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Relationship between fertility and spermatozoal transcriptome characteristics in stallion

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DEDICATION

I dedicate this work to
my husband Bassel and my daughter Ghazal,
who have been my source of inspiration,
who continually provide their moral, spiritual and emotional support

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List of abbreviations

AI	Artificial insemination
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
BCF	Beat-cross frequency
BTB	Blood-testis barrier
CASA	Computer assessed semen analysis
CDKs	Cyclins and cyclin-dependent kinases
cDNA	Complementary DNA
cRNA	Complementary RNA
Ct	Gene expression levels
Ct_{target}	Gene expression levels for the target gene
$Ct_{reference}$	Gene expression levels for the reference gene
CS	Compound symmetry type
DAVID	Database for annotation, visualization and integrated discovery
DE	Differentially expressed gene list for experiment 3
DEG	Differentially expressed genes list for experiment 2
DEPC water	Diethyldicarbonat water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide solution mix
DTT	Dithiothreitol
EU	European union
EUR	Euro
FAK	Focal adhesion kinase
FC	Fold change
FDR	False discovery rate
FSH	Follicle stimulating hormone
FSHB	FSH beta
GnRH	Gonadotropin-releasing hormone
GRTH	Gonadotrophin-regulated testicular RNA helicase

IPA	Ingenuity pathways analysis
GCOS	Genechip operating software
GO	Gene ontology
G1	First gap phase
G2	Second gap phase
KEGG	Kyoto encyclopedia of genes and genomes
LH	Luteinizing hormone
LIN	Linearity
LSM	Least-squares means
m	Mean
M	Mitosis phase
mRNA	Messenger RNA
MAS5	Microarray suit 5
NCBI	National center for biotechnology information
One-way ANOVA	One-way analysis of variance
PBS	Phosphate-buffered saline
PLIER	Probe logarithmic intensity error
RNA	Ribonucleic acid
RBPs	RNA-binding proteins
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
R^2	Correlation coefficients
S	Synthesis phase
SAS	Statistical analysis system
SD	Standard deviation
SE	Standard errors
SEM	Standard error of mean
STR	Straightness
UN	Unstructured covariance type
UV	Ultraviolet
VAP	Average path velocity
VCL	Curvilinear velocity
VEGF	Vascular endothelial growth factor

VSL	Straight line velocity
WHO	World health organization
WOB	Wobble
WT	Whole transcript

Symbols used

Gene Symbol	Explanation/ Gene name
ACE	Angiotensin-converting enzyme
ACTB, ACTG1	Actin, gamma 1; beta actin
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
CCNT1	Cyclin T1
CD134	Homologue: tumor necrosis factor receptor superfamily, member 4
CD27	Ligand: CD70 molecule
CHK proteins	Checkpoint kinase
CSF1	Colony-stimulating factor 1
CSF3R	Colony stimulating factor 3 receptor (granulocyte)
DAZAP1	Deleted in azoospermia associated protein-1
EEF1G	Eukaryotic translation elongation factor 1 gamma
FOXO1	Forkhead box protein O1
FOXO3	Forkhead box protein O3
FOXO4	Forkhead box protein O4
FOXO6	Forkhead box protein O6
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPRT	Hypoxanthine phosphoribosyltransferase
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL13	Interleukin-13
IL17A	Interleukin 17A
IL22RA1	Interleukin 22 receptor, alpha 1
MSY2	Mouse Y-box protein 2
NcoA4	Nuclear receptor activator A4
NFAT	Nuclear factor of activated T-cells
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa
OAS2	2',5'-oligoadenylate synthetase 2, 69/71kDa

PGK2	Phosphoglycerate kinase 2
PTK2	protein tyrosine kinase 2
P53	Tumor protein P53
RPL32	Ribosomal protein L32
RPL5	Ribosomal protein L5
SAM68	Src-associated in mitosis 68 kDa
SF9	Tumor necrosis factor receptor superfamily, member 9
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SPA17	Sperm autoantigenic protein 17
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)

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1 Introduction

1.1 General background

The horse industry in the European Union (EU) is of economic importance in many European countries. Horses played an important role in the history of man and civilizations by providing the means for transport, agriculture, wars and service of utility. The number of horses has increased continuously in Europe during the last 20 years and horses are mainly used for leisure and sport activities.

The largest number of horses in the EU exists in Germany and Great Britain. In 2012, 3 500 sport-horse sires and 60 000 broodmares with 28 000 sport-horse foals were registered in Germany. Another 3 800 pony and small-horse sires, 23 000 pony and small-horse broodmares with 9 000 foals were registered in the same year (DKB Bundeschampionate, 2012). Liljenstolpe (2009) demonstrated that the annual total expenses in the German horse sector were approximated to 2.6 billion EUR and the total sales were nearly five billion EUR. Furthermore, each 3-4 horses in Germany create one fulltime job. Horse industry business-chances for farmers include feed, equipment, livery stables, training of horses and riders in equestrian sport as well as various veterinary and other equine health care services (Liljenstolpe, 2009).

Horses were domesticated first in the areas of China and Mesopotamia (Morel, 2008), and the domestication of horses was a great revolution bringing the power of horses to communications, transportation, farming and warfare.

Horses belong to the species Equus caballus that have different breeders some of which are Arabian, Quarter Horse, Thoroughbred, Tennessee Walker, Morgan, Miniature Horse, Warmblood, Andalusian, Standardbred and Icelandic Pony.

Among farm animals, horses have the lowest reproductive rate due to their breeding selection over hundreds of years because of the use of horses as war, work and sport animals. Unlike other domestic species, the selection of equines for breeding is based on their pedigree, athletic prowess and conformation characteristics with no consideration of fertility potential during selection (Colenbrander et al., 2003). However, excellent sport or race performance and noble appearance are not related to great fertility of the stallion. Therefore, reproductive traits usually have low heritability estimates and have been selected on horses by indirect ways (Taveira and Mota, 2007). Additionally, superior performing stallions with decreased fertility might be more attractive to breeders of sport horses. However, due to the subfertility of such stallions, they will result in a low number

of foals, which leads to loss of money in the equine industry because of the inability to regular production of foals. Therefore, improved fertility is desirable and the heritability of fertility values is needed for horses that are more fertile in future. In addition to the selection factor, there are many factors that led to decrease the conception rate in the herd such as maintenance of older mares and stallions due to the superior sporting performance of their progeny, and the handling and breeding of horses as sport animals, which differ from those that are exclusively used only as breeding animals.

Unlike bulls, stallions have been not selected by the artificial insemination industry for many years and generations based on semen production and sperm quality. This explains that there is a wide variation in semen characteristics among individuals and the semen quality of stallion is remarkably not sufficient (Kavak et al., 2004).

In the last decades, the use of artificial insemination in equine industry and the possibilities to transport cooled and frozen semen samples have made many changes in this industry, which offered many advantages over natural service. Some of these advantages are the safety for both stallion and mare and the reduced risk of infectious disease transmission. In addition, more insemination doses represent a higher income for the breeder of horses or artificial insemination company.

The fertility of stallion described in many races, countries and purposes is relatively low compared to other domestic animal. Stallion fertility can be determined in different ways including considering parameters reflecting breeding or reproduction success rates such as pregnancy rate, foaling rate and non-return rate, which show immense individual variations. Male fertility may also be assessed using sperm characteristics, which are also highly variable (Hamann et al., 2005).

Conventional semen evaluation is very subjective mainly based on sperm concentration and movement of the spermatozoa. Sperm morphology and progressive motility are the most commonly parameters used in the evaluation of stallion semen in both laboratories and stud farms because such evaluation of sperm motility and morphology is easy and rapid to perform (Katila, 2001). The use of different semen analysis methods, subfertile and infertile stallions would be identified and the reason for decreased conception rates may be revealed (Colenbrander et al., 2003). Moreover, molecular genetic markers associated with fertility traits may be useful as early indicator for stallion fertility and improvement programs.

1.2 Objective and statement of the problem

Horse is a seasonal polyestrous breeder with a natural breeding season lasting from April to September in the northern hemisphere. Seasons influence the reproductive system of the stallion and the reproductive activity is stimulated by long days and short nights (Palmer and Guillaume, 1992).

Fertility of stallion is of increasing importance in the horse industry with a complex environmental and genetic background. The low reproductive rate of horses is reflected in the low per cycle pregnancy rates which range from 43 - 60 % (Morris and Allen, 2002) when compared to boars 85 - 90 % (Colenbrander et al., 1993) and rams 80 - 90 % (Menzies, 1999). This low reproductive rate of stallion is due to the selection of equine for breeding according to their pedigree, noble appearance and sport performance (Colenbrander et al., 2003). In addition to the selection factor, the relatively low fertility may be influenced with breeding and care, which are different between animals used for sport and those that are exclusively used in breeding (Jackson, 1971).

Breeding values for different breeds are calculated for important traits such as sportive results, movement, conformation and health. Stallion fertility rates are influenced strongly by non-stallion factors such as breeding year and season, age of mares, type of covering (natural, artificial insemination) and type of sperm (fresh, fresh and shipped, frozen/thawed) (Hamann et al., 2005). Male fertility may also be assessed using sperm characteristics such as progressive sperm motility and sperm morphology, which are also highly variable. Prediction of fertility based on sperm characteristics has been studied in bull (Kastelic and Thundathil, 2008), pig (Gadea, 2005), ram (O' Meara et al., 2008) and human (Sigman and Zini, 2009). Sperm features evaluated included total and progressive motility (Jasko et al., 1992; Dowsett and Pattie, 1982), morphology (Jasko et al., 1990) and Deoxyribonucleic acid (DNA) quality (Morrell et al., 2008).

In stallion, attempts to find a relationship between fertility and sperm characteristics have failed or at least yielded disappointing results. Hirano et al. (2001) and Jasko et al. (1990) found a significant correlation between sperm features and fertility in stallion.

In contrast, Dowsett and Pattie (1982) and Voss et al. (1981) reported no correlation between sperm morphology, motility and fertility in stallion. Moreover, animal-based intrinsic, genetic and physiological data sets associated with male fertility are largely unknown.

In recent years, increased attention has focused on the analysis and characterization of sperm Ribonucleic acid (RNAs) and their role in the regulation of spermatogenesis, fertilization and early embryo development (Boerke et al., 2007).

Male germ cells are highly specialized cells, adapted to the functions of transport and delivery of the paternal genetic material to the female gamete. Spermatozoa contain a large population of RNAs including messenger RNA (mRNA), interference RNA, antisense RNA and micro-RNA (Hosken and Hodgson, 2014; Dadoune, 2009). In the study of Zhao et al. (2006), a large number of RNAs was detected in human spermatozoa. The quality and quantity of these RNAs as well as their potential roles in male fertility are still largely unknown.

It was first believed that RNAs in spermatozoa are remnants and their sole purpose was to transfer the paternal genome to the oocyte during fertilization (Krawetz, 2005).

The sperm transcriptome has been studied in human (Ostermeier et al., 2002), boars (Yang et al., 2009; Kempisty et al., 2008) and bull (Feugang et al., 2010; Bissonnette et al., 2009; Gilbert et al., 2007). Moreover, the utility of sperm RNA as markers for infertility has been explored (Steger, 2001; Miller, 2000) in which differences in transcript levels in sperm of different motility (Bissonnette et al., 2009; Lambard et al., 2004) as well as between normal and abnormal sperm samples (Platts et al., 2007; Steger et al., 2003), have been reported.

Krawetz (2005) demonstrated that some of the spermatozoal RNAs could be delivered to the oocyte during fertilization.

Martins and Krawetz (2005) identified protamine 2 and clusterin in zygotes using zona-free hamster egg and human spermatozoa, which were incubated together in a culture medium. These transcripts were detected in human spermatozoa and zygotes, but not in unfertilized oocytes. These transcripts could have a role in the oocyte at the time of fertilization and during early embryonic development (Bukowska et al., 2013; Carrell, 2008; Lalancette et al., 2008; Boerke et al., 2007).

Recently, Das et al. (2013) characterized the global transcriptome in the spermatozoa of fertile stallions and explored the important role of these transcripts in male fertility.

These findings provided a better concept of the biological importance of sperm RNAs allowing the identification of biomarkers of stallion fertility.

Despite the advances achieved in transcriptional analysis of spermatozoa in several species, genetic studies of stallion spermatozoa are still limited.

The aim of the present study was to characterize the seasonal variations in the quality of semen derived from fertile and subfertile stallions. On the other hand, molecular processes that are associated with different seasonal conditions as well as with male fertility were identified. The results of the present study might be helpful in prediction of stallion fertility as well as in the selection of future breeding stallions.

1.3 Aims of the study

The aim of this study is threefold:

- to evaluate different sperm quality parameters in a group of fertile and subfertile stallions during the breeding and non-breeding season in order to determine their relationship to fertility as measured by pregnancy rate and to characterize seasonal variations in stallion semen quality, namely monitoring ejaculate volume, sperm concentration, total sperm count, sperm motility and sperm morphology.
- to investigate cellular processes, functional networks and biological functions in response to different seasonal conditions by comparing microarray-derived sperm transcriptomes of stallions between breeding season (April to September) and non-breeding season (October to March).
- to identify molecular processes that are associated with male fertility by comparing microarray-derived sperm transcriptomes of stallions that were clearly assigned to either a fertile or subfertile group based on reproductive success and sperm characteristics.

2 Lecture view

2.1 Stallion semen

Stallion semen consists of seminal plasma and spermatozoa. Seminal plasma is the fluid portion of semen in which the spermatozoa is suspended. Seminal plasma is rich in nutrients, secreted by the epididymis and the accessory sex glands and mixed with spermatozoa during ejaculation.

Sperm production of the stallion depends on age and testicular volume by which extrinsic factors play an important role such as nutrition, medication, breeding season, temperature and frequency of ejaculation (Pickett, 1993).

The average volume of equine semen is 70 ml (30 - 300 ml) and the sperm concentration ranges between 30×10^6 and 800×10^6 sperm ml^{-1} (Samper, 2009).

The seminal plasma plays an essential role in the transport, protection and nutrition of the spermatozoa in the female genital tract. It affects also the environment in the female sex organs and thereby enhances the viability and fertilization capacity of the sperm cells in addition to its role as a vehicle transporting ejaculated spermatozoa through the female genital tract. Furthermore, seminal factors regulate sperm capacitation, acrosome reaction and sperm-oocyte interaction (Töpfer-Petersen et al., 2005).

Seminal plasma contains different components such as ions, energy substrates, organic compounds, free amino acids, monosaccharides, lipids, prostaglandins, polyamines and steroid hormones (Katila and Kareskoski, 2006; Töpfer-Petersen et al., 2005).

Stallion semen is composed of three identifiable fractions including pre-sperm, sperm-rich, and post-sperm fractions (Morel, 2008). The pre-sperm fraction is the initial fraction, which is watery in consistency and contains no sperm. The function of this fraction is to clean the urethra from stale urine and bacteria prior to ejaculation.

The sperm-rich fraction is the major deposit by the stallion and its volume normally ranges between 40 - 80 ml. Spermatozoa compose the main part of this fraction. However, other cells like nucleated epithelial cells of reproductive tracts, immature spermatozoa, lymphocytes, leucocytes are also found in this fraction as well as proteins, lipids, pigment particles and prostatic amyloids (Pickett, 1993). The third fraction is the post-sperm or gel fraction. Its volume varies enormously from none at all to 80 ml. The gel volume depends on breeding season, libido and age of the stallion and the function of this fraction is still unclear (Morel, 2008).

Stallion semen differs in sperm concentration and composition of seminal plasma between species and among and within individual stallions.

2.2 Sperm physiology in stallion

Stallion spermatozoon is a highly specialized cell and can be divided anatomically into two structural regions; a head and a flagellum (tail) as shown in Figure 1. The average length of equine spermatozoon ranges between 61 µm and 86 µm. This length is remarkably smaller than that of mouse with spermatozoon length of 123 µm, hamster spermatozoon with 189 µm and rat spermatozoon with 190 µm (Varner et al., 2014; Brito, 2007; Varner and Johnson, 2007). The plasma membrane overlies the total of spermatozoal structures, which consists of lipids as major component, proteins, carbohydrates and cholesterol (Langlais and Roberts, 1985).

The sperm head

Stallion sperm head is relatively flat, which is thick in the posterior portion of the sperm head (Varner et al., 2000; Johnson 1991).

In average, the length of stallion spermatozoon head ranges between 5.33 µm and 6.62 µm and its width is 2.79 µm to 3.26 µm (Brito, 2007).

The head can be subdivided into acrosomal region, equatorial segment, post acrosomal region and posterior ring, which demarcates the junction between the head and the flagellum (Varner et al., 2014; Varner and Johnson, 2007). The head contains *the nucleus*, which consists of highly condensed chromatin associated tightly with the nuclear proteins known as protamines, *the acrosome*, which plays an essential role during the fertilization process as well as *the male genome*, which consists of the X or Y chromosome and haploid number of somatic chromosomes (Evenson et al., 1995).

The contents of the nucleus are separated from the surrounding cytoplasm by the double-lipid-layered nuclear envelope. The acrosome is located between the plasma membrane and the nuclear envelope. It has two membranes; the outer and the inner membranes, which enclose the acrosomal matrix between them (Parks et al., 1987). The acrosome contains an abundant supply of active molecules such as hydrolytic enzymes, which are important for the adhesion to the zona pellucida of the oocyte and to penetrate it during the fertilization process (Brito, 2007).

After the sperm-oocyte fusion, the spermatozoa undergo the acrosome reaction in which the outer membrane of the acrosome fuses with the overlying plasma membrane to create hybrid vesicles and pores, which leads to release of hydrolytic enzymes from the

acrosome matrix. These enzymes are necessary to penetrate the zona pellucida of the oocyte during fertilization (Abou-Haila and Tulsiani, 2000).

