles D (2007): Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution. Dev Biol 304, 317-325

Rutten S, Morrison R, Reicks D (2000): Boar stud production analysis. Swine Health Prod 8, 11-15

Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH (2000): Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim Reprod Sci 60-61, 663-677

Safranski TJ (2008): Genetic selection of boars. Theriogenology 70(8), 1310-1316

Sales K, Milne S, Williams A, Anderson R, Jabbour H (2004): Expression, localization, and signaling of prostaglandin F2 receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the epidermal growth factor receptor and mitogen-activated protein kinase signaling pathways. J Clin Endocrinol Metab 89, 986-993

Salsabili N, Mehrsai A, Jalalizadeh B, Pourmand G, Jalaie S (2006): Correlation of sperm nuclear chromatin condensation staining method with semen parameters and sperm functional tests in patients with spinal cord injury, varicocele, and idiopathic infertility. Urol J 3, 32-37

Sathananthan AH, Ratnam SS, Ng SC, Tarin JJ, Gianaroli L, Trounson A (1996): The sperm centriole: its inheritance, replication and perpetuation in early human embryos. Hum Reprod 11(2), 345-356

Sato S, Oyamada Y, Atsuji K, Nade T, Sato S-i, Kobayashi E, Mitsuhashi T, Nirasawa K, Komatsuda A, Saito Y, et al. (2003): Quantitative trait loci analysis for growth and carcass traits in a Meishan x Duroc F2 resource population. J Anim Sci 81, 2938-2949

Saunders C, Larman M, Parrington J, Cox L, Royse J, Blayney L, Swann K, Lai F (2002): PLCz: a sperm-specific trigger of ca^{2+} oscillations in eggs and embryo development. Development 129, 3533-3544

Saunders C, Swann K, Lai F (2007): PLCzeta, a sperm specifc plc and it potential role in fertilization. Biochem Soc Symp 74, 23-26

Schell C, Frungieri MB, Albrecht M, Gonzalez-Calvar SI, Köhn FM, Calandra RS, Mayerhofer A (2007): A prostaglandin D2 system in the human testis. Fertil Steril 88(1), 233-236

Schlatt S (2003): Testicular tissue grafting: breakthrough in a long-known technique. Biol Reprod 60(1), 104

Schlenker G, Jugert L, Mudra K, Pohle M, Heinze C (1984): Semen quality of boars with differing halothane susceptibility. Monatshefte für Veterinärmedizin 39(22), 760-763

Schultz N, Hamra FK, Garbers DL (2003): A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci U S A 100(21), 12201-12206

See MT (2000): Selection for AI Stud traits, Proceeding of In: Proceedings of National Swine Improvement Federation, vol. 25, Nashville, Tennessee, USA,7–8 December, 25

Simmons D, Blotting R, Hla T (2004): Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 56, 387-437

Singleton WL (2001): State of the art in artificial insemination of pigs in the United States. Theriogenology 56, 1305-1310

Sirianni R, Chimento A, De Luca A, Zolea F, Carpino A, Rago V, Maggiolini M, AndÃ² S, Pezzi V (2009): Inhibition of cyclooxygenase-2 down-regulates aromatase activity and decreases proliferation of Leydig tumor cells. J Biol Chem 284(42), 28905-28916

Sironen AI, Uimari P, Serenius T, Mote B, Rothschild M, Vilkki J (2009): Effect of polymorphisms in candidate genes on reproduction traits in Finnish pig populations. J Anim Sci 88, 821-827

Soberman RJ, Christmas P (2003): The organization and consequences of eicosanoid signaling. J Clin Invest 111(8), 1107-1113

Sone Y, Ito M, Shirakawa H, Shikano T, Takeuchi H, Kinoshita K, Miyazaki S (2005): Nuclear translocation of phospholipase C-zeta, an egg-activating factor, during early embryonic development. Biochem Biophys Res Commun 330, 690-694

Sousa M, Mendoza C, Barros A, Tesarik J (1996): Calcium responses of human oocytes after intracytoplasmic injection of leukocytes, spermatocytes and round spermatids. Mol Hum Reprod 2, 853-857