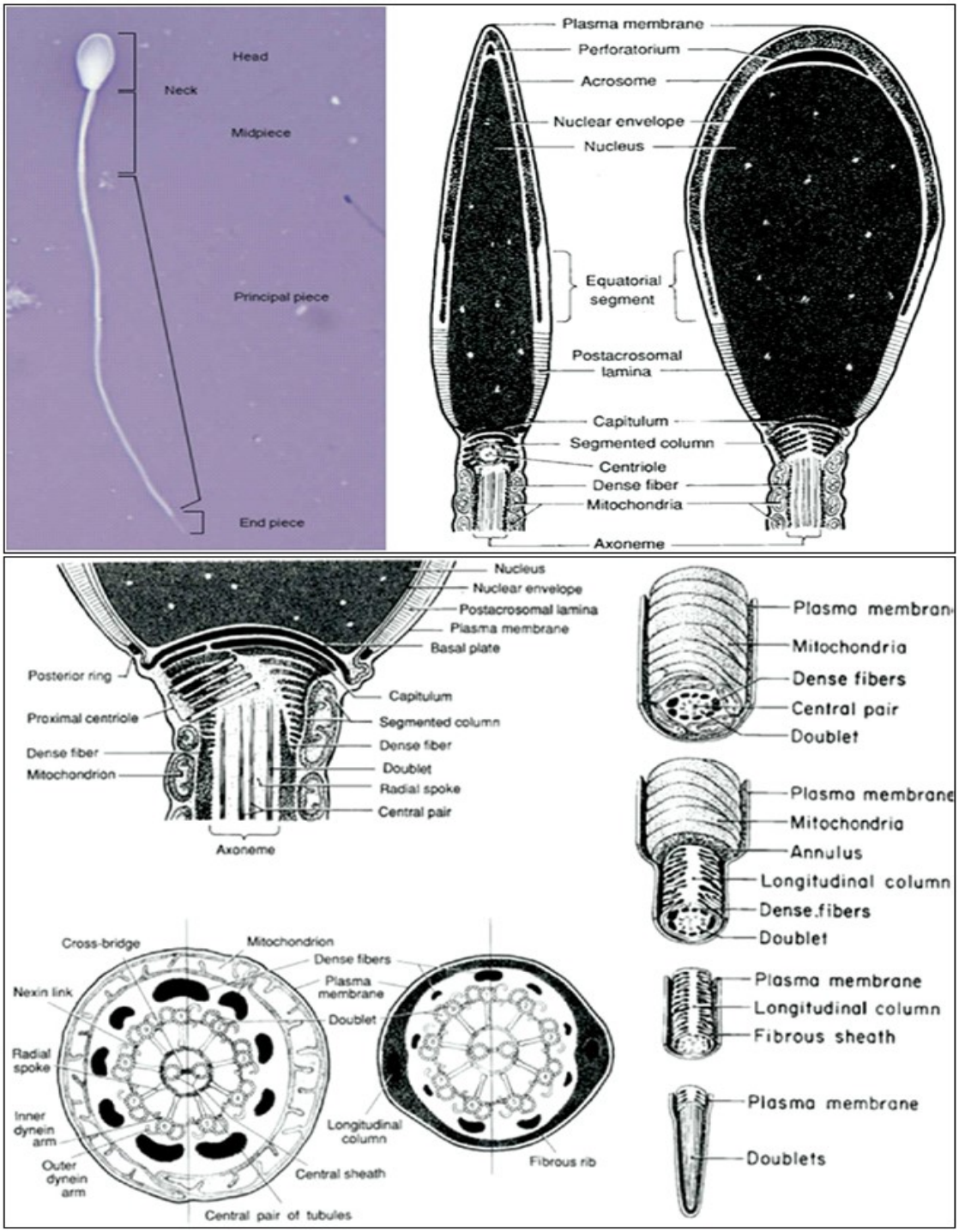

Figure 1: Structure of stallion spermatozoon (Brito, 2007; Amman and Graham, 1993) The equine spermatozoon can be divided anatomically into a head and a flagellum (tail). The sperm head is relatively flat and thick in the posterior portion of the sperm head and can be subdivided into acrosomal region, equatorial segment, post acrosomal region and posterior ring. The sperm tail is the longest part of the spermatozoon and can be subdivided into the neck or connecting piece, middle piece, principal piece and end piece

The sperm tail

The sperm tail or flagellum is the longest part of the spermatozoon and can be subdivided into the neck or connecting piece, middle piece, principal piece, and end piece (Varner and Johnson, 2007). The flagellum provides the sperm with the force, which is important to propel the sperm through the female genital tract during fertilization process (Samper, 2009). The neck is the piece, which connects the head and the middle piece and it consists of the capitulum, segmented columns and the proximal and distal centrioles, which are involved in the development of the neck and the axoneme (Amann and Graham, 1993). The middle piece is characterized with the presence of a large population of mitochondria overlying the dense fibers and the axoneme (Varner and Johnson, 2007). The equine spermatozoon contains 40 - 50 mitochondrial gyres. Mitochondria produce and store energy in the form of adenosine triphosphate (ATP) (Samper, 2009).

The produced energy in form of ATP is necessary for sperm function and sperm motility. The annulus is the connecting piece between the principal piece and the middle piece. The principal piece is composed of the axoneme and the fibrous sheath, which provides support for the sperm axoneme and contains protein kinases that are essential for sperm capacitation prior to fertilization (De Jonge and Barrat, 2006). Protein kinases regulate the intracellular Ca^{2+} during sperm capacitation and acrosome reaction and cause a rapid increase in the cytosolic calcium, which lead to membranes fusion and acrosome reaction. The end piece contains axonemal doublets, the ends of outer dense fibers as well as the end of fibrous sheath (De Jonge and Barrat, 2006).

2.3 Spermatozoa formation

Spermatozoa formation (spermatogenesis) is a complex organized process, which occurs in the seminiferous epithelium of the testis. Spermatogenesis is controlled by a chain of processes acting through endocrine, paracrine and autocrine pathways (Varner and Johnson, 2007). Spermatogenesis involves multiplication and differentiation of the germ cells and results in the formation of highly specialized mature spermatozoa. Spermatogenesis begins at puberty and continues until old age and takes approximately 64 days in humans and 40-60 days in domestic animals. In the stallion, spermatozoa formation lasts 57 days from the first division of stem cell until the formation and release of the mature spermatozoon (Johnson, 1991).

The ultimate purpose of spermatogenesis is to guarantee and maintain daily output of fully differentiated spermatozoa that ranges from > 200 million in man to 2 - 3 billion in bull (De Jonge and Barrat, 2006) and 5 billion in stallion (Gebauer et al., 1974).
Spermatozoa formation can be divided into three developmental phases:

- Spermatogoniogenesis
- Spermatogenesis
- Spermiogenesis

2.3.1 Spermatogoniogenesis

Spermatogoniogenesis is the first stage of spermatozoa formation, which takes 14.9 days in the stallion (Morel, 2008). Spermatogoniogenesis is the development of diploid undifferentiated spermatogonia into primary spermatocytes.
Spermatogonial stem cells are subdivided into A-type spermatogonia, which do not have any heterochromatin in their nuclei and B-type spermatogonia with abundant heterochromatin in their nuclei (Abou-Haila and Tulsiani, 2000).
Spermatogoniogenesis starts with the mitotic division of A1-type spermatogonia to renew their own number providing a continuous source of stem cells for continuing the process of sperm formation in future, and to produce daughter cells, which differentiate to primary spermatocytes and subsequently to spermatozoa (Samper, 2009; De Jonge and Barratt, 2006). The most A1 spermatogonia multiply to produce A2- and A3-spermatogonia, which differ in their function from the A1-spermatogonia. A3-spermatogonia, in turn, divide to produce B1- and B2-spermatogonia, which differ in their function and morphology from the A3-spermatogonia. In the last stage of spermatogoniogenesis, the B2-spermatogonia divide to produce primary spermatocytes (Morel, 2008).

2.3.2 Spermatogenesis

Spermatogenesis is the longest phase in spermatozoa formation, which lasts 18.7 days in stallion (Morel, 2008). The purpose of this process is to build a population of fully differentiated spermatozoa. In this process, B2-spermatogonia divide by mitosis to produce primary spermatocytes (Figure 2), which enter the first division of meiosis to produce spherical secondary spermatocytes (Samper, 2009; Holstein et al., 2003). The first meiotic division involves the multiplication and exchange of genetic material and results in two diploid secondary spermatocytes, which divide during the second meiotic division to produce spermatids. The resulting spermatids have an elongated cellular shape

and consists of condensed nucleus, midpiece and tail for motility (Samper, 2009; Holstein et al., 2003). In the second division, the genetic material is halved and thereby, two haploid spermatids per single secondary spermatocyte are produced. This process takes 0.7 days (Morel, 2008).

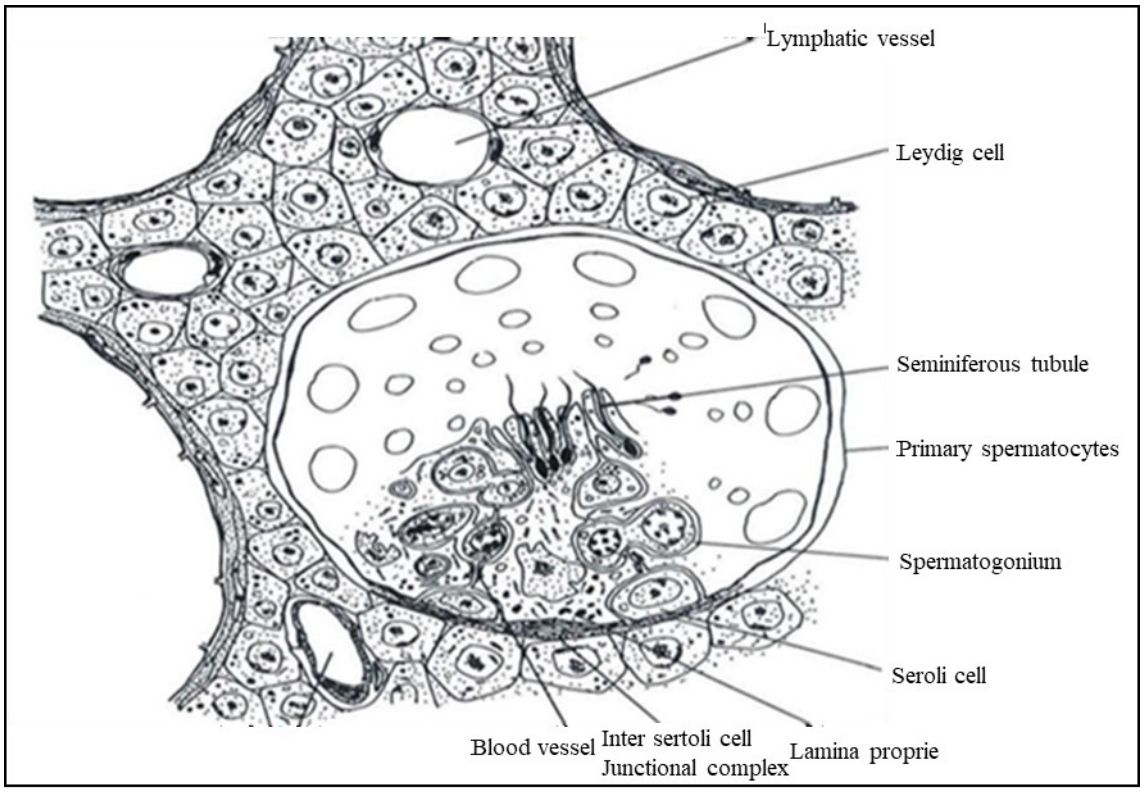

Figure 2: Across-sectional view through a seminiferous tubule within the testis of the stallion. Spermatogonial stem cells divide to produce primary spermatocytes, which enter the first division of meiosis to produce spherical secondary spermatocytes. These in turn divide during the meiotic division to produce spermatids. The differentiation and maturation of haploid round spermatid into mature sperm occur in the last stage of spermatogenesis (Morel, 2008)

2.3.3 Spermiogenesis

This process refers to the dramatic differentiation and maturation of haploid round spermatids into mature spermatozoa. This differentiation process includes cell size and cell morphology. Spermiogenesis takes 18.6 days (Morel, 2008). During spermiogenesis, the nuclear chromatin markedly condenses to about one tenth of the volume of an immature spermatid because of the largely replacement of histones first by the transition proteins and subsequently with sperm-specific-protamines. Furthermore, formation of the acrosome by the Golgi apparatus and formation and development of flagellum structures, occur in this phase (Holstein et al., 2003). Spermatid is round in form and non-motile whereas spermatozoon has an elongated shape, motile and has specialized components

and surface molecules (Holstein et al., 2003). These morphological changes are essential for the production of viable mature spermatozoa and male fertility. This process takes 9-14 days in the stallion (Brito, 2007). The motility of spermatozoa depends on the normal development of the axoneme structures, the presence of mitochondrial sheath and the implantation of the flagellum at the nucleus by the both centrioles (Samper, 2009).

2.4 RNA in sperm

Mammalian sperm cell is considered to be a dormant cell with the sole purpose of delivering the paternal genome into the oocyte during fertilization (Galeraud-Denis et al., 2007). Some reports over the past decade have provided accumulated evidence that mature mammalian sperm contain complex populations of RNAs (Carreau et al., 2007; Krawetz, 2005; Ostermeier et al., 2005; Ostermeier et al., 2002).

It was firstly believed that RNA in spermatozoa has no functions and it is simply residues of spermatogenesis reflecting the events, which occurred during their formation in the testis (Krawetz, 2005). Recently, several experiments have shown that RNA might have active functions both within the cell itself and in the oocyte after delivering during fertilization, where it remains constant until the activation of the expression of the embryonic genome. Moreover, the analysis of spermatozoal RNA could provide a deep understanding for the gene expression during male germ cell development and the events surrounding the fertilization process.

Krawetz (2005) suggested that some of the mRNA, which were detected in the sperm nucleus, are delivered into the oocyte during fertilization. Martins and Krawetz (2005) identified protamine 2 and clusterin in zygotes yielded from hamster oocytes and human spermatozoa. However, these transcripts were not detected in unfertilized hamster oocytes. The role of these transcripts is still discussed but they are involved in spermatozoal development, chromatin repackaging, fertilization as well as zygotic and early embryonic development (Bukowska et al., 2013; Carrell, 2008; Lalancette et al., 2008; Boerke et al., 2007; Miller et al., 2005; Ostermeier et al., 2004).

Recently, many researches have demonstrated that mammalian spermatozoa play more important roles than just deliver their paternal genome into the oocyte during fertilization. During spermatozoa formation, the structure of the male genome is constantly modified. Histones, transition proteins and protamines seem to play a crucial role in this process. The histones in haploid round spermatids are replaced by transition proteins.

The second step of this process occurs in elongating spermatids and involves replacement of transition proteins with sperm-specific-protamines (Griffin et al., 2019; Jha et al.,2017; Balhorn et al., 1984), which play crucial roles in DNA condensation, DNA stabilization and regulation of gene expression (Barone et al., 1994). As a result of these changes gene expression is silenced and the DNA in the nucleus starts to condense extremely. During this process, many redundant organelles are shed from the maturing spermatid, so that the spermatid obtains its elongated sperm-like shape (Boerke et al., 2007).

The absence of protein synthesis in maturing spermatids coincides with a progressive reduction in RNA content. The transcription of DNA into RNA is also canceled in spermatids just before and during the replacement of transition proteins with sperm-specific-protamines and finally the interaction of DNA with these protamines (Ward, 1993). In the later phases of spermatid differentiation, the protaminated DNA becomes super condensed and forms DNA toroid structures (Figure 3).

The purposes of such chromatin-condensation process are to facilitate the transport of spermatozoa through the female genital tract and to save the paternal genetic information as well as to protect the DNA from exogenous oxidative stressors (Griffin et al., 2019; Ni et al., 2016). Any disruption in this chromatin-condensation process will have profound effects on the integrity of DNA and subsequently on the fertilization potential and embryonic development (Griffin et al., 2019; Zhang et al., 2006).

Therefore, minimal amounts of RNA are present in these cells and the sperm nucleus is transcriptionally inert, and do not contain sufficient ribosomal RNA (rRNAs) to support translation processes (Miller et al., 1999).

New investigations using various techniques such as reverse transcription polymerase chain reaction (Saunders et al., 2007) and microarray technology (Zhao et al., 2006) discovered specific populations of RNAs in mature spermatozoa.

Sperm transcriptome has been studied in different species including human (Ostermeier et al., 2002), boars (Yang et al., 2009; Kempisty et al., 2008) and bull (Feugang et al., 2010; Bissonnette et al., 2009; Gilbert et al., 2007) and the utility of sperm RNA as markers for infertility has been explored (Steger, 2001; Miller, 2000).

The presence of different spermatozoa transcripts in different sperm population related to different motility and normal or abnormal sperm morphology could provide information about the surrounding milieu in the testis in which such sperm subpopulations are produced (Miller and Ostermeier, 2006).

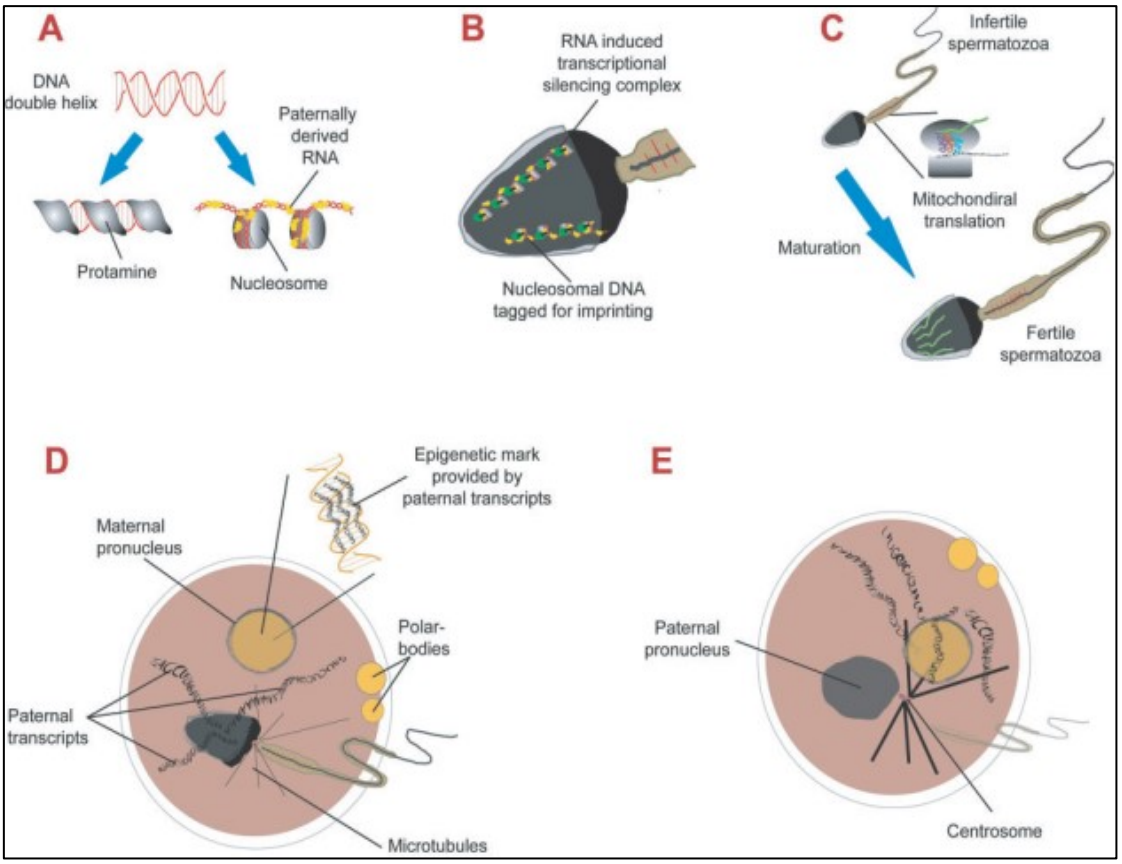

Figure 3: "Predicted functionality of spermatozoal RNAs. (A) Paternal chromatin packaging: the vast majority of DNA within the spermatozoon nucleus is packaged by highly charged protamines. (B) Imprinting: there is mounting evidence that antisense RNAs play a critical role in establishing silenced chromatin domains. (C) Sperm maturation: under certain conditions, some cytoplasmic mRNAs are apparently translated de novo, possibly on mitochondrial polysomes. (D) Spermatozoal transcripts may be functionally important to the zygote by actively promoting post-fertilization development. (E) Embryonic spatial patterning: paternal transcripts may play a key role in spatial patterning of the developing zygote" (Miller and Ostermeier, 2006)

2.5 Seasonality in horses

In a variety of domestic animals, reproduction is strongly influenced by climate change and environmental factors such as daylight and temperature in order to optimize the survival of their progeny. Domestic animals are classified as seasonal and non-seasonal breeders depending on the number of times they breed during the year. Seasonal breeders have specific period in the year in which they actively breed, whereas non-seasonal breeders can be breed throughout the year (Gerlach and Aurich, 2000).

In seasonal breeding females, ovulatory cycles occur only during one part of the year; and in the male, testicular size, sperm production and reproductive behavior are downregulated in the non-breeding season (Gerlach and Aurich, 2000).

Likewise, horses show increased reproductive activity during spring and summer months in which the temperature and daylight are very suitable and the food is available. In contrast, horses exhibit a decreased reproductive capacity in the winter months, which reflects in sexual behavior, hormonal level in the blood as well as in the quantitative and qualitative characteristics of semen and sperm (Pickett et al., 1976).

Moreover, Janett et al. (2003) reported in their study that the volume of semen, total sperm number and sperm motility of fresh stallion semen were higher in summer compared to the winter.

In addition to photoperiod, exogenous factors such as age, reproductive stage, nutrition, and environmental temperature affect the seasonal reproductive activity (Aurich, 2011).

The environmental signals such as temperature and photoperiod interact with the endogenous mechanisms in which the reproductive capacity of the seasonal breeders is affected. The most widespread environmental factors, which affects the reproductive activity is photoperiod (Gerlach and Aurich, 2000).

Melatonin is an indole amine and considered to be one of the key elements in the regulation of seasonal reproductive activity, which regulates the circannual rhythms in reproductive process (Aurich, 2011). By decreasing light, the pineal gland produces more melatonin, which inhibits the release of gonadotropin-releasing hormone (GnRH) resulting in a decrease in the gonadotropins and testicular activity (Samper, 2009). In contrast, increasing the period of light inhibits melatonin synthesis, in turn, GnRH, LH and follicle stimulating hormone (FSH) are released and testicular activity resumes (Samper, 2009).

2.6 Evaluation of stallion semen

The general aim of semen evaluation is to assess the prospects of fertility of individual stallions or individual semen samples (Malmgren, 1997; Amann and Hammerstedt, 1993; Jasko, 1992). The conventional parameters to evaluate stallion fertility are usually including either quantitative parameters such as gel and gel free volume, total semen volume, sperm concentration per milliliter and total number of spermatozoa in the ejaculate or qualitative parameters such as percentage of motile and progressive motile spermatozoa, sperm morphology, longevity of sperm motility after cooled storage and bacteriological status (Brito, 2007; Love et al., 2000; Voss et al., 1981).

Many factors such as seasons, collection technique and frequency of collection play an important role in the process of semen evaluation.

Although these evaluations of quantitative and qualitative parameters of semen provide abundant and valuable information about semen quality. However, they give only crude information about the fertility of the stallion (Samper, 2009).

2.6.1 Semen volume

Volume of stallion ejaculate depends on age, testicular volume and sperm reserve capacity of the epididymis. It is also influenced by extrinsic factors such as nutrition, temperature, breeding season, frequency of ejaculation and medication (Pickett, 1993).
The semen consists of two main parts: cellular elements 5 - 10 % and seminal plasma 90 - 95 %. The cellular elements contain spermatozoa as the main part. In addition, other cells like nucleated epithelial cells of reproductive tracts, immature spermatozoa, lymphocytes and leucocytes are found in this fraction.
Seminal plasma is secreted by the epididymis and it is rich in nutrients such as proteins, ions, amino acids, lipids, monosaccharides, prostaglandins and steroid hormones (Bladh, 2009).
The volume of an ejaculate in non-human species is determined by pouring the semen from the collection bottle into a sterile, pre-warmed and graduated cylinder after removing the gel, which can be separated immediately after semen collection or during the collection process (Samper, 2009; Juhász et al., 2000; Jasko, 1992).
In stallion, the total volume of an ejaculate should be 60 - 120 ml and the gel-free volume 30 - 100 ml (Samper, 2009; Rodríguez-Martinez, 1996). The appearance of semen gives a rough estimate of concentration. Semen with low concentration seems to be transparent in consistency when compared to the semen with high concentration, which has creamy consistency (Samper, 2009).
Color changes of the ejaculate can give an indication of the presence of abnormal cells or contamination (Lopate et al., 2003). Semen contaminated with blood has a pink-tinged to red hue. Semen contaminated with white blood cells or purulent material has a thick green to yellow appearance (Samper, 2009; Lopate et al., 2003).

2.6.2 Sperm concentration

Sperm concentration can be defined as the number of sperm per milliliter of seminal fluid and this parameter is an accurate measure of spermatogenesis. Therefore, sperm concentration is considered to be one of the most critical determinants of male subfertility (Steigerwald and Krause, 1998; Centola and Ginsburg, 1996).

In addition, determination of sperm concentration in the gel-free portion of the ejaculate is important to determine the correct amount of artificial insemination dose or to calculate the volume of sperm samples for different assays (Juhász et al., 2000).

In the stallion, concentrations of $100-350\times10^6$ ml^{-1} are common, which usually fall in the range of $50-150\times10^6$ ml^{-1} when regular semen collections are carried out. Several factors also influence the concentration of sperm in an ejaculate such as season, nutrition, medication and ejaculation frequency. Traditionally sperm concentration is determined by means of cell-counting chambers (hemocytometer) upon recommendations of the World Health Organization (WHO) (Samper, 2009). In accordance with the WHO, the improved Neubauer hemocytometer could be the chamber of preference (Varner, 2008). Alternatively, a Bürker hemocytometer can be used (Kuisma et al., 2006). An additional possibility to measure sperm concentration is the use of computer assessed semen analysis (CASA). This system can determine sperm concentration and sperm motility with sufficient accuracy (Samper, 2009).

NucleoCounter SP-100 is a new device, which has been developed to measure sperm concentration. This device counts mammalian cell nuclei stained with propidium iodide, which enters the lysed sperm cell after dilution with the SP-100 reagent. This dye is used to estimate the concentration of non-viable cells and the concentration of total cells in a suspension (Samper, 2009; Hansen et al., 2006).

2.6.3 Sperm motility

The evaluation of sperm motility is considered to be a fundamental laboratory parameter to assess the fertilizing ability of spermatozoa in an ejaculate. Moreover, sperm motility is one of the most important parameters in the establishment of correlations between sperm quality and fertility because of the important role of motility for the transport of sperm through the female reproductive tract during fertilization. Motility analysis still remains the easiest assessment and is a major component of every evaluation procedure of semen quality. Spermatozoal motility can be estimated subjectively using light microscope or objectively using automated devices.

2.6.3.1 Light microscopy

The purpose of sperm motility analysis using light microscope is to determine the percentage of total sperm motility and the proportion of progressively moving spermatozoa. Subjective motility analysis is inexpensive and simple to do in which a fixed volume of sperm droplet (2-5 µl) is delivered onto a pre-warmed clean glass slide covered

with a warm cover-slide. Then, total and progressive motility can be determined using a phase contrast microscope at a magnification of 150 - 200× (Samper, 2009). This method of sperm motility analysis is inaccurate and depends on subjective estimates of sperm motility characteristics using a microscope with a great variation caused by differences between examiners (Samper, 2009; Katila, 2001; Davis and Katz, 1993).

Furthermore, environmental conditions should be optimized for this analysis to obtain an accurate assessment of sperm motility (Katila, 2001). This means that all materials that can have contact with the semen such as microscope glass slides, pipettes for transfer the semen and coverslips should be stored in an incubator at 37 °C because of the correlation between sperm motility and temperature (Samper, 2009). Before assessment of sperm motility, the sperm sample should be extended to a concentration of $25 - 50 \times 10^6$ sperm ml^{-1} using an extender that does not alter the motility because of the strong relationship between sperm concentration and subjective evaluation of sperm motility (Van Duijn and Hendriske, 1968). In addition to that, the extension of semen inhibits the agglutination of raw sperm, which affects the movement of individual sperm especially in the case of high sperm concentration (Samper, 2009).

2.6.3.2 Computer assessed semen analysis (CASA)

Subjective evaluation of sperm motility has the disadvantage that the estimation of sperm motility can vary among the examiners. Therefore, an experienced person for the subjective assessment of sperm motility is required and recommended in order to obtain adequate results.

Several different techniques have been developed in order to obtain an objective estimation of sperm motility. The best known method for objectively sperm motility estimation is the analysis by means of CASA. This technique allows the objective evaluation of sperm motility parameters, sperm concentration and sperm head morphology (Verstegen et al., 2002). CASA has the advantages that it is capable to provide additional information that would not be attained through subjective assessments. The most important parameters measured using CASA are total and progressive motility, velocity, linearity, straightness and lateral oscillatory movement of sperm cells (Juhász et al., 2000).

These parameters may provide valuable information relative to sperm motility, which plays an important role in the assessment and prediction of male fertility (Mortimer and Mortimer, 1990; Bongso et al., 1989).

2.6.3.3 CASA parameters

CASA is an automated method that measures several specific motility parameters and provides a reliable evaluation of the fertilizing ability of the spermatozoa.

CASA describes the movements of spermatozoa and provides more detailed data on sperm motility (Youn et al., 2011). With CASA, various measures of velocity are taken and reported in micrometers per second (μm sec^{-1}) (Mortimer, 2000). These include:

- Curvilinear velocity (VCL) refers to the total distance that the sperm head covers in the observation period in micrometers per second (Figure 4).
- Straight line velocity (VSL) is determined from the straight line distance between the first and the last points of the trajectory in micrometer per second.
- Average path velocity (VAP) is the distance, which the spermatozoon has traveled in the average direction of movement in the observation period in micrometers per second.
- Amplitude of lateral head displacement (ALH) is the width of the lateral movement of the sperm head. It is calculated as the total width of the head trajectory and is expressed in micrometers (David et al., 1981).

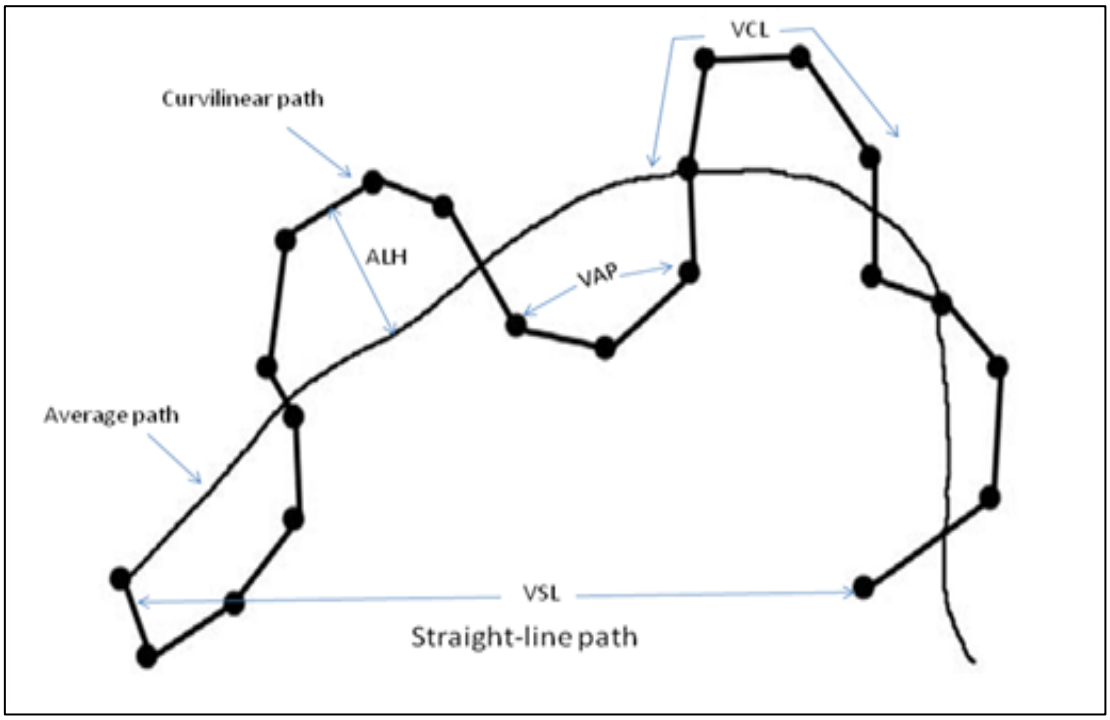

Figure 4: An illustration of different sperm motility parameters using CASA system showing average path, curvilinear path and straight path as well as VSL, ALH, VAP and VCL (WHO, 1999)

- The beat-cross frequency (BCF) is the number of times the sperm head crosses the direction of movement and is expressed in hertz. BCF is calculated by counting the number of times in which the curvilinear path of the sperm crosses its average path per second.

In addition, some values are calculated automatically using the measurements, which are measured using CASA such as:

- Linearity (LIR) is the linearity of the curvilinear path and calculated as the ratio of VSL compared to VCL (VSL/VCL) X 100.
- Straightness (STR) is the linearity of the average path and calculated as the ratio of VSL compared to VAP (VSL/VAP) X 100.
- Wobble (WOB) is the degree of oscillation of the sperm head about the average path and calculated as the ratio of VAP compared to VCL (VAP/VCL) X 100.

The importance of these parameters in association with fertility has not been extensively evaluated. However, hyperactivation of stallion spermatozoa has been described by increases in VCL and ALH (McPartlin et al., 2009; Rathi et al., 2001).

2.6.4 Sperm morphology

A major part of any breeding soundness examination is the evaluation of sperm morphology. Sperm morphology is one of the most important indicators of male fertility potential. A sperm cell considered to be normal if it confines to the criteria classifying the normal morphology of a sperm such as the shape and size of the sperm head, the neck and the tail (Haidl and Schill, 1993).

The standard evaluation of sperm morphology is performed with phase and/or light microscopy (Kenney et al., 1983). The computer assisted methods have also been used (Casey et al., 1997; Ball and Mohammed, 1995). However, available computer assisted methods can only evaluate the sperm head, but cannot evaluate morphological abnormalities of acrosomes, midpieces and tails (Kavak et al., 2004).

Sperm morphology is considered to be as excellent biomarker of sperm dysfunction and determining the source of male subfertility or even infertility (Dominguez et al., 1999; Tasdemir et al., 1997).

A variety of morphological evaluation methods and classification systems have been developed involving the visualization of a semen smear under a microscope. During such evaluation precess, the structure of sperm cell such as shape and size of the sperm head, acrosome, midpiece and tail, can be closely examined. Features of sperm morphology can

be evaluated by examining unstained, buffered-formal saline fixed suspensions or stained smears (Juhász et al., 2000). The morphology or structure of spermatozoa is typically examined with a light microscope at 1000× magnification. Standard bright-field microscope optics can be used to examine unstained samples fixed in buffered-formal saline or air dried semen smears prepared by specific stain methods. At least 100 sperm should be examined and classified.

2.6.4.1 Wet mounts without staining

Sperm morphology can be evaluated by examining wet mount preparations of unstained samples fixed in buffered-formal saline under phase-contrast microscopy. This method allows excellent visualization of sperm defects. However, the use of 1000× phase-contrast objective is recommended. The use of wet mount preparations seemed to reduce the induction of some artifacts such as detached sperm heads but increased others such as bent midpiece (Brito, 2007).

2.6.4.2 Staining methods

Many different staining methods have been used for examining sperm morphology in stallion. Unlike to the wet mount preparations, the use of staining methods facilitates the observation of some sperm defects such as acrosome defects, nuclear vacuoles and cytoplasmic droplets.

On the other hand, the use of different staining techniques could influence the outcome of morphologically normal spermatozoa (Gago et al. 1998; Meschede et al., 1993).

The most frequently used staining techniques are the Eosin-nigrosin (Dott and Foster, 1972), the Spermac (Oettle, 1986) and the Giemsa (Graham, 1996).

Eosin-nigrosin is probably the most frequently staining method used for animal semen because it is simple to use, effective and the sperm cell is clearly visualized.