Spotter A, Drogemuller C, Hamann H, Distl O (2005): Evidence of a new leukemia inhibitory factor-associated genetic marker for litter size in a synthetic pig line. J Anim Sci 83(10), 2264-2270

Stein K, Primakoff P, Myles D (2004): Sperm-egg fusion: events at the plasma membrane. J Cell Sci 117, 6269-6274

Stice SL, Robl JM (1990): Activation of mammalian oocytes by a factor obtained from rabbit sperm. Mol Reprod Dev 25, 272-280

Su G, Lund MS, Sorensen D (2007): Selection for litter size at day five to improve litter size at weaning and piglet survival rate. J Anim Sci 85, 1385-1392

Sun X, Funk C, Deng C, Sahu A, Lambris JD, Song W-C (1999): Role of decay-accelerating factor in regulating complement activation on the erythrocyte surface as revealed by gene targeting. Proc Natl Acad Sci USA 96, 628-633

Swann K (1990): A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster oocytes. Development 110, 1295-1302

Swann K, Saunders CM, Rogers N, Lai FA (2006): PLCζ(zeta): a sperm protein that triggers ca2+ oscillations and egg activation in mammals. Sem Cell Dev Biol 17, 264-273

Swierstra EE, Dyck GW (1976): Influence of the boar and ejaculation frequency on pregnancy rate and embryonic survival in swine. J Anim Sci 42(2), 455-460

Taylor JF, Everett RW (1985): Estimation of variance components by the expectation-maximization algorithm for restricted maximum likelihood in a repeatability model for semen production. J Dairy Sci 68(11), 2948-2953

Tegeder I, Pfeilschifter J, Geisslinger G (2001): Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 15, 2057-2072

Terman A, Kmiec M, Polasik D (2006): Estrogen receptor gene (ESR) and semen characteristics of boars. Arch Tierz Dummerstorf 49, 71-76

Thery C, Zitvogel L, Amigorena S (2002): Exosomes: composition, biogenesis and function. Nat Rev Immunol 2, 569-579

Umesiobi DO (2008): Effects of sexual restraint and stimulation of boars on fertility and fecundity rate in sows. PAS 91(1), 379–385

Virts E, Raschke W (2001): The role of intron sequences in high level expression from CD45 cDNA constructs. J Biol Chem 276, 19913-19920

Vyt P, Maes D, Dejonckheere E, Castryck F, Van SA (2004): Comparative study on five different commercial extenders for boar semen. Reprod Domest Anim 39, 8-12

Waberski D, Meding S, Dirksen G, Weitze KF, Leiding C, Hahn R (1994): Fertility of long-term-stored boar semen: Influence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. Anim Reprod Sci 36(1-2), 145-151

Walter FP (2003): Medical physiology: a cellular and molecular approaoch ISBN: 101-4160-2328-4163

Wang J, Bégin L, Bérubé N, Chevalier S, Aprikian A, Gourdeau H, Chevrette M (2007): Down-regulation of CD9 expression during prostate carcinoma progression Is associated with CD9 mRNA modifications. Clin Cancer Res 13(8), 2354-2361

Waterhouse R, Ha C, Dveksler G (2002): Murine *CD9* is the receptor for pregnancy-specific glycoprotein 17. J Exp Med 195, 277-282

Wehbrink D, Hässig M, Ritter N, Zerbe H, Bleul U, Boos A (2008): Immunohistochemical demonstration of cyclooxygenase-2 (COX-2) and prostaglandin receptors EP2 and FP expression in the bovine intercaruncular uterine wall around term. Anim Reprod Sci 106(3-4), 241-254

Wekerle L (1982): The inheritance of some boar semen anomalies. In: Allattenyésztési és takarmányozási kutatóközpont közleményei 25-28

Wichmann L, Isola J, Tuohimaa P (1994): Prognostic variables in predicting pregnancy A prospective follow up study of 907 couples with an infertility problem. Hum Reprod 9, 1102-1108

Wilson ER, Johnson RK, Wettemann RP (1977): Reproductive and testicular characteristics of purebred and crossbred boars. J Anim Sci 44, 939-947

Wilson ME (2002): Feeding and managing the boar for optimal reproductive capacity. J Anim Sci 80(2), 196