Eosin is a supravital stain, which does not penetrate cells with intact membrane. Live sperm exclude the stain and appear white, whereas dead sperm or sperm with defect membrane take up the stain and appear pink in color.

Therefore, unstained sperm have intact membrane (live) and those staining red have disrupted membranes (dead) (Brinsko, 2011). Nigrosin provides a purple background, which allows visualization of unstained sperm. In addition, sperm defects will be excellent visualized (Brito, 2007).

2.7 Classification systems of sperm abnormalities

Sperm abnormalities have long been associated with male subfertility and sterility in most species depending on the type and frequency of the abnormalities. The causes of defective sperm structure may be environmental, genetic or a combination of both (Chenoweth, 2005). Sperm abnormalities impair fertilization rate, subsequent embryonic development and pregnancy outcome. A number of classification systems have been developed for sperm abnormalities, which include:

2.7.1 Primary, secondary and tertiary sperm defects

In this system, sperm abnormalities have been classified according to their presumptive origin. Primary sperm defects are testicular in origin and reflect a failure of spermatogenesis caused by pathological processes in the seminiferous epithelium (Chenoweth, 2005). Primary defect includes such defects as nuclear vacuoles, pyriform heads, microcephalic sperm, dag-like defect and mitochondrial sheath defect.

Secondary sperm defects occur during sperm maturation as well as during their passage and storage in the epididymis (Samper, 2009). An example of a secondary defect is a distal midpiece reflex.

Tertiary sperm defects are not assumed to be more deleterious to fertility than primary and secondary sperm defects. These defects could be induced by a variety of improper semen collection or handling procedure such as high ambient temperature and/or improper slide preparation for sperm morphology examination (Barth and Oko, 1989; Blom, 1977). This classification system has the disadvantage that the anatomical origin of some spermatozoal morphologic abnormalities is unknown and some defects could be considered as primary or secondary.

For example, detached sperm heads may be due to a defect of the basal plate, which connects the sperm head to the midpiece (primary) or it may be due to abnormal function of the epididymis (secondary) or it can be artifact produced by smearing the semen (tertiary) (Varner, 2008; Barth, 1994).

2.7.2 Major and minor sperm defects

In this system, sperm defects are classified as either major or minor sperm defects according to their important to fertility. Major sperm defects are considered to be associated with impaired fertility and cause early embryonic death or prevent fertilization

(Chenoweth, 2005). These defects include abnormal head, abnormal midpiece, proximal droplets and double forms, which might be have a greater impact on fertility.

Minor sperm defects are considered to be of less importance with regard to fertility such as distal plasma droplets or simple bent tail. These defects alter sperm motility and therefore, the spermatozoon can not reach the oocyte during fertilization (Samper, 2009). Minor sperm defects considered to be of minor consequence of male fertility.

However, they may cause a serious reduction in fertility when present in very high numbers (Barth and Oko, 1989; Blom, 1977).

2.7.3 Compensable and non-compensable sperm abnormalities

Another system for determining spermatozoal defect is the compensable and non-compensable sperm abnormalities. In this system, sperm defects have been classified in terms of their ability to overcome the presence of sperm abnormalities and to increase the number of spermatozoa in the dosage in order to achieve optimal fertility.

Compensable sperm abnormalities such as knobbed acrosome and bent tail, either do not reach the site of fertilization or the defective spermatozoa reach the oocyte but not capable to penetrate the zona pellucida and therefor, the cortical reaction is not induced.

Sperm defects, which lead to failed fertilization or early pregnancy loss, are termed non-compensable (Saacke et al., 2000). An example of a non-compensable defect is a defect in chromatin condensation or diadem defect (Card, 2005; Barth, 1994).

2.7.4 Classification of sperm abnormalities by recording the number of specific morphologic defects

Recently, more simple classification of abnormalities is developed, which includes seven basic categories of abnormalities including abnormal head, abnormal neck, abnormal connecting piece, abnormal tail, isolation of head and tail, duplication of any parts and deficiency of head and tail (Nishikawa, 1959).

This method of classification is considered to be superior compared to the traditional system because it reveals more specific information regarding a population of sperm. Moreover, false assumptions about the origin of sperm defects are avoided using this classification system (Varner, 2008).

In the situation where a spermatozoon has more than one defect, the Society for Theriogenology guidelines suggests the most proximal defect is identified on each spermatozoon (Kenney et al., 1990).

In this morphology evaluation system, defects are prioritized based on the assumption that certain defects are more important or more deleterious to fertility than others (Brito, 2007). When abnormalities are counted on each spermatozoon, only one defect is recorded when more than one defect is identified. Using this classification system, sperm abnormalities can be classified as following:

2.7.4.1 Acrosome defect

The acrosome is a cup-like structure, developed from the Golgi apparatus during spermatogenesis and exists on the anterior half of the sperm head.

Acrosome defects include either complete absence of the acrosome or non-specific alterations in the formation of the acrosome such as knobbed, roughed and detached acrosome. The most common defect of the acrosome is the knobbed acrosome, which appears as an excess of acrosomal matrix on the apex of the sperm head (Brito, 2007). The acrosome defect appears under microscope examination as a bead-like thickening or roughing on the sperm head apex. This defect has been associated with infertility in bulls (Barth, 1986) and rams (Soderquist, 1998). In most cases, acrosomal abnormalities occur in combination with other sperm defects, which suggests impaired spermatogenesis and subsequently subfertility or infertility in stallion (Hurtgen and Johnson, 1982) and bull (Thundathil et al., 2002; Blom and Birch-Andersen, 1962). Sperm containing knobbed acrosome either lack the ability to attach to the oocyte during fertilization or have reduced capability to do that (Chenoweth, 2005).

The knobbed acrosome can be caused by environmental factors such as stress, toxins and increased testicular temperature, but can also be of genetic origin (Chenoweth, 2005).

2.7.4.2 Head defect

Head defects include microcephalic (small, underdeveloped or dwarf), macrocephalic (large or giant), pyriform (narrow at the base), nuclear vacuoles and tapered sperm head. Sperm head defect considered to be one of the most defects found in stallion ejaculate (Brito, 2007). Sperm with tapered and pyriform heads have reduced ability to bind the zona pellucida. However, sperm with such defects do not lose their ability to penetrate the zona pellucida and to fertilize the oocytes after reaching the site of fertilization (Thundathil et al., 1999). Microcephalic and macrocephalic sperm have an uneven distribution of nuclear chromatin content after abnormal cell division (Brito, 2007).

Nuclear vacuoles are usually found at the apex of the nucleus and appear as dark dots in eosin-nigrosin stained smears and can be observed anywhere on the sperm head (Brito,

2007). Vacuolated spermatozoa have been shown to be transported normally to the oviduct and are able to penetrate oocytes, but are incompatible with embryonic development (Barth, 1994).

Many factors are involved in the incidence of sperm vacuoles such as stress, illness, feed shortage and abnormal climatic conditions (Barth and Oko, 1989).

In the stallion, nuclear vacuoles are identified in high proportion and the increase of sperm with head abnormalities has been reported to be related to decreased fertility potential (Brito et al., 2011; Janett et al., 2003). Furthermore, Jasko et al. (1990) observed a negative correlation between the percentage of sperm head defects and fertility in stallion. Love et al (2000) also observed in stallion an association between sperm head defects and fertility.

2.7.4.3 Detached sperm head

It is common to find a few detached heads in a sample of an ejaculate. However, this sperm defect might be present in very high numbers in the case of sperm accumulation in the excurrent tract (Card, 2005; Love et al., 2000; Jasko et al., 1990). The common causes for large number of spermatozoa with detached heads in a semen sample are testicular hypoplasia, testicular degeneration, senescence of spermatozoa due to sexual inactivity and sometimes specific conditions such as decapitated sperm defect (Menon et al., 2011; Brito, 2007).

2.7.4.4 Proximal and distal plasma droplet

Sperm cytoplasmic droplets are normal remnants of the spermatid residual cytoplasm, which remain attached to neck region of sperm after release into the seminiferous tubules and appear as small spherical masses attached to the neck region in the proximal plasma droplet and to the distal region of the neck in distal plasma droplet (Brito, 2007).

Sperm cytoplasmic droplets are often the most prevalent defect in the ejaculate of stallions. However, proximal cytoplasmic droplets are considered to be more associated with impaired fertility than the distal cytoplasmic droplets (Card, 2005; Love et al., 2000; Jasko et al., 1990). Sperm with proximal plasma droplet are not capable to bind and penetrate the zona pellucida during fertilization. Therefore, proximal cytoplasmic droplets are classified as major defects (Brito, 2007).

In stallion, Jasko et al. (1990) found a negative correlation between the percentages of proximal cytoplasmic droplet and per cycle pregnancy rates, which was greater than the correlation with distal droplets.

In boars, the proportion of spermatozoa with distal cytoplasmic droplet in stored semen had a negative correlation with pregnancy rates (Waberski et al., 1994).

2.7.4.5 Midpiece defect

Midpiece defects include distal midpiece reflex, segmental aplasia of the mitochondrial sheath, dag-like defect, pseudo-droplet defect, corkscrew midpiece defect, disrupted sheet and stump tail (Brito, 2007).

The most common midpiece defect is distal midpiece reflex, which appears in light microscopy as a bend in the distal region of the midpiece and the dag-like defect, which appears as folding of the midpiece or the entire tail with partly or completely loss of the mitochondrial sheet. In the case of disrupted spermatogenesis in bull, a small percentage of distal midpiece reflex have been observed (Brito, 2007).

In stallion, segmental aplasia of the mitochondrial sheet might be observed in a low percentage in varying degrees; some sperm lack a small part of the sheet, whereas others seem to miss the mitochondrial sheet completely (Brito, 2007). This defect creates a point of structural weakness because of the tendency to fracture when the sperm acquires motility.

Pseudo-droplet defect was characterized by a local thickening on the midpiece. This defect is also more likely to be irregular in shape and more visually dense than droplets (Chenoweth, 2005).

The tail stump is a rare genetic defect and was first reported in bulls (Williams and Savage, 1925). It has also been observed in mouse, rabbit, dog, stallion and man (Barth and Oko, 1989). This defect consists in an anomaly of development of the distal centriole and affects 80 % to 100 % of sperm causing sterility (Chenoweth, 2005).

This defect might occasionally be observed in a low percentage of sperm in stallions. Close examination using light microscopy reveals that the tail is replaced by a small stump (Brito, 2007).

2.7.4.6 Sperm tail defect

Simple coiled tail with or without retention of cytoplasmic material is among the most common sperm defects and results in impaired motility. In this defect the tail is folded forming a loop at the point where it is bent (Chenoweth, 2005). Sometimes, a cytoplasmic droplet is trapped in the loop. This defect is usually associated with distal midpiece reflex. Simple coiled tail defect appears to develop during the store of spermatozoa in the

epididymis and might be caused by exposure of the sperm to abnormal secretions in the epididymis (Brito, 2007).

Furthermore, hypotonic or cold shock may cause a similar type of bend without a trapped droplet. Urine contamination of the semen may also induce hypotonic shock, which consequently led to a bent midpiece and/or principal piece (Barth, 1994).

Love et al. (2000) observed no correlation between simple coiled tail defect and fertility in stallion.

2.7.4.7 Duplicated heads or tails

Duplications in sperm include two or more tails on a single sperm head as well as two or more heads with one tail. Duplication of the tail is an uncommon defect that is associated with duplication of the implantation fossa and replication of the distal centriole. Sperm with multiple heads and tails might have normal head structure with normal DNA content. However, abnormalities of nuclear shape and abnormal DNA condensation in one or more heads can be observed. These sperm originate from multinucleated spermatids and the duplications of sperm head might be the result of incomplete cell dissociation during spermatogenic divisions (Brito, 2007).

Figure 5 represents drawings of normal and abnormal sperm morphologic features, as viewed by differential-interference contrast microscopy of fixed and unstained wet-mount sperm.

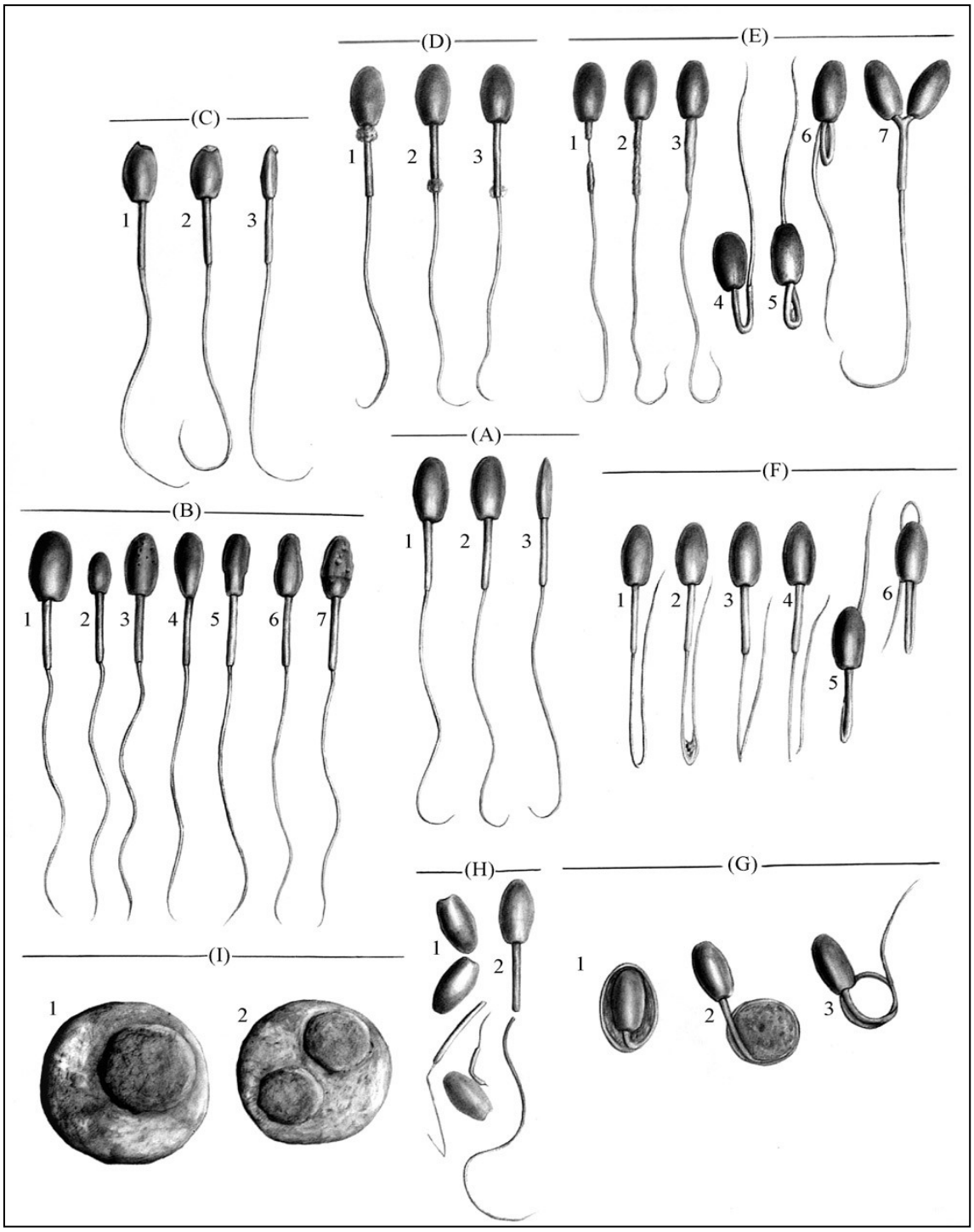

Figure 5: "Normal and abnormal sperm morphologic features (A): normal sperm; (B): Abnormal head morphology (macrocephalic B1, microcephalic B2, nuclear vacuoles or crater defects B3, tapered head B4, pyriform head B5, hour-glass head B6, degenerate head B7; (C): Acrosomal defects /knobbed acrosome; (D): cytoplasmic droplets (Proximal cytoplasmic droplets D1, distal cytoplasmic droplets D2 and D3); (E): Midpiece abnormalities (segmental aplasia of the mitochondrial sheath E1, corkscrew defect E2, enlarged mitochondrial sheath E3, bent midpiece E4, E5, and E6, double midpiece/double head E7); (F): tail defects (bent principal piece F1, F2, F3, and F4, single bend involving the midpiece-principal piece junction F5, double bend involving the midpiece-principal piece junction F6); (G): Coiled tail; (H): Fragmented sperm /detached heads or tailless heads; (I): Premature germ cells, round spermatids" (Varner, 2008)

2.8 Fertility of the stallion

2.8.1 Fertility date

Fertility of stallion is of economic importance for the horse industry. Evaluation of stallion fertility potential is an important part of breeding management. Unlike other domestic species, the selection of equines for breeding is based on their pedigree, athletic prowess and conformation characteristics with no consideration of fertility potential during selection (Colenbrander et al., 2003).

Stallion fertility is most accurately defined by mating/inseminating a high number of mares, evaluating pregnancy rate and finally measuring the outcome of foals in relation to the number of mares mated (Rodríguez-Martínez, 2003). However, this method is time consuming and expensive. Furthermore, many factors such as management of the stallion (nutrition, housing, semen collection, semen processing and storage), management of the mares (optimal time of insemination, reproductive status and conditions of the mares) as well as handling and processing techniques of the semen samples may have a large impact on the success of insemination and overall fertility of the stallion (Bedford-Guaus, 2007). Fertility of stallion can be characterized using five parameters including foaling rate, per season pregnancy rate, per cycle pregnancy rate, first cycle pregnancy rate and non-return rate. *Foaling rates* can be defined as the percentage of foals born out of the total number of mares bred in the previous year. This rate is often considered the ultimate measures of stallion fertility, but the use of this parameter has several disadvantages. It does not take into account the number of cycles that are needed to get the mares pregnant and it is markedly influenced by quality of management (McDowell et al., 1992). Furthermore, foaling rate is influenced strongly by non-stallion factors such as breeding year and season, age of mares, type of covering (natural, artificial insemination) and type of sperm (fresh, fresh and shipped, frozen/thawed) (Hamann et al., 2005).

Per season pregnancy rate is total number of mares pregnant divided by the total number of mares bred during the year. Seasonal pregnancy rate is used to evaluate stallion fertility. However, this rate is strongly influenced by the reproductive status, veterinary and housing management of breeding mares (Love, 2011). Furthermore, this rate should be above 85 % in well-managed herds. (Card, 2010).

A relatively high seasonal pregnancy rate can be achieved with multiple mattings in several cycles during the breeding season, whereas *first cycle pregnancy rate* is more sensitive indicator of stallion fertility than seasonal pregnancy rate, which can be defined

as the number of mares pregnant to the first breeding of the year divided by the total number of mares pregnant in that year (Love, 2011). The use of this rate instead of the all cycles or seasonal rates will remove some of the factors attributable to mare subfertility.
A quicker and most practical way of assessing fertility is to examine the *per cycle pregnancy rate*, which is the total number of mares pregnant divided by the total number of breeding cycles. Detected pregnancy by ultrasound or later by healthy foal born or early embryonic loss and abortion are also counted as a positive pregnancy result in this system. Moreover, this parameter has the advantage that the result is available in a shorter interval and gives indication of fertility during the breeding season (Colenbrander et al., 2003). *The non-return rate* is the percentage of mares that did not return for breeding within a specific time after last insemination (Van Buiten et al., 1999).
Van Buiten et al. (1999) reported that non-return rate at 28 days is a valuable parameter for the assessment of stallion fertility. However, the disadvantage of this rate is that mares may not be returned for mating for another reasons than becoming pregnant.

2.8.2 Relationship between sperm quality and male fertility

The general aim of semen evaluation is to assess the prospects of fertility of individual stallion or individual semen samples (Samper, 2009).
Conventional laboratory tests for assessment of spermatozoal quality in relation to male fertility can be divided into three groups of parameters including *quantitative and qualitative parameters*, which include sperm concentration in ml, total sperm number in the ejaculate, total and progressive motility, proportion of morphological abnormal sperm and live / dead ration; *parameters describing function of accessory sex gland*, which include ejaculate volume, gel free volume, PH value of the ejaculate; and *parameters describing sexual behavior and libido of the stallion* for example number of jumps, time required for mating and semen collection. In these cases, the correlation between sperm quality and fertility is not proven. However, the prediction of fertility based on quantitative and qualitative parameters has been studied extensively in male of several species and their correlation to male fertility has given conflicting results (Pesch et al., 2006; Colenbrander et al., 2003; Amann and Hammerstedt, 1993).

2.8.2.1 Sperm motility and stallion fertility

Sperm motility is commonly believed to be one of the most important characteristics for evaluating the fertility potential of ejaculated spermatozoa. Evaluation of spermatozoal

motility in both raw and extended forms is considered to be a fundamental laboratory test for assessing the fertilizing capacity of spermatozoa in an ejaculate (Varner, 2008).

Good progressive sperm motility is an indicator of both unimpaired metabolism and intactness of membranes (Johnson et al., 2000).

Using frozen stallion spermatozoa, Krik et al. (2005) found that progressive motility and straightness of the sperm were significantly correlated to first cycle fertility and no single parameter was significantly correlated with seasonal fertility. In contrast, Pesch et al. (2006) reported no significant correlation between total and progressive motility and fertility. Jasko et al. (1992) identified in their study the relationship between conventional semen quality parameters and fertility in stallion. Although, they found a weak correlation between the percentages of motile, progressively motile and morphologically normal sperm with fertility.

2.8.2.2 Sperm morphology and stallion fertility

Sperm morphology is recognized as an excellent biomarker of sperm dysfunction and it has been shown to be the most stable parameter and has the advantage of being predictive of fertility success. Evaluation of sperm morphology is part of the stallion breeding soundness evaluation, which provides clinician invaluable information for assessing the breeding soundness of a stallion and the potential fertility of individual semen samples.

In stallion, attempts to correlate the percentage of morphologically normal or abnormal spermatozoa with fertility have given conflicting results. Jasko et al. (1990) reported that the rate of spermatozoa with normal morphology correlates positively with fertility to various degrees, while Dowsett and Pattie (1982) and Voss et al. (1981) did not find any relationship between sperm morphology and fertility.

In addition, Kavak et al. (2004) reported that many morphological sperm abnormalities might be acceptable for normal stallions.

In the study of Einarsson et al. (2009), it was demonstrated that highly fertile stallions in regular use usually have a low frequency of morphologically abnormal spermatozoa.

3 Materials and methods

3.1 Experimental design

Experiments were performed using eight healthy warmblood stallions which belong to two different insemination centers in Germany. A total of 64 ejaculates were collected from the stallions. Each ejaculate was assessed individually for volume, sperm concentration, sperm motility and sperm morphology. The individual assessment of each ejaculate is found in Appendix I.

The aim of the first experiment was to evaluate different semen quality parameters in a group of fertile and subfertile stallions namely monitoring ejaculate volume, sperm concentration, total sperm number, sperm morphology as well as CASA derived sperm movement characteristics during the breeding and non-breeding season in order to investigate semen quality parameters associated with high fertility in stallion as measured by pregnancy rate and to characterize the seasonal variations in these semen quality parameters between breeding and non-breeding seasons.

For this study, eight stallions with known fertility were chosen. Animals were divided into two groups based on their pregnancy rate (total number of mares pregnant divided by the total number of mares bred during the year): group 1, fertile stallions (80-90 % pregnancy rate, n= 4; stallion A, B, C, D) and group 2, subfertile stallions (40-60 % pregnancy rate, n= 4; stallion E, F, G, H).

A total of 64 ejaculates (4 per animal in breeding season, 4 per animal in non-breeding season) were conventionally analyzed.

After semen collection, semen samples were assessed individually for motility using CASA and for morphology using eosin-nigrosine stain. Semen quality parameters associated with high fertility were identified and the seasonal variations in semen quality parameters for both fertile and subfertile stallion groups was determined.

After that, RNA was isolated from spermatozoa of each stallion and the gene expression analysis was performed using two different approaches.

The first gene expression approach in the second experiment included expression analysis depending on the different seasonal conditions in which the sperm samples were collected. The aim of the first gene expression in this experiment was to investigate cellular processes, functional networks and biological functions in stallion in response to different seasonal conditions associated with increase of daylight and temperature during the breeding season. This approach was performed by comparing the microarray-derived

sperm transcriptomes of the stallions (A, B, E, F) as the breeding season group and the stallions (C, D, G, H) as non-breeding group. The stallions were so divided in two groups for this gene expression analysis to insure that the fertility of stallions used has no effect on this analysis. Therefore, each season group consists of two fertile and two subfertile stallions. However, the collection of semen was performed in breeding season for the first group and in non-breeding season for the second group.

The isolated RNA from sperm of each stallion was used to prepare the microarray probes. The principal component analysis was used to detect transcript with different abundance between breeding and non-breeding season.

A 1.5-fold difference in transcript abundance was set as the threshold among differentially expressed genes (DEG). Finally, the associations between the genes were evaluated using Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) and Ingenuity Pathways Analysis (IPA) (www.Ingenuity.com) to identify functional networks, biological functions and significant canonical pathways associated with the differentially expressed genes.

The second gene expression approach in the third experiment included expression analysis depending on the different fertility between the stallion groups. The aim of this experiment was to identify molecular processes that are associated with male fertility by comparing microarray-derived sperm transcriptomes of stallions that were clearly assigned to either fertile group (stallions A, B, C) or subfertile group (stallions E, F, G) based on reproductive success and sperm characteristics. Semen samples sused for this experiment were the samples, which were collected in breeding season for both fertile and subfertile stallion groups to ensure that season has no effect of this analysis.

The isolated RNA from sperm of each stallion was used to prepare the microarray probes. Afterwards, the analysis of variance (one-way ANOVA) was performed to detect transcripts with different abundance between the fertile and subfertile stallions.

A 1.2-fold difference in transcript abundance was set as the threshold among differentially expressed genes (DE). Finally, the associations between the genes were evaluated using DAVID and IPA to identify biological functions and significant canonical pathways associated with the differentially expressed genes as explained in Figure 6.

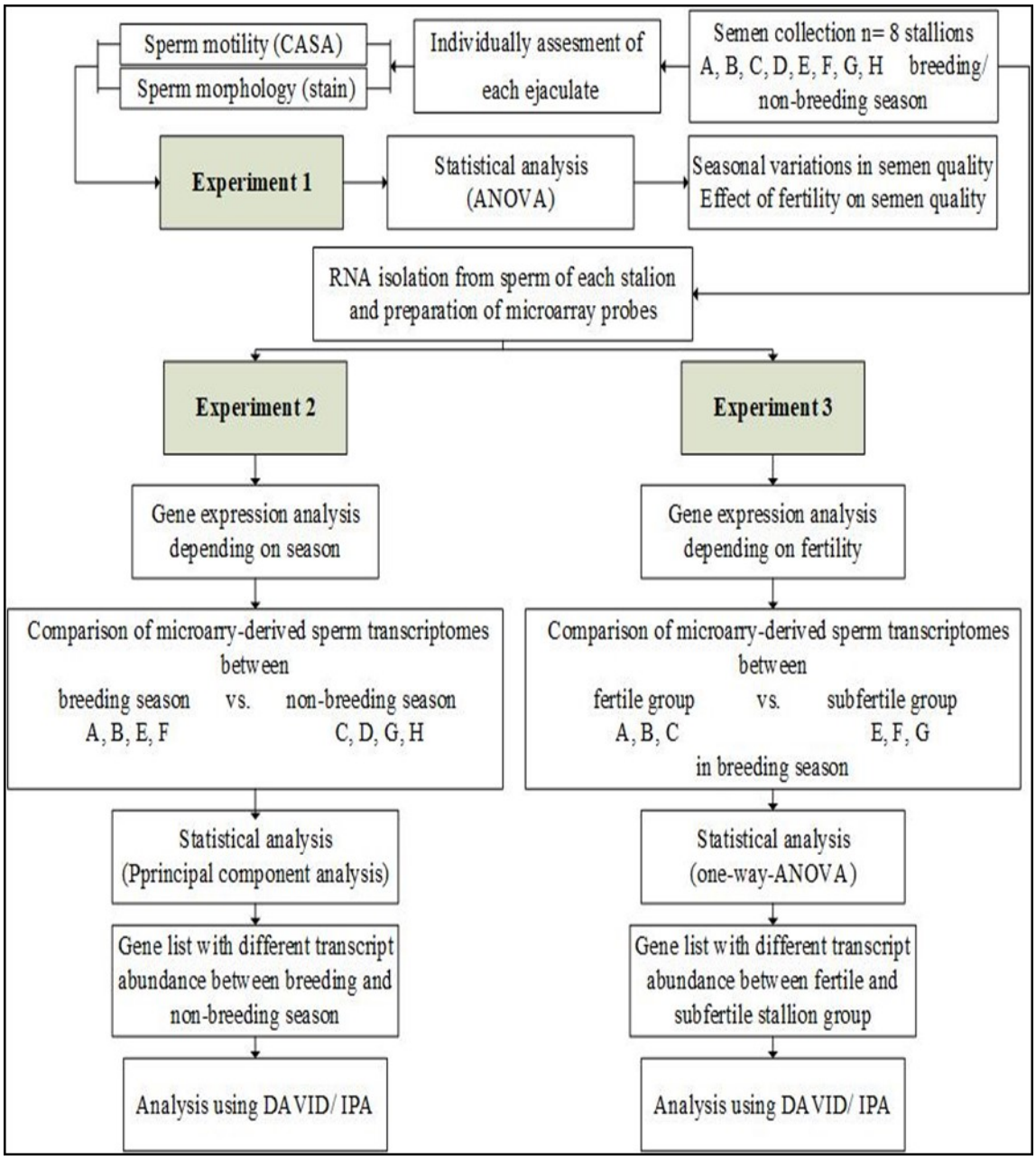

Figure 6: Experimental design

3.2 Semen collection and preparation of semen samples

All animal procedures were performed in relation to the general terms and conditions of the EU-stallion station of different German state studs. No particular ethical issues had to be considered because the semen samples used in the present study had been collected as part of routine breeding procedures. The stallions were properly housed and fed and their surroundings were kept in sanitary conditions in accordance with the requirements to operate an artificial insemination (AI) station within the EU. Experiments were performed using eight healthy warmblood stallions (Hanoverian, Mecklenburg and Oldenburg

Warmblood). The age of the stallions ranged from seven to 12 years. Stallions were selected from more than 150 active stallions with a minimum covering number of 10 mares per season. Available seasonal breeding reports were analyzed by the stud manager examining results after AI with extended fresh semen.

All stallions were part of a regular semen collection regimen during the breeding and non-breeding period. A total of 64 ejaculates were collected from the stallions (eight ejaculates from each one; four in breeding season and four in non-breeding season) for all experiments as described in experimental design.

All stallions were collected 2 - 3 times within the week prior to sample collection. Semen samples were collected using an artificial vagina (Hannover model, Minitüb, Germany) by having the stallion mount a phantom mare. After each stallion was collected, the filter containing the gel from the ejaculate was removed and the volume of the ejaculate was determined by pouring the semen from the collection bottle into a pre-warmed graduated cylinder and the sperm concentration was measured using a photometer (Minitüb, Germany). Then total sperm number in gel-free semen was estimated by multiply the semen volume and sperm concentration.

As soon as an ejaculate is collected, the semen samples were diluted using an appropriate pre-warmed extender (Minitüb, Germany) to a constant concentration of 100×10^6 sperm ml^{-1}. Shortly following semen dilution, the extended semen was divided into 10 ml tubes, loaded into transport container and cool transported at 4 °C to the laboratory in Dummerstorf.

3.3 Stallion semen processing and examination

3.3.1 Sperm motility

Stallion sperm motility was estimated using Computer Assessed Semen Analyzer (CASA, Minitüb, Germany). CASA allows fast, accurate and objective measurement of motion characteristics taken from tracks of large numbers of spermatozoa. Various measures of total and progressive motility ratio as well as sperm velocities including VCL, VSL, and VAP in micrometers per second (µm sec^{-1}) were taken and reported with CASA. In addition to sperm velocities amplitude of ALH in micrometers, BCF in hertz, linearity (LIN), straightness (STR) and wobble (WOB) are measured and reported by CASA.

For motility analysis, diluted semen was further diluted in the laboratory to obtain sperm concentration of 25 - 50 x 10^6 sperm ml^{-1} using the same extender, which was used in the collection station to dilute the semen samples before being transported to the laboratory. After that diluted semen samples were stored at room temperature for 10 - 15 min. Then approximately 2.8 µl of fresh semen was loaded into a single pre-warmed Leja chamber with a 10 µm depth and then the slide was placed on a stage 37 °C. This was subsequently analyzed under a microscope (Nikon SMZ800, Japan) using X40 objective. A total of 15 microscopic fields and a minimum of 1000 sperm cells were examined per semen sample.

3.3.2 Sperm morphology

Sperm morphology was assessed using eosin-nigrosin stain (Nidacon, Sweden). The percentages of morphologically normal sperm and percentages of sperm with specific morphological defects were determined using a microscope (Nikon UFX-DX, Japan) with (× 1000) fold magnification. Semen smears were prepared by mixing 20 µl semen with the same volume of the eosin-nigrosin stain. After that, the mixture was put on glass slide. To make the stained smear, a second slide held at 30 - 40 º angle is needed, which was pushed against the drop of stained semen and pulled back slowly. Finally, the smear let then air dried. A total of 100 sperm cells in each ejaculate were examined and classified.

Sperm abnormalities were classified by recording the number of specific morphologic defects into: acrosome defect (knobbed, roughed and detached acrosome), sperm head defect (microcephalic, macrocephalic, pyriform, nuclear vacuoles and tapered sperm head), detached sperm head, midpiece defect (distal midpiece reflex, segmental aplasia of the mitochondrial sheath, dag-like defect, pseudo-droplet defect, corkscrew midpiece defect, disrupted sheet and stump tail), proximal plasma droplet, distal plasma droplet, tail defect (simple coiled tail with or without retention of cytoplasmic material) and head or tail duplications.

Some of normal and abnormal sperm morphology features from own laboratory work is showed in Figure 7. Semen smears were prepared as described above and the spermatozoa were analyzed at random on different areas of the slide. As shown in Figure 7, the equine sperm cell is divided anatomically into a head and a flagellum (tail). *Normal sperm* head is flattened and thicker in the posterior part of the head (Figure 7 A). The most common *acrosome defect* is the knobbed acrosome, which consists of an excess of acrosomal matrix located at the apex of the sperm head (Figure 7 B). *Head defects* include:

macrocephalic (large or giant) (Figure 7 C1), microcephalic (small, underdeveloped or dwarf) (Figure 7 C2), extremely abnormal shape (Figure 7 C3), and pyriform (narrow at the base) (Figure 7 C4). *Detached sperm head* (Figure 7D) it is common to find a few detached sperm heads in a sample of an ejaculate.