Wimmers K, Lin CL, Tholen E, Jennen DGJ, Schellander K, Ponsuksili S (2005): Polymorphisms in candidate genes as markers for sperm quality and boar fertility. Anim Genet 36(2), 152-155

Winnall WR, Ali U, O'Bryan MK, Hirst JJ, Whiley PAF, Muir JA, Hedger MP (2007): Constitutive expression of prostaglandin-endoperoxide synthase 2 by somatic and spermatogenic cells is responsible for prostaglandin E2 production in the adult rat testis. Biol Reprod 76(5), 759-768

Xing Y, Ren J, Ren D, Guo Y, Wu Y, Yang G, Mao H, Brenig B, Huang L (2009): A whole genome scanning for quantitative trait loci on traits related to sperm quality and ejaculation in pigs. Anim Reprod Sci 114, 210-218

Xu X, Pommier S, Arbov T, Hutchings B, Sotto W, Foxcroft G (1998): In vitro maturation and fertilization techniques for assessment of semen quality and boar fertility. J Anim Sci 76, 3079-3089

Yamaguchi K, Ishikawa T, Kondo Y, Fujisawa M (2008): Cisplatin regulates Sertoli cell expression of transferrin and interleukins. Mol Cell Endocrinol 283(1-2), 68-75

Yoda A, Oda S, Shikano T, Koucki Z, Awaji T, Shirakawa H, Kinoshita K, Miyazaki S (2004): Ca^{2+} oscillation-inducing phospholipase c zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. Dev Biol 268, 245-257

Yoneda A, Kashima M, Yoshida S, Terada K, Nakagawa S, Sakamoto A, Hayakawa K, Suzuki K, Ueda J, Watanabe T (2006): Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase C[zeta]. Reproduction 132(3), 393-401

Yoon S-Y, Fissore RA (2007): Release of phospholipase C [zeta] and [Ca2+]i oscillation-inducing activity during mammalian fertilization. Reproduction 134(5), 695-704

Yoshida N, Amanai M, Fukui T, Kajikawa E, Brahmajosyula M, Iwahori A, Nakano Y, Shoji S, Diebold J, Hessel H, et al. (2007): Broad, ectopic expression of the sperm protein PLCZ1 induces parthenogenesis and ovarian tumours in mice. Development 134(21), 3941-3952

Yubero N, Jiménez-Marín A, Lucena C, Barbancho M, Garrido J (2010): Immunohistochemical distribution of the tetraspanin CD9 in normal porcine tissues Mol Biol Rep DOI: 10.1007/s11033-010-0198-8

Yubero NJ-M, A. Yerle,M. Morera,L. Barbancho,M.J. Llanes,D. Garrido,J.J. (2003): Molecular cloning, expression pattern and chromosomal mapping of pig CD9 antigen. Cytogenet Genome Res 101(2), 143-146

Zamaratskaia G, Squires EJ, Babol J, Andersson HK, Andersson K, Lundstrom K (2005): Relationship between the activities of cytochrome P4502E1 and P4502A6 and skatole content in fat in entire male pigs fed with and without raw potato starch. Livest Prod Sci 95, 83 – 88

Zhu G, Miller B, Boucheix C, Rubinstein E, Liu C, Hynes R, Myles D, Primakoff P (2002): Residues SFQ (173-175) in the large extracellular loop of *CD9* are required for gamete fusion. Development 129, 1995-2002

Ziyyat A, Rubinstein E, Monier-Gavelle F, Barraud V, Kulski O, Prenant M, Boucheix C, Bomsel M, Wolf J (2006): *CD9* controls the formation of clusters that contain tetraspanins and the integrin [alpha]6[beta]1, which are involved in human and mouse gamete fusion. J Cell Sci 119, 416-424

Zou CX, Yang ZM (2000): Evaluation on sperm quality of freshly ejaculated boar semen during in vitro storage under different temperatures. Theriogenology 53(7), 1477-1488

9 Appendix

Appendix 1: Association between PLCz genotypes with sperm quality traits in the COMBINED group