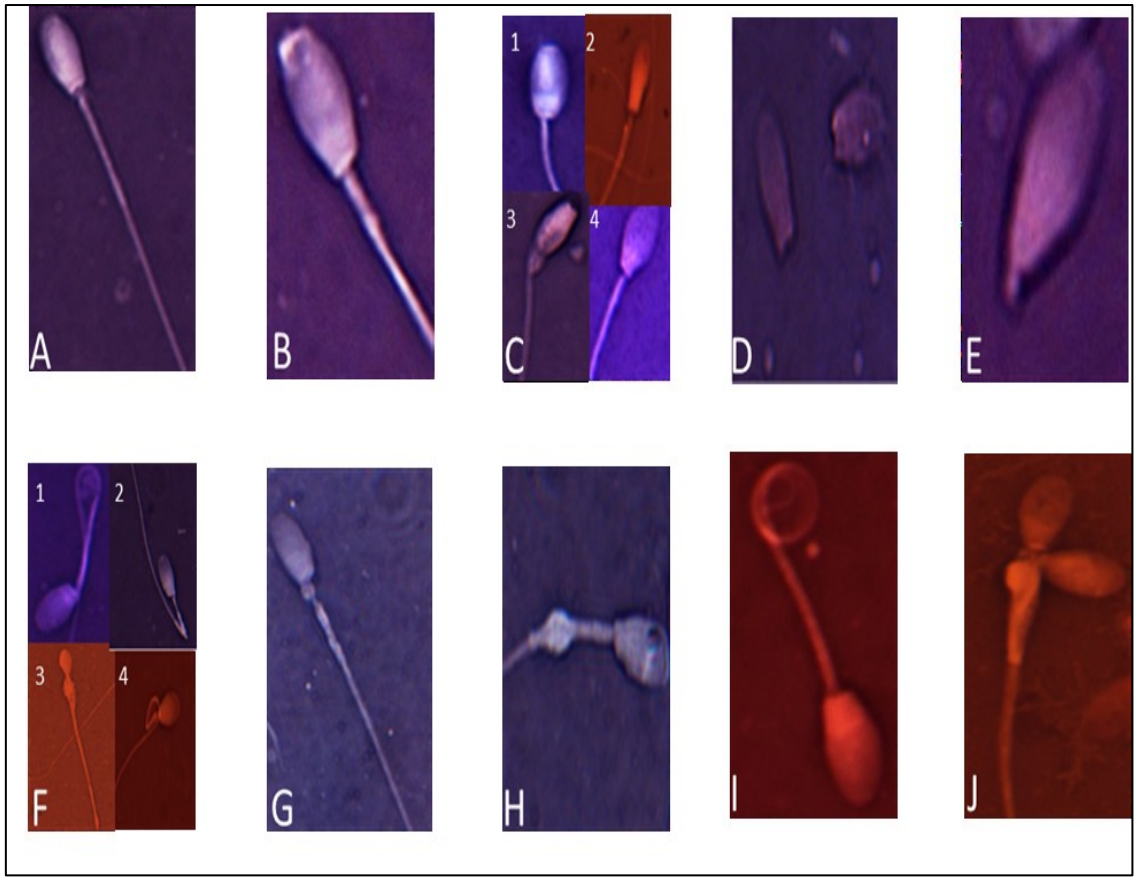

Figure 7: An overview of common abnormalities in stallion sperm morphology. Stained smears were prepared using eosin-nigrosin stain and the analysis was performed with (× 1000) fold magnification

The tail stump (Figure 7 E) is defect, which consists in an anomaly of development of the distal centriole. Close examination using light microscopy shows that the tail is replaced by a small stump. This defect considered in this study as midpiece defect. *Midpiece defects* include: bend midpiece with retention of cytoplasmic droplet (Figure 7 F1). The most common midpiece defect is distal midpiece reflex, which appears as a bend in the distal region of the midpiece (Figure 7 F2), corkscrew midpiece defect this defect consist in abnormalities of the mitochondrial sheet (Figure 7 F3). Coiling of the midpiece without retention of cytoplasmic material (Figure 7 F4). *Proximal plasma droplet* defect appears as small spherical masses attached to the neck region of sperm (Figure 7 G). *Distal plasma droplet* defect appears as small spherical masses attached to the distal region of the neck

(Figure 7 H). *Tail defects* including simple coiled tail with or without retention of cytoplasmic material are the most common sperm tail defects. In the Figure 7 I is simple coiled tail without retention of cytoplasmic material. *Duplication* of sperm head includes two or more heads on a single sperm tail (Figure 7 J).

3.4 Gene expression analysis using Affymetrix microarray GeneChip

3.4.1 Total RNA isolation from stallion spermatozoa

RNA is easily degraded due to the broad distributions of RNases in the environment. Care and attention should be taken both during and after the isolation of RNA to avoid degradation of RNA isolated from stallion spermatozoa.

Prior to RNA extraction, the lab bench, pipettes and gloves were cleaned with RNaseZap solution to avoid any RNase contamination during RNA extraction.

Total RNA of spermatozoa was extracted with TRIzol® RNA isolation reagent (Invitrogen), according to the manufacturer's protocol with minor modifications. Sperm samples were thawed on ice and then centrifuged for 5 min at 5 000 xg at 4 °C. The supernatant was discarded and each sperm pellet resuspended with 3 ml hypotonic solution (99 ml DEPC water supplemented with 0.5 ml 0.1 % SDS and 0.5 ml 0.5 % Triton X-100). Samples were incubated for 15 min on ice for lysis of somatic cells.

After a centrifugation step of 5 min at 5 000 xg at 4 °C, the hypotonic solution was discarded and each pellet was washed three times with phosphate-buffered saline (PBS). The samples were mixed in TRIzol (1 ml for 100×10^6 sperm in suspension) by aspirating and releasing 20 - 30 times with a 27-gauge needle attached to a 5 ml syringe.

After 15 min of incubation at room temperature (with vortexing every 5 min) the homogenized samples were centrifuged at 12 000 xg for 10 min at 4 °C. This resulting pellet consists of insoluble material such as membranes, polysaccharides and high molecular weight DNA.

The supernatant, containing the RNA, was transferred to a fresh tube, which is already refilled on 1 ml TRIzol. Afterwards 200 µl of chloroform was added to each sample. The samples were homogenized by vortexing for 30 secs and incubated for 5 min at room temperature. This was followed by centrifugation at 12 000 xg for 15 min at 4 °C. Following centrifugation, the RNA remains in the aqueous phase (top phase), DNA in the interphase and proteins in the organic phase (bottom phase).

The aqueous phase in each sample was transferred to a fresh tube with care taken not to touch the DNA interphase and mixed with one volume isopropanol (100 %), and 1 µl glycogen. The samples were incubated for 30 min at room temperature to precipitate the RNA and then centrifuged at 20 000 xg for 30 min at 4 °C.

The supernatant was removed and the pellets washed three times with 75 % ethanol. Samples were centrifuged at 12 000 xg for 5 min at 4 °C. The ethanol was discarded and the pellets dried 5 to 10 min at room temperature. For each RNA sample, the RNA pellet was dissolved in 20 µl water and a heating step for 15 min at 58 °C was performed. Samples were then frozen at -80 °C until further analysis.

3.4.2 RNA concentration and quality assessment

3.4.2.1 Quantification of RNA by NanoDrop spectrophotometer

The concentration of RNA samples was determined by measuring the absorbance at 260 nm (A260) in Nano-Drop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). A conversion factor of 40 was used for RNA and 50 for DNA.

Measuring A260 is a reliable way to determine RNA concentration. Using this method, the contamination of RNA sample with genomic DNA, protein or phenol can be detected. RNA and DNA absorb at 260 nm, proteins and phenols absorb light at 280 nm, and carbohydrates and salts absorbs at 230 nm. The ratio of 260/280 provides an estimate of the purity of RNA. Pure RNA has a ratio 260/280 of 1.8 to 2.1. A lower ratio indicates the presence of phenols, proteins or other contaminants that absorb at or near 280 nm.

The 260/230 ratio is another measure of purity, a ratio below 2.0 indicates contaminants that absorb at 230 nm. Since all RNA samples were dissolved and diluted in RNase free water then this ratio is determined in water. 260/230 ratio between 1.5 and 2.0 are considered to be sufficiently pure for further experiments.

3.4.2.2 Purity assessment of spermatozoal RNA by real time-PCR (RT-PCR)

Genomic DNA contamination of the samples was tested by RT-PCR (Light cycler 480, Roche, Germany). RNA cannot serve as templates for RT-PCR. Instead, RNA must be converted into complementary DNA (cDNA) in order to detect genomic DNA contamination of RNA samples. This can be performed by a reverse transcription reaction using the reverse transcriptase enzyme.

cDNA synthesis

Firstly, all components needed to synthesize cDNA from total RNA that were stored at -20 °C were put on ice to thaw before use. Two reverse transcription master mixes must be prepared and stored on ice. Table 1 shows the volume of each component needed to prepare each reverse master mix.

Table 1: Components of the reverse transcription master mixes and the volume in µl for one reaction

Master mix (1)		**Master mix (2)**	
Component	Volume [µl]	Component	Volume [µl]
random hexamer primer (0.2 µg μl^{-1}, Fermentas)	2.5	5XFirst Strand buffer (Invitrogen)	4
Oligo (dt)13 Primer (0.5 µg μl^{-1})	1	DTT (0.1M, Invitrogen)	1
RNase inhibitor (40 u μl^{-1}, Promega)	1	dNTPs Mix (10mM, Invitrogen)	1
RNase free water	7	RNase free water	0.5
1 µl of total RNA	1	SuperScript III Reverse Transcriptase (200 u μl^{-1}, Invitrogen)	1
Total	12.5		7.5

The reaction for cDNA synthesis was carried out in 20 µl reaction volume. Firstly, 1 µl total RNA was mixed well with the first master mix to yield 12.5 µl mixture followed by mixing the sample well by pipetting up and down a few times and then the tubes were sealed properly. After sealing each tube, the tubes were briefly centrifuged to remove any air bubbles from the samples. Then, the tubes were incubated at 68 °C for 5 min and put on ice for 5 min. After that, the second reverse master mix (7.5 µl) was added to the first master mix, which contains the RNA, and the sample was mixed well by pipetting up and down for a few times and incubated at 25 °C for 5 min. After that, samples were incubated at 50 °C for one hour and at 75 °C for 15 min. The thermal cycler program used to perform the reverse transcription reactions is shown in Table 2. Finally, cDNA samples were stored at -20 °C until use.

Table 2: Reverse transcription thermal cycler program for cDNA synthesis

	Step (1)	**Step (2)**	**Step (3)**	**Step (4)**	**Step (5)**	**Step (6)**
Temperature	68 °C	on ice	25 °C	50 °C	75 °C	4 °C
Time	5 min	5 min	5 min	1 h	15 min	∞

Quantitative RT-PCR

The purity of RNA samples was further checked by RT-PCR. RT-PCR enables quantification of the PCR products in real time during each PCR cycle. The PCR products are labelled and detected by using a fluorescently tagged substrate during the amplification process.

The SYBR technology was used to label and detect RT-PCR products. SYBR is an intercalating dye that has a fluorescent effect when binding to double-stranded DNA. Reactions for RT-PCR were performed in a final volume of 12 µl using 96-well plate. RT-PCR master mix was made using the components listed in Table 3.

Table 3: Reaction mix for RT-PCR

Component	Volume per well [µl]
Primer forward (CGGGAGCTACTACCGCTACA)	0.6
Primer reverse (GCCTTCTGCATCTTCTCCTC)	0.6
Kapa SYBR Fast (KAPA BIOSYSTEMS, Germany)	6
Distilled water	2.8
cDNA	2
Total	12

The protamine 2 gene was targeted to detect the presence of genomic DNA contamination in RNA samples (Das et al., 2010). After that, 10 µl from the prepared RT-PCR master mix was pipetted to each reaction well following addition of 2 µl of cDNA templet. In addition to cDNA samples, a negative control (2 µl distilled water instead of cDNA in only one well) was performed to ensure that no DNA contaminations exist in the water that used to prepare RT-PCR master mix, and a positive control (2 µl equine genomic DNA instead of cDNA in only one well) was made to facilitate the detection of DNA in RNA samples. The thermal cycling conditions for RT-PCR are showed in Table 4.

Table 4: Thermal cycling conditions of RT-PCR

Step	Temperatur	Time
Initial denaturation	95 °C	10 min
3-step cycling (45 cycles)		
Denaturation	95 °C	15 s
Annealing	60 °C	10 s
Extension	72 °C	15 s

Agarose gel electrophorese

RT-PCR products were separated via 2 % agarose gel electrophorese. Gel electrophoresis is a technique used to separate DNA fragments according to their size. The gel was prepared by dissolving 1 g agarose gel in 50 ml 1 x TAE buffer. The suspension was then heated until all the agarose was melted. Then 2 µl ethidium bromide was added to the gel agarose solution to visualize the DNA. After the gel was melted, the suspension was poured into a horizontal gel chamber. Each DNA sample was mixed with 0.5 volume of agarose gel loading buffer before loading into the wells of solid agarose gel. A 100 bp DNA ladder was used as a molecular weight standard. One well in the gel is reserved for a negative control, which contains all essential components of the amplification reaction except the template. The use of negative control enables the detection of contamination with foreign DNA in the sample tested. Another well is also reserved for positive control, which consists of a segment of equine genomic DNA of known size. Finally, electrophoresis was performed in 1 x TAE buffer at 120 V cm^{-1} and the ethidium bromide-DNA complexes were visualized under UV light. This method allowed the detection of genomic DNA contamination in RNA samples. After RNA purity assessment, all RNA samples, which belong to each individual stallion, were polled together and stored at -80 °C for later use in microarray approach.

3.4.3 Microarray experiments and data analysis (for experiment 2 and 3)

For microarray experiments, samples of each of the eight stallions were hybridized on catalogue genome-wide Equine Gene 1.0 ST Array covering 30 559 transcript clusters (Affymetrix, Santa Clara, CA, USA).

For each hybridization, 500 ng of total RNA was reverse-transcripted into cDNA using the GeneChip® WT PLUS Reagent Kit (Affymetrix) and this was transcripted into complementary RNA (cRNA). The hydrolysis of cRNA was performed to generate biotin-labeled antisense cDNA target according to the manufacturer's instructions.

The biotin-labeled antisense cDNA targets were fragmented and individually hybridized onto microarrays. After staining and washing steps, the arrays were scanned and raw data were obtained using Affymetrix GeneChip operating software (GCOS 1.1.1).

The microarray raw data were quality controlled by the microarray suite 5 (MAS5) and normalized by the probe logarithmic intensity error (PLIER) algorithm using Affymetrix Expression Console 1.1 software (Affymetrix, St. Clara, CA, USA).

According to the MIAME standard, the microarray data has been deposited in the database of the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

3.4.4 Bioinformatics analyses of differentially expressed genes using software tools

To identify gene-associated molecular functions that were overrepresented in the gene lists generated by comparative analysis of gene expression between breeding and non-breeding season (DEG) and between fertile and subfertile stallions (DE), DAVID and IPA software tools were used in this study. These tools enabled the extraction of biologically meaningful information from the long list of differentially expressed genes.

3.4.4.1 Identification of overrepresented functions using DAVID

To identify overrepresented functions of genes whose transcripts were significantly differentiated between breeding and non-breeding season as well as between fertile and subfertile stallions, gene lists (DEG and DE) were submitted to DAVID.

DAVID functional classification tool uses 14 functional annotation sources to create similarity matrix between target genes. A set of fuzzy classification algorithms is used to group genes based on their co-occurrences in annotation terms and ranks the gene groups using an internal (EASE).

The gene functional classification tool in DAVID builds clusters of genes with significantly similar ontology as tested against the whole list of genes in the equine genome array. The grouping of genes based on functional similarity can systematically enhance biological interpretation of the large gene list obtained by the comparative gene expression analysis between breeding and non-breeding season in experiment 2 and between fertile and subfertile stallions in experiment 3.

3.4.4.2 Identification of biological functions and canonical pathways using IPA

The associations between the genes were further evaluated using the ingenuity pathway analysis to identify significant biological functions and canonical pathways associated with the DEG and DE genes. This analysis is very useful since it is known that genes do not work alone but have networks of interactions and their effects are combined. Canonical pathway analysis identified the pathways from the IPA library that were most significant to the data set.

Genes from the submitted list that were associated with a canonical pathway in the IPA knowledge base were considered for the analysis.

The significance of biological functions and canonical pathways was determined by Fisher's exact test with a cut-off set at 0.05. p-values from Fisher's exact test were adjusted for multiple testing with the Benjamini-Hochberg multiple testing corrections. Canonical pathways were also ordered by the ratio value, which is the number of genes in a given list that mapped to the pathway divided by total number of genes that make up that pathway.

3.4.5 Validation of the microarray results using RT-PCR assay

3.4.5.1 Primer design

Primers play an important role for the success of validation experiments. Optimal primer design is critical for efficient amplification of target sequences. In this study four different genes from the most affected biological functions (OAS1, OAS2, IL13 and IL22RA1) were selected and validated using RT-PCR. Two reference genes (RPL32 and HPRT) were used to normalize the data.

The primers were designed using mRNA accession number and the web site of NCBI. The primers used were ordered from Sigma-Aldrich®. The list of primer sequences is provided in Table 5.

Table 5: Primers used for Validation of microarray results; F forward; R reverse primer

Gene Symbol	Gene Name	Primer Sequence 5´-3´	mRNA Accession Number
RPL32	ribosomal protein L32	**F**: GGAGGTGCAGCCATCTACTC **R**:GCGCACCCTATTGTCAATGC	CX_594263.1
HPRT	hypoxanthine phosphoribosyl transferase	**F**: GCGTCGTGATTAGTGATGATGAA **R**:TGATGGCCTCCCATCTCCTT	AY_372182
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	**F**: TATCTCTTGCCAGACACACGG **R**:GAGCCACCCTTCACCACTTTG	NM_001082489
OAS2	2',5'-oligoadenylate synthetase 2, 69/71kDa	**F**: AGAACCAGGCCCGTGATCTTG **R**:GCACACTCCGGATGACATTTCT	NM_001081773
IL13	interleukin-13	**F**: CTCAGCCGGGCAGGTTTCTA **R**:CCACATGCTTTCCACCGTGA	NM_001143791
IL22RA1	interleukin 22 receptor, alpha1	**F**: TCGCTGCAACACACTACCAT **R**:CGTGGAGCTCTAAGCGGTAG	XM_001501288

The designing of primers is very important and so that needs to do very carefully. There are several factors that need to be considered during designing of primers such as:

- elimination of primer-dimers due to complementary sequence between primers
- melting temperature should be between 58 °C and 60 °C
- the differences between the melting temperature of the forward and reverse primers should be less than 2 °C
- primer sequences length should be short 18 - 27 bp
- low GC content, which ranges between 50 - 65 %

3.4.5.2 Reaction setup and relative quantification of expression levels

To quantify differences in the expression level of the selected genes from experiment 3 between fertile and subfertile stallions, relative quantification method was used. To obtain accurate relative quantification of mRNA target, it is also recommended to evaluate the expression level of an endogenous control. To reach this goal two common housekeeping genes (RPL32 and HPRT) were selected and used to normalize the data.

Firstly, PCR master mix was made for each target and reference gene using the components listed in Table 3.

RT-PCR for each target and reference gene was prepared and purified to remove primers excesses and nucleotides using PCR purification kit (Qiagen) according to the manufacturer's instructions.