Trait	PLCz genotype (μ±SE)			Effect (μ±SE)	
	AA	AC	CC	Additive	Dominance
Nr. of observations[#]	20382	17451	2831		
SCON (10^8/ml)	3.04±0.05	2.96±0.06	3.08±0.14	-0.02±0.07	0.11±0.09
VOL (ml)	256.99±4.30	261.73±4.41	260.79±10.76	-1.90±5.70	-2.84±7.15
MOT (%)	84.74±0.31	84.71±0.32	85.22±0.77	-0.24±0.41	0.26±0.51
PDR (%)	5.93±0.24	5.75±0.24	5.19±0.60	0.37±0.32	-0.19±0.39
ASR (%)	6.51±0.26	6.39±0.27	6.47±0.65	0.02±0.34	0.10±0.43

[#]repeated meansurement

Appendix 2: Association between PLCz genotypes with sperm quality traits in the PI population

Trait	PLCz genotype (μ±SE)			Effect (μ±SE)	
	AA	AC	CC	Additive	Dominance
Nr. of observations[#]	15371	11910	2065		
SCON (10^8/ml)	2.97±0.09	2.99±0.06	2.96±0.08	0.01±0.06	-0.03±0.08
VOL (ml)	246.00±6.17	244.40±4.08	245.87±5.70	0.07±4.20	1.53±5.85
MOT (%)	85.11±0.46	85.38±0.30	84.82±0.42	0.15±0.31	-0.42±0.43
PDR (%)	5.44±0.35	5.15±0.23	5.40±0.33	0.02±0.24	0.27±0.34
ASR (%)	6.33±0.38	6.22±0.25	6.39±0.35	-0.03±0.26	0.14±0.37

[#]repeated meansurement

Appendix 3: Association between COX-2 genotypes with sperm quality traits in the COMBINED group

Trait	COX-2 genotype (μ±SE)			Effect (μ±SE)	
	GG	GA	AA	Additive	Dominance
Nr. of observations[#]	9127	24031	13598		
SCON (10^8/ml)	2.98±0.08	3.04±0.05	2.99±0.06	-0.01±0.05	-0.05±0.07
VOL (ml)	260.60±6.51	257.01±3.83	261.04±5.01	-0.22±3.98	3.81±5.37
MOT (%)	84.74±0.46	84.96±0.27	84.46±0.36	0.14±0.28	-0.36±0.38
PDR (%)	5.88±0.36	5.60±0.21	5.78±0.27	0.05±0.22	0.23±0.29
ASR (%)	6.25±0.40	6.35±0.23	6.61±0.30	-0.18±0.24	0.08±0.33

[#]repeated meansurement

Appendix 4: Association between COX-2 genotypes with sperm quality traits in the PI population

Trait	COX-2 genotype (μ±SE)			Effect (μ±SE)	
	GG	GA	AA	Additive	Dominance
Nr. of observations[#]	7095	15235	7016		
SCON (10^8/ml)	2.97±0.09	2.99±0.06	2.96±0.08	0.01±0.06	-0.03±0.08
VOL (ml)	246.00±6.17	244.40±4.08	245.87±5.70	0.07±4.20	1.53±5.85
MOT (%)	85.11±0.45	85.38±0.30	84.82±0.42	0.15±0.31	-0.42±0.43
PDR (%)	5.44±0.36	5.15±0.23	5.40±0.33	0.02±0.24	0.27±0.34
ASR (%)	6.33±0.38	6.22±0.25	6.39±0.35	-0.03±0.26	0.14±0.36

[#]repeated meansurement

Appendix 5: Association between COX-2 genotypes with sperm quality traits in the PIHA population

Trait	COX-2 genotype (μ±SE)			Effect (μ±SE)	
	GG	GA	AA	Additive	Dominance
Nr. of observations[#]	679	5835	3501		
SCON (10^8/ml)	2.88±0.21	3.14±0.08	3.06±0.10	-0.17±0.14	-0.09±0.12
VOL (ml)	277.03±19.41	266.50±7.69	276.39±9.71	0.32±10.85	10.21±13.30
MOT (%)	84.48±1.37	84.63±0.54	84.34±0.68	0.07±0.76	-0.22±0.94
PDR (%)	6.22±0.97	5.91±0.38	5.93±0.48	0.14±0.54	0.16±0.67
ASR (%)	5.21±1.17	6.46±0.47	6.79±0.59	-0.79±0.66	-0.46±0.81