A dilution series of RT-PCR product was carried out to obtain the six reference points of the standard curve (10^1, 10^2, 10^3, 10^4, 10^5 and 10^6). The RT-PCR reaction was performed in a final volume of 12 µl using 96-well plate. 2 µl of each dilution was then added as cDNA templet, in triplicate, for each gene in the reaction plate. Finally, the plate was covered with an adhesive cover and centrifuged at 4 000 xg for 30 secs at 4 °C to spin down the content and to remove air bubbles.

The thermal cycling conditions for RT-PCR are shown in Table 4. The plates were then placed in the light cycler system (Light cycler 480, Roche, Germany) for amplification and melt curve analysis. During the RT-PCR run, the fluorescence was monitored providing an amplification curve. Gene expression levels were listed as Ct values that match the number of cycles where the fluorescence signal was detected above the threshold line.

In this study, the expression level of mRNA was measured using relative quantification. This method determines the changes in steady-state mRNA level of the target gene and

expresses it relative to the level of the reference gene. Expression level was calculated by comparing the Ct value between target gene and reference gene to obtain normalized Ct value $\Delta Ct = Ct_{target} - Ct_{reference.}$ Then t-test was used to compare the gene expression between the fertile and subfertile stallion groups using the normalized Ct value for each stallion in both groups; $\Delta Ct_{group\ (1)} = Ct_{target} - Ct_{reference\ median}$ versus $\Delta Ct_{group\ (2)} = Ct_{target} - Ct_{reference\ median}$ ($Ct_{reference\ median}$ is calculated as the average of Ct_{RPL32} and Ct_{HPRT}). Differences between the groups were considered significant at $p \leq 0.05$.

3.4.5.3 RT-PCR amplification efficiency

In order to define the RT-PCR efficiency the standard curve method was used in which a gene-specific standard curve was generated for each gene using a RT-PCR amplicon quantified by spectrophotometry (260/280) converted into number of molecules and serially diluted to produce the six reference points of the calibration curve (10^1 - 10^6 copies). For each dilution, Ct values were plotted against the log value of the input DNA. The highest quality PCR run with an efficiency of 2.0, meaning that the number of target molecules doubles with every PCR cycle. The correlation between Ct and the log of transcript copy number per PCR was described by the coefficient of determination, denoted R^2 (1.0 indicated perfect correlation).

To reliably compare gene expression between treatments, equal amplification of RT-PCR reactions is one important criterion. The correlation coefficients (R^2) and slope values can be obtained from the standard curve (Efficiency= $10^{(-1/slope)} - 1$).

The RT-PCR efficiency for the target and the reference genes should be between 90 % - 110 % (a slope between -3.1 and -3.6).

3.5 Statistical analysis

Statistical analysis for the first experiment were performed using the statistical analysis system (SAS) software, Version 9.3 for Windows. Copyright, SAS Institute Inc., Cary, NC, USA. Descriptive statistics and tests for normality were calculated with the UNIVARIATE procedure of Base SAS software.

The data of the semen quality parameters were evaluated by repeated measurement ANOVA using the MIXED procedure of SAS/STAT software.

The model included the fixed effects fertility (fertile group, subfertile group), season (breeding season, non-breeding season) and the interaction fertility*season. Repeated measures (season and ejaculate) on the same stallion were taken into account by the

REPEATED statement of the MIXED procedure using an unstructured covariance type (UN) for season and a compound symmetry type (CS) for ejaculate to calculate the blocks of the block diagonal residual covariance matrix as UN@CS where @ is the direct product.

Least-squares means (LSM) and their standard errors (SE) were computed for each fixed effect in the model, and all pairwise differences between LS-means were tested by the Tukey-Kramer procedure.

The SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LS-means for the two-way interactions. Effects and differences were considered significant if $p < 0.05$.

In the second experiment the principal component analysis (PCA) was used to detect transcript with different abundance between breeding and non-breeding seasons.

The data from microarray experiment is considered high dimensional data and contains a large number of interrelated variables.

PCA takes the high dimensional data and produces a new data set called principal components (PCs), which consists of fewer variables than the original data set, but retaining as much as possible of the information. Herewith, it is possible to deduct the biological meaning of the data and the complexity of the analysis can be reduced.

The uncorrelated PCs are extracted by linear transformation of the original variables using the statistical analysis system (SAS) software, Version 9.3 for Windows, so that the first two PCs (PC1 and PC2) retain most of the variation present in all of the original variables.

In PCA, each variable (gene) is represented by a point and the distances between the points preserve the distances between the high dimensional expression profiles.

After that, the genes are grouped into more manageable homogeneous variance-dominating PCs.

The PCA was performed on this variance-dominating PCs again using the criteria $p < 0.05$, $FC > 1.5$ to identify genes that are significantly differentiated between breeding and non-breeding seasons.

One-way analysis of variance (ANOVA) was performed to detect transcript with different abundance between fertile and subfertile stallions in experiment 3 using the statistical analysis system (SAS)/ Genetics software, Version 9.3 for Windows. Copyright, SAS Institute Inc., Cary, NC, USA.

ANOVA is a hypothesis-testing technique, which can handle minor violation of the normality, as well as some differences in variances between the treatments.

ANOVA used to test the hypothesis that all means of treatments are equal, assuming homogeneity of the variance. Homogeneity of variance tests the null hypothesis H_0, which assumes that there are no differences between two or more variances as well as the H_1 hypotheses, which assumes that there are differences between the variances.

In all cases, p value ≤ 0.05 are considered as statistically significant. When the level is significant $p \leq 0.05$, the H_0 hypothesis should be rejected and the H_1 hypothesis should be supported.

4 Results

4.1 Experiment 1: Investigation of semen quality parameters associated with high fertility in stallion and the seasonal variations of these parameters between breeding and non-breeding seasons

4.1.1 Investigation of semen quality parameters associated with high fertility in stallion as measured by pregnancy rate

To investigate whether stallions presenting different fertility rates (as measured by pregnancy rate) also differ in their semen quality profile, spermatozoal qualitative and quantitative parameter were compared between two groups of stallion, which differ in pregnancy rate (fertile group 80 - 90 % vs. subfertile group 40 - 60 %) during the breeding and non-breeding seasons.

As shown in Table 6 no significant differences were observed in ejaculate parameters including ejaculate volume, sperm concentration and total sperm number between the fertile and subfertile stallion group in both breeding and non-breeding seasons.

Table 6: LS-Means (LSM±SE) of measured parameters of fresh semen for ejaculates collected from fertile and subfertile stallions during breeding and non-breeding season

Ejaculate parameter	Breeding season		Non-breeding season	
	Fertile group (n= 4)	Subfertile group (n= 4)	Fertile group (n= 4)	Subfertile group (n= 4)
Ejaculate volume [ml]	45.6±4.8	43.6±4.8	30.5±3.4	27.4±3.4
Sperm concentration [10^6 ml^{-1}]	275.3±55.9	233.2±55.9	373.3±50.4	347.1±50.4
Total sperm number [10^9]	12.02±1.9	9.5±1.9	10.6±1.4352	9.5±1.4

Parameters for sperm morphologic characteristics of fertile and subfertile stallions in breeding and non-breeding seasons are summarized in Table 7. The percentage of morphologically normal spermatozoa was significantly higher in fertile group when compared with subfertile group (67.1±4.9 vs. 37.7±4.9, $p < 0.05$), respectively. This significant difference was observed only in breeding season and only in the proportion of morphologically normal spermatozoa.

No significant differences in morphologic characteristics were found between fertile and subfertile stallions in non-breeding season.

Based on motility analysis using CASA system for fertile and subfertile stallions in breeding season, there were significant differences in total and progressive motility between fertile and subfertile stallions (80.97±3.6 vs. 61.5±3.6 and 68.2±3.7 vs. 46.5±3.7, $p < 0.05$), respectively as presented in Table 8.

Table 7: LS-Means (LSM±SE) of morphological parameters of spermatozoa for ejaculates collected from fertile and subfertile stallions during breeding and non-breeding season

Morphology parameter [%]	Breeding season		Non-breeding season	
	Fertile group (n= 4)	Subfertile group (n= 4)	Fertile group (n= 4)	Subfertile group (n= 4)
Normal sperm	67.1±4.9[a c]	37.7±4.9[b]	47.3±6.02 [d]	30.2±6.02
Acrosome defect	3.5±1.4	6.4±1.4	3.9±1.05	4.6±1.05
Head defect	4.1±1.4	5.5±1.4	3.6±1.6	6.3±1.6
Detached sperm head	1.6±0.9	3.8±0.9	2.5±1.95	4.4±1.95
Midpiece defect	16.8±5.2	33.5±5.2	31.1±5.7	44.3±5.7
Proximal plasma droplet	3.3±1.8	4.8±1.8	5.4±2.7	3.7±2.7
Distal plasma droplet	1.3±1.7	4.0±1.7	1.3±1.2	3.1±1.2
Tail defect	1.9±0.7	3.7±0.7	4.4±1.2	2.9±1.2
Duplications	0.4±0.2	0.6±0.2	0.4±0.2	0.4±0.2

Means with different letters (a, b between fertile and subfertile groups); (c, d between breeding and non-breeding seasons) are significantly different ($p < 0.05$)

In addition, there is a difference in velocities of sperm movement VAP and VCL, which were significantly higher in fertile group in comparison to subfertile group (77.4±2.3 vs. 65.8±2.3 and 129.2±4.1 vs. 111.2±4.1, $p < 0.05$), respectively. Furthermore, no significant differences in VSL, STR, LIN, WOB, ALH and BCF were observed between fertile and subfertile stallions in breeding season. In non-breeding season, no significant differences were found between fertile and subfertile stallions in all CASA derived sperm movement characteristics as shown in Table 8.

Table 8: LS-Means (LSM±SE) of CASA derived sperm movement parameters of spermatozoa for ejaculates collected from fertile and subfertile stallions during breeding and non-breeding season

Motility parameter	Breeding season		Non-breeding season	
	Fertile group (n= 4)	Subfertile group (n= 4)	Fertile group (n= 4)	Subfertile group (n= 4)
Total motility [%]	80.97±3.6[a]	61.5±3.6[b]	64.4±4.7	45.8±4.7
Progressive motility [%]	68.2±3.7[a]	46.5±3.7[b]	48.2±4.4	27.9±4.4
VAP [µm s^{-1}]	77.4±2.3[a c]	65.8±2.3[b c]	57.4±3.8[d]	49.2±3.8[d]
VCL [µm s^{-1}]	129.2±4.1[a]	111.2±4.1[b]	99.5±7.3	86.4±7.3
VSL [µm s^{-1}]	55.9±2.7[c]	46.8±2.7[c]	39.9±3.4[d]	32.1±3.4[d]
STR [%]	71.4±1.8	70.4±1.8	68.1±2.2	64.6±2.2
LIN [%]	42.4±1.4	41.4±1.4	39.1±1.2	36.6±1.2
WOB [%]	59.4±0.8	58.6±0.8	57.3±1.2	56.7±1.2
ALH [µm]	3.7±0.1	3.6±0.1	3.4±0.2	3.3±0.2
BCF [HZ]	32.3±0.5[c]	32.3±0.5[c]	29.9±0.7[d]	28.6±0.7[d]

Means with different letters (a, b between fertile and subfertile groups); (c, d between breeding and non-breeding seasons) are significantly different ($p < 0.05$)

4.1.2 Seasonal variations in quantitative and qualitative sperm characteristics of fertile and subfertile stallion groups

To investigate the effects of seasons on stallion semen characteristics, the differences in various quantitative and qualitative parameters of stallion sperm for each fertility group in both breeding and non-breeding seasons were examined.

No significant seasonal variations were observed in ejaculate volume, sperm concentration and total sperm number between breeding and non-breeding seasons for fertile and subfertile stallion groups as shown in Table 6.

In order to investigate the seasonal changes of stallion sperm morphology, 100 sperm from each ejaculate were assessed and classified using eosin-nigrosine stain under phase-contrast microscopy oil immersion (x 1000). Stallions in fertile group showed a great morphological similarity between the breeding and non-breeding season, where the proportions of defect spermatozoa in fresh stallion semen including acrosome defect, head defect, detached sperm head, midpiece defect, proximal and distal plasma droplets, tail defect, and duplications showed no significant differences between the both seasons.

However, the total proportion of morphologically intact spermatozoa in fresh semen was significantly higher in breeding season (67.1±4.9 vs. 47.3±6.02, $p < 0.05$).

No significant differences between breeding and non-breeding seasons were observed in proportion of morphologically normal spermatozoa as well as in all sperm defects in subfertile group as presented in Table 7.

Furthermore, the seasonal variations in CASA derived sperm movement characteristics were also investigated. For each fresh semen sample, the percentage of motile spermatozoa and velocities of sperm movement were evaluated using CASA system.

The percentage of total and progressive motility suggested no significant differences between breeding and non-breeding seasons in both fertile and subfertile stallions. However, the pattern of motility and velocities of sperm movement was changed. Detailed analysis by CASA system demonstrated a significantly higher percentage of VAP, VSL and BCF in the breeding season when compared to non-breeding season in both fertile and subfertile stallions. Moreover, no significant differences were found between the breeding and non-breeding season for VCL, STR, LIN, WOB and ALH in fertile and subfertile stallions as summarized in Table 8.

4.2 Experiment 2: Investigation of cellular processes, functional networks and biological functions in stallion in response to different seasonal conditions

The aim of this experiment was to investigate cellular processes, functional networks and biological functions in stallion in response to different seasonal conditions associated with increase of daylight and temperature during the breeding season. This approach was performed by comparing the microarray-derived sperm transcriptomes between the breeding and non-breeding season in which the isolated RNA from sperm of each stallion was used to prepare the microarray probes. The principal component analysis was used to detect transcript with different abundance between breeding and non-breeding season.

4.2.1 Total RNA extraction from stallion semen and quality assessment

Total RNA was extracted from semen of eight different stallions and the purity of RNA samples were checked by RT-PCR. Agarose gel electrophoresis images showing RT-PCR results are presented in Figure 8. Approximately 400 - 600 ng of total RNA was isolated from each sperm pellet of 100×10^6 sperm as measured by NanoDrop spectrophotometer. The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA. All RNA samples had values between 1.5 and 1.7 for absorbance ration 260/280 but lower values were observed for the ratio of 260/230, between 1.2 - 1.5.

The purity of RNA samples was further checked by RT-PCR. The PRM2 gene was targeted to detect the presence of genomic DNA contamination in RNA samples.

As shown in Figure 8 and after comparing the bands in the samples (lanes 1 - 13) to the DNA ladder (lane 16), the approximate size of the samples can determine. All cDNA samples (Lanes1 - 13) had a single band of 167 bp. The positive control (lane 15), which contains a known amount of equine genomic DNA, is used to check that the primer set works and the reaction has been set up correctly as well as to check the quality of RNA and the absence of genomic DNA contamination in all RNA samples, which were used to prepare the microarray probes. The negative control (lane 14) did not give any amplicons indicating that no primer-dimers were generated from the negative control PCR reaction, which contains only both primer sets and distilled water, which was used to prepare all RT-PCR reactions.

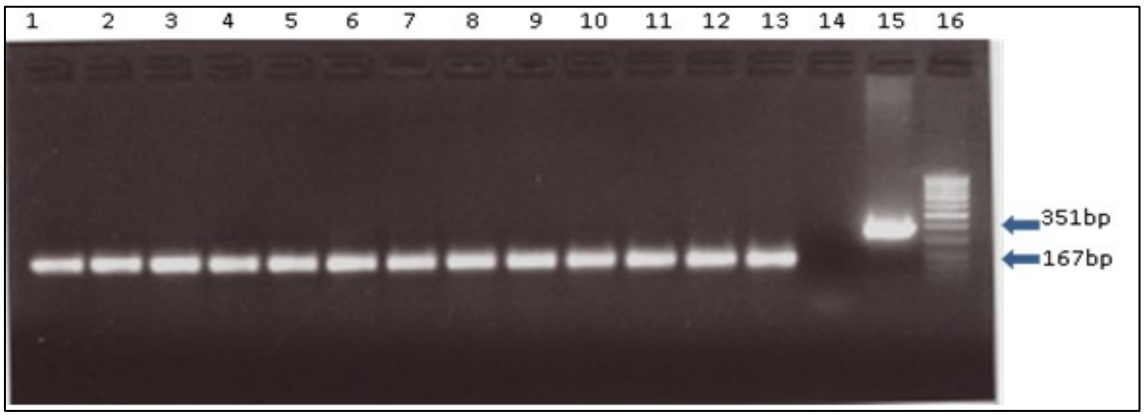

Figure 8: Agarose gel electrophoresis images showing RT-PCR results; lanes (1 - 13) RNA samples from the eight stallions (some replicates), which were used in experiment 2 and 3; lane 14 water as negative control; lane 15 genomic equine DNA as positive control; lane 16 DNA ladder 100 bp

4.2.2 Bioinformatic analysis of DEG genes using software tools

4.2.2.1 Functional annotation analysis of DEG genes using DAVID database

A total of 581 genes were found having significantly different abundance of transcripts between breeding and non-breeding seasons.

To group genes based on functional similarity, the list of differently expressed genes DEG from this analysis was submitted to DAVID.

DAVID clustering algorithm classifies highly related genes into functionally related groups. The gene ontology (GO) classification of the DEG using DAVID was explored. The GO consists of three structured categories that describe gene products in term of their associated biological processes, molecular function and cellular component.

DAVID uses a novel agglomeration algorithm to condense a list of genes into organized classes of related genes; called biological modules. DAVID was used in this study with its default options and the highest classification stringency cut off to group the genes into functionally related clusters. Grouping genes based on their functional similarity enhances biological interpretation of large list of genes derived from microarray studies. In this experiment, 581 genes were found having significantly different abundance of transcripts between the two stallion groups in response to different seasonal conditions. DAVID assigned 255 transcripts of them to GO and according to its clustering analysis, revealing differentially expressed transcripts were categorized in only one cluster with enrichment score of 0.97. This cluster shows significant enrichment of 5 genes in GO term protein acetylation with p= 0.0024 as presented in Table 9.