[#]repeated meansurement

Appendix 6: Association between CD9 genotypes with fertility traits in the COMBINED group

Trait	CD9 genotype (μ±SE)			Effect (μ±SE)	
	AA	AT	TT	Additive	Dominance
Nr. of boars	248	28	64		
NRR42 (%)	0.52±0.45	-0.63±1.19	1.24±0.83	-0.36±0.44	1.51±1.23
NBA (per litter)	0.04±0.04	0.06±0.10	-0.05±0.07	0.04±0.04	-0.06±0.11

Appendix 7: Association between CD9 genotypes with fertility traits in the PI population

Trait	CD9 genotype (μ±SE)			Effect (μ±SE)	
	AA	AT	TT	Additive	Dominance
Nr. of boars	175	18	38		
NRR42 (%)	0.32±0.55	-0.08±1.67	0.82±1.17	0.65±1.73	-0.25±0.62
NBA (per litter)	0.07±0.04	0.09±0.14	-0.05±0.10	-0.07±0.14	0.06±0.05

Appendix 8: Association between CD9 genotypes with fertility traits in the PIHA population

Trait	CD9 genotype (μ±SE)			Effect (μ±SE)	
	AA	AT	TT	Additive	Dominance
Nr. of boars	73	10	26		
NRR42 (%)	0.71±0.57	-1.70±1.33	1.74±0.93	-0.51±0.48	2.93±1.42
NBA (per litter)	-0.01±0.07	0.02±0.16	-0.02±0.12	0.01±0.06	-0.03±0.17

Appendix 9: Association between PLCz genotypes with fertility traits in the COMBINED group

Trait	PLCz genotype (μ±SE)			Effect (μ±SE)	
	AA	AC	CC	Additive	Dominance
Nr. of boars	142	178	20		
NRR42 (%)	0.71±0.54	0.28±0.56	1.21±1.30	-0.25±0.69	0.68±0.86
NBA (per litter)	-0.02±0.05	0.05±0.05	0.13±0.12	-0.08±0.06	0.01±0.08

Appendix 10: Association between PLCz genotypes with fertility traits in the PI population

Trait	PLCz genotype (μ±SE)			Effect (μ±SE)	
	AA	AC	CC	Additive	Dominance
Nr. of boars	99	118	14		
NRR42 (%)	0.69±0.68	-0.44±0.75	0.89±1.71	-0.10±0.91	1.24±1.16
NBA (per litter)	0.01±0.06	0.10±0.06	0.22±0.14	-0.10±0.08	0.02±0.10

Appendix 11: Association between PLCz genotypes with fertility traits in the PIHA population

Trait	PLCz genotype (μ±SE)			Effect (μ±SE)	
	AA	AC	CC	Additive	Dominance
Nr. of boars	43	60	6		
NRR42 (%)	0.14±0.67	1.04±0.67	1.66±1.02	-0.76±0.88	-0.14±1.08
NBA (per litter)	-0.03±0.09	0.04±0.08	-0.06±0.21	0.01±0.11	-0.09±0.14

Appendix 12: Association between COX-2 genotypes with fertility traits in the COMBINED group

Trait	COX-2 genotype (μ±SE)			Effect (μ±SE)	
	GG	GA	AA	Additive	Dominance
Nr. of boars	62	186	92		
NRR42 (%)	0.59±0.83	0.81±0.49	0.01±0.67	0.29±0.50	-0.51±0.66
NBA (per litter)	-0.04±0.07	0.03±0.04	0.02±0.06	-0.03±0.04	-0.04±0.06

Appendix 13: Association between COX-2 genotypes with fertility traits in the PI population

Trait	COX-2 genotype (μ±SE)			Effect (μ±SE)	
	GG	GA	AA	Additive	Dominance
Nr. of boars	52	124	55		
NRR42 (%)	0.12±0.97	0.70±0.63	-0.25±0.94	0.18±0.64	-0.76±0.89
NBA (per litter)	-0.03±0.08	0.06±0.52	0.07±0.08	-0.05±0.05	-0.04±0.07

Appendix 14: Association between COX-2 genotypes with fertility traits in the PIHA population

Trait	COX-2 genotype (μ±SE)			Effect (μ±SE)	
	GG	GA	AA	Additive	Dominance
Nr. of boars	10	62	37		
NRR42 (%)	2.06±1.34	0.74±0.61	0.09±0.77	0.98±0.74	0.34±0.90
NBA (per litter)	0.07±0.17	0.01±0.07	-0.03±0.09	0.05±0.09	0.01±0.11

Acknowledgments

I would like to express my greatest appreciation and my deepest sincere to Prof. Dr. Karl Schellander, director of the Animal Breeding and Husbandry group, Institute of Animal Science, University of Bonn for accepting me as a Ph.D student and giving me an opportunity to conduct scientific research in the institute. He is a great supervisor to work with, very kind, generous, enthusiastic. I am really grateful to his valuable advice, thoughtful comments and enthusiastic supervision making it possible for me to finish my study. I wish to express my sincere thanks to Prof. Dr. Brigitte Petersen, Department of Prevention Health Management, University of Bonn, member of examination committee for willingness and assistance as counterpart-supervisor of this study.

I would like to thanks Dr. Chirawath Phatsara and Dr. Elisabeth Jonas for their kind cooperation. Thanks for your collaboration, help, careful guidance and making available all facilities. I extend my sincere thanks to Dr. Dawit Tesfaye, Dr. Ulas Mehmet Cinar and apl. Univ. Prof. Dr. Christian Looft for their kind help, kept an eye on the progress of my work and always was available when I needed his advices, thoughtful comments and valuable advice during the period of this study. I am extremely grateful to Mr. Muhammad Jasim Uddin for his unlimited help, close guidance, sustainable advice during the course of my thesis work in addition to proof reading of the manuscript. I wish to thank Dr. Ernst Tholen and Ms Christine Große- Brinkhaus for their kindness and willingness to help in data analysis. I am also grateful to Ms. Maren Pröll and Ms. Christiane Neuhoff for their kind cooperation to write the German version of the abstract of this dissertation.

My sincere thanks go to other colleages at the Institute of Animal Science for helping me in one way or another to successfully accomplish this task. I would like to thank all administrative members of the Institute of Animal Science, particularly Ms. Bianca Peters and Ms. Ulrike Schröter for their kind helps with all documents. Thanks also go to Mr. Peter Müller for his really useful help in computer technique, and Ms. Nadine Leyer, Mrs. Helga Brodeßer, Birgit Koch-Fabritius, Mrs. Claudia Müller and Mr. Heinz Biörnsen for offering me everything during lab work of this work. My sincere thanks go to Mr. Bernhard Gehrig, institute of Biochemistry, University of Bonn for a western blotting technique, he is the excellent technician and very kind to give me the information of this experiment. I will never forget my beloved Muka Memedovska who used to give me many cups of best coffee in the morning and for giving the feeling of being her kindness as well as my mother at home (may her soul rest in peace).

I would like to thank Prof. Dr. Therdchai Vearasilp and Prof. Dr. Suchada Vearasilp, my former advisor at Chiang Mai University, for their encouragement and valuable advices, also supporting me for the doctoral study in Germany. I would like to express my special thanks to all of member of TSKB (Thai Students in Köln-Bonn), especially, Dr. Saowaluck Yammuen-art, Dr. Parinya Wilaiphan, Dr. Watchara Laenoi, Dr. Autchara Kayan not only for their being my superb colleagues but for teaching a basic of animal science and molecular genetic and also for many great things that they have shared with me during the years I lived far away from home. My special thanks go to Mr. Sutthiphong Ruangchante, it would be a very hard and difficult time for me accomplishing my life in Germany without him. My heartfelt gratitude and special thanks go to Mr. Chayakorn Netramai, Ms. Caroline Nehls and Ms. Greta Gandolfi for good time we had together and also for the warmth and kindness they always offer.

My deepest gratitude goes to my family for their unflagging love and support throughout my life, no words can satisfactorily express my deep feeling of thankful. I am indebted to my parents and my sister for their endless encouragement and inspiration in all my studies. They have put all their effort to create the best possible environment for me to reach at this level. I have no suitable word that can fully describe their everlasting effort.

Finally I would like to thank and appreciate all those who contribute in one way or the other and not mentioned namely to the realization of this work.