Table 9: Significant enrichment of DEG genes according to DAVID

Category	Gene Symbol	Gene Name
SP_PIR_KEYWORDS Term: Acetylation	ACTB, ACTG1	actin, gamma 1; beta actin
	CCNT1	cyclin T1
	EEF1G	eukaryotic translation elongation factor 1 gamma
	PGK2	phosphoglycerate kinase 2
	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)

4.2.2.2 Identification of biological functions and canonical pathways associated with DEG genes using IPA

The associations between the differently expressed genes were further evaluated using the Ingenuity pathways analysis software (IPA) for biological functions, canonical pathways and networks analysis. IPA analyzes various bioinformatics tools including functional annotation, clustering and network discovery based on Ingenuity Knowledge Base, which is the core technology of all IPA systems.

The p-value is developed from Right-tailed Fisher's exact test, which used to interpret the interaction and functions of the differentially expressed genes.

The individual enriched functions in IPA were filtered by removing redundant functions with overlapping genes as well as functions that were not directly relevant to the present study.

In this study, a total of 581 probe-sets with low or high transcript abundance (1.5-fold change) were used to identify biological functions and pathways association by IPA tool. The pathway analysis methods used enable the extraction of biologically meaningful information from a long list of differentially expressed genes. IPA functional analysis of DEG revealed that the top significantly affected biological functions by change of environmental conditions between the breeding and non-breeding season include: cellular assembly and organization, cellular function and maintenance, cellular compromise, carbohydrate metabolism, lipid metabolism, organ morphology, organismal development, small molecule biochemistry, cell cycle, cell to cell signaling and interaction, cellular movement, embryonic development, tissue morphology, cell death and survival and cellular development. Figure 9 shows the biological functions that were found to be significant to the target genes. The X-axis on the left indicates -log (p-value) and the orange horizontal line denotes the threshold for significance ($p = 0.05$). Each individual blue bar is a particular biological function and the functions are listed from most significant to least significant.

Further analysis conducted in IPA was also to understand the enriched and significant canonical pathways of genes identified in this dataset. The pathway analysis using IPA identifies enriched canonical pathways and scores directional changes based on gene expression. For this analysis IPA calculates two different statistics as part of a core analysis *the p-value*, which is calculated using a Right-Tailed Fisher's Exact Test. This test was used to calculate the probability of which each functional gene set was enrichment. Only the biological functions/pathways (Bonferroni's corrected p-value < 0.05) were considered significant enrichment. *The activation Z-score*, which represents the bias in gene regulation that predicts whether the upstream regulator exists in an activated or inactivated state. Z-score takes into account the directional effect of one molecule on another molecule or on a process and the direction of change of molecules in the data set. In practice, Z-score > 2 or < -2 is considered significant.

By means of this analysis a total of 27 significantly enriched canonical pathways were identified using the p-value threshold of 0.05 as shown in Figure 10.

This analysis identified the pathways from the IPA library of canonical pathways that were most significant to the DEG list. As shown in Figure 10 the top selected significant canonical pathways include FAK signaling, clathrin-mediated endocytosis signaling, regulation of actin-based motility by Rho, VEGF signaling, actin cytoskeleton signaling,

Gap junction signaling, paxilin signaling, ID-myo-inositol hexakisphosphate biosynthesis II, D-myo inositol $(1,3,4)$-trisphosphate biosynthesis and RhoGDI signaling. The X-axis on the left in Figure 10 indicates -log (p-value). Orange line represents the ratio of genes from the DEG list divided by the total number of genes that make up the pathway, and the orange horizontal line denotes the threshold for significant ($p = 0.05$). Each individual colored bar is a particular pathway and the color indicates its predicted state: increasing (orange) or decreasing (blue). Gray bar means that the pathway currently ineligible for a prediction and no prediction can be made. Darker colors indicate higher absolute Z-scores.

IPA was also used to generate metabolic pathways that were most significant to the target genes (DEG). The most significant metabolic pathways affected by the change of environmental conditions between breeding and non-breeding season are ID-myo-inositol hexakisphosphate biosynthesis II, D-myo-inositol $(1,3,4)$-triphosphate biosynthesis, pyridoxal 5′-phosphate salvage pathway, super pathway of D-myo-inositol $(1,4,5)$-triphosphate metabolism, gluconeogenesis I and salvage pathway of pyrimidine ribonucleotides as presented in Figure 11 in which each individual bar is a particular metabolic pathway and the pathways are listed from most significant to least significant. The X-axis indicates -log (p-value) and the orange horizontal line denotes the threshold for significant ($p = 0.05$). The ratio (orange dots connected by a line) represents the ratio of genes from the submitted DEG list divided by the total number of genes that make up this pathway.

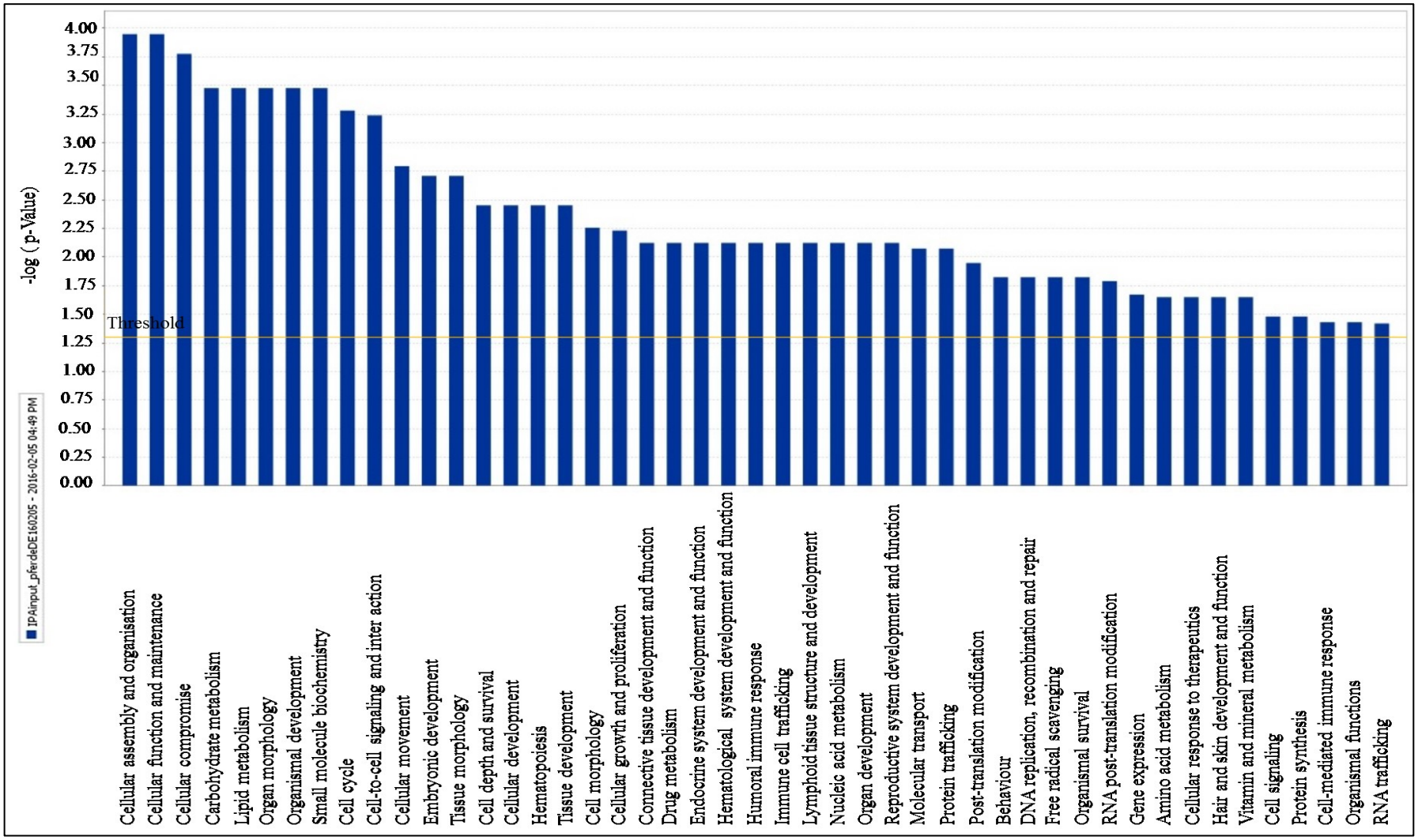

Figure 9: Representative graph displaying biological functions associated with DEG genes. The IPA blue bars displays biological functions along the X-axis. The Y-axis displays the (log) significance. Functions are listed from most significant to least significant. The orange horizontal line denotes the threshold for significant (p= 0.05)

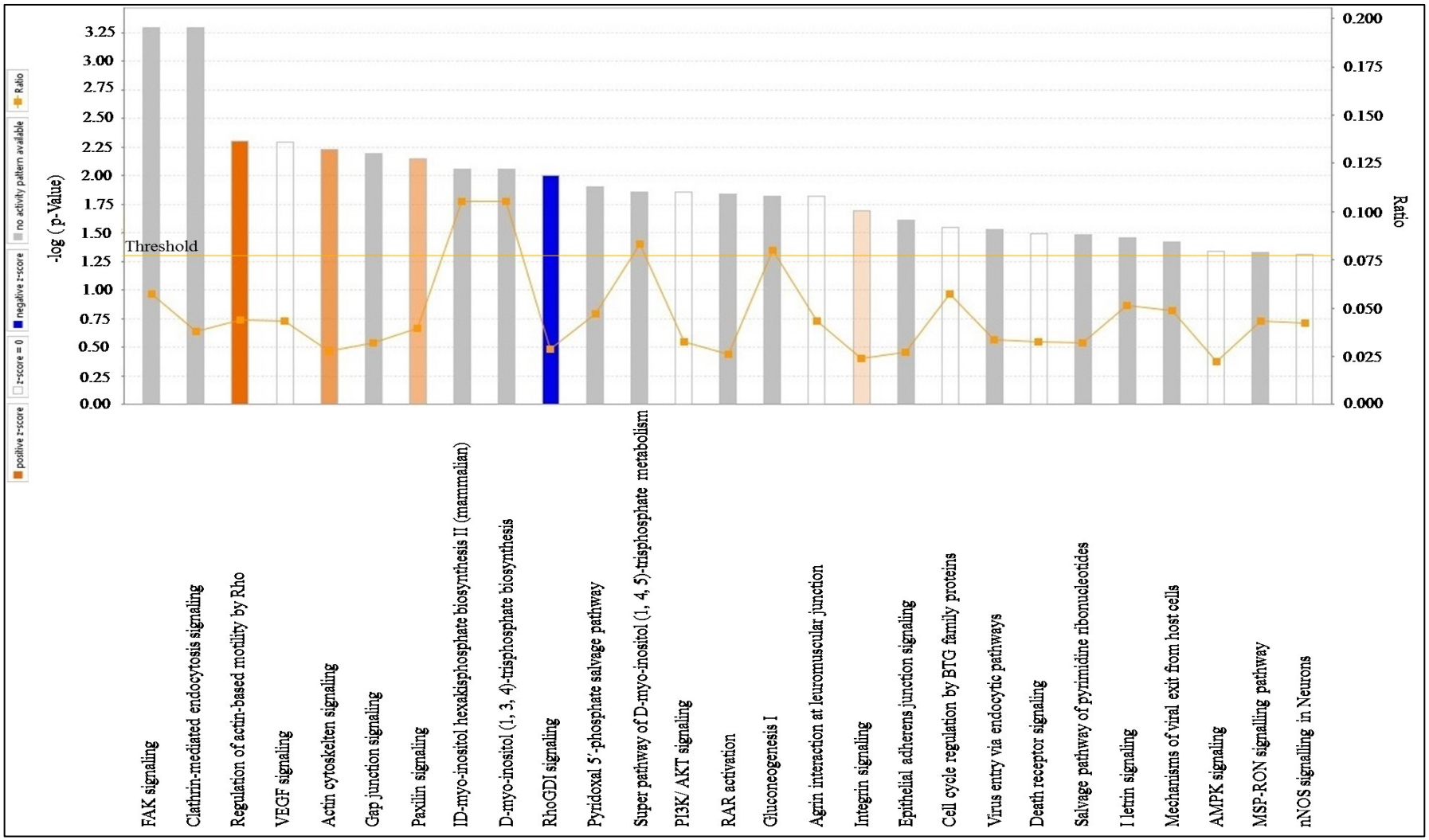

Figure 10: Significantly canonical pathways associated with DEG genes using Benjamin-Hochberg multiple testing correction [-log (B-H P-value)] as generated by IPA. Orange line represents the ratio of genes from the DEG list divided by the total number of genes that make up the pathway and the orange horizontal line denotes the threshold for significant (p= 0.05)

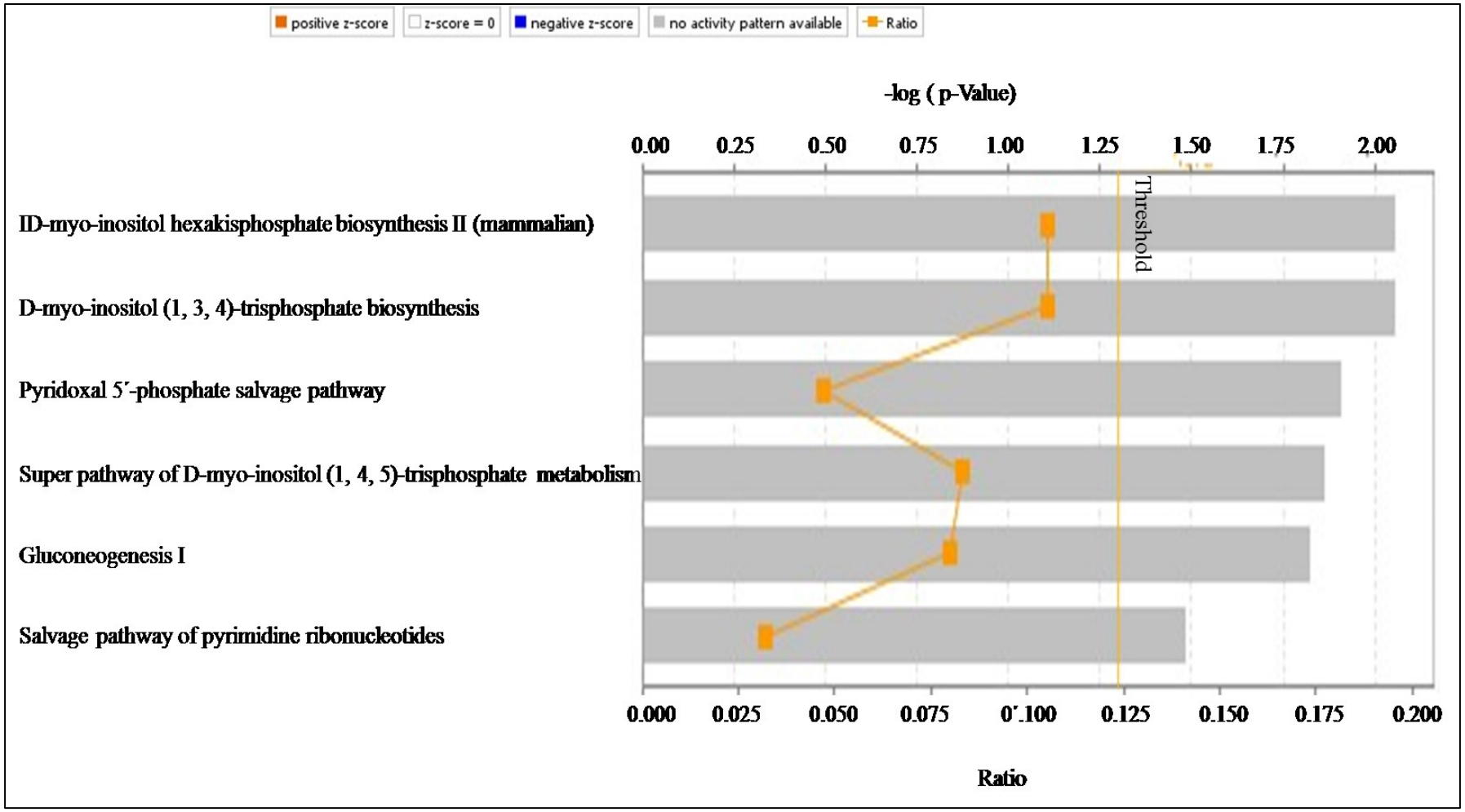

Figure 11: Significantly enriched metabolic pathways associated with DEG genes as generated by IPA. Orange line represents the ratio of genes from the submitted gene list divided by the total number of genes that make up the pathway and the orange horizontal line denotes the threshold for significant (p= 0.05)

Network analysis by IPA was carried out to draw the connection of focus molecules presenting relationships between the interacting genes. For network analysis, the mapped identifiers were overlaid onto a global molecular network developed from information contained in the IPA Knowledge Base. Networks of focus genes were then algorithmically generated based on their connectivity and assign a score.

The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset. It takes into account the number of focus genes in the network and the size of the network to approximate the relevance of the network to the original list of focus genes. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that network is due to chance alone. IPA network analysis revealed 10 top networks display a score ranged between 14 and 44. The first molecular network (score= 44) converged on cardiovascular disease, organismal injury and abnormalities as well as skeletal and muscular disorders. The second molecular network (score= 44) converged on embryonic development, tissue morphology and cellular development. The third molecular network (score=27) represents cancer, organismal injury and abnormalities and cell death and survival. The forth molecular network (score=25) represents cancer, organismal injury and abnormalities and cellular function and maintenance. The fifth molecular network (score=25) converged on inflammatory disease, neurological disease and connective tissue development and function. Furthermore, two networks were found to be enriched for biological functions, which related to development of reproductive system and gonadogenesis as shown in Figure 12. These networks represent molecular relationships between genes or gene products. Genes or gene products are represented in the network graph as nodes and the biological relationship between two nodes is represented as a line (solid lines represent direct interactions while dashed lines represent indirect interactions). Nodes are displayed using various shapes that represent the functional class of the gene product such as chemical or drug, cytokine, enzyme, growth factor, kinase, ion channel, peptidase, phosphatase, transcription regulator, translation regulator, transporter or other products. The color of the nodes indicates the up and down regulation of the molecules in the network (red indicates up-regulation and green down-regulation). The lines connecting the nodes are colored orange when leading to activation of the downstream node, blue when leading to its inhibition and yellow if the findings underlying the relationship are inconsistent with the state of the downstream node.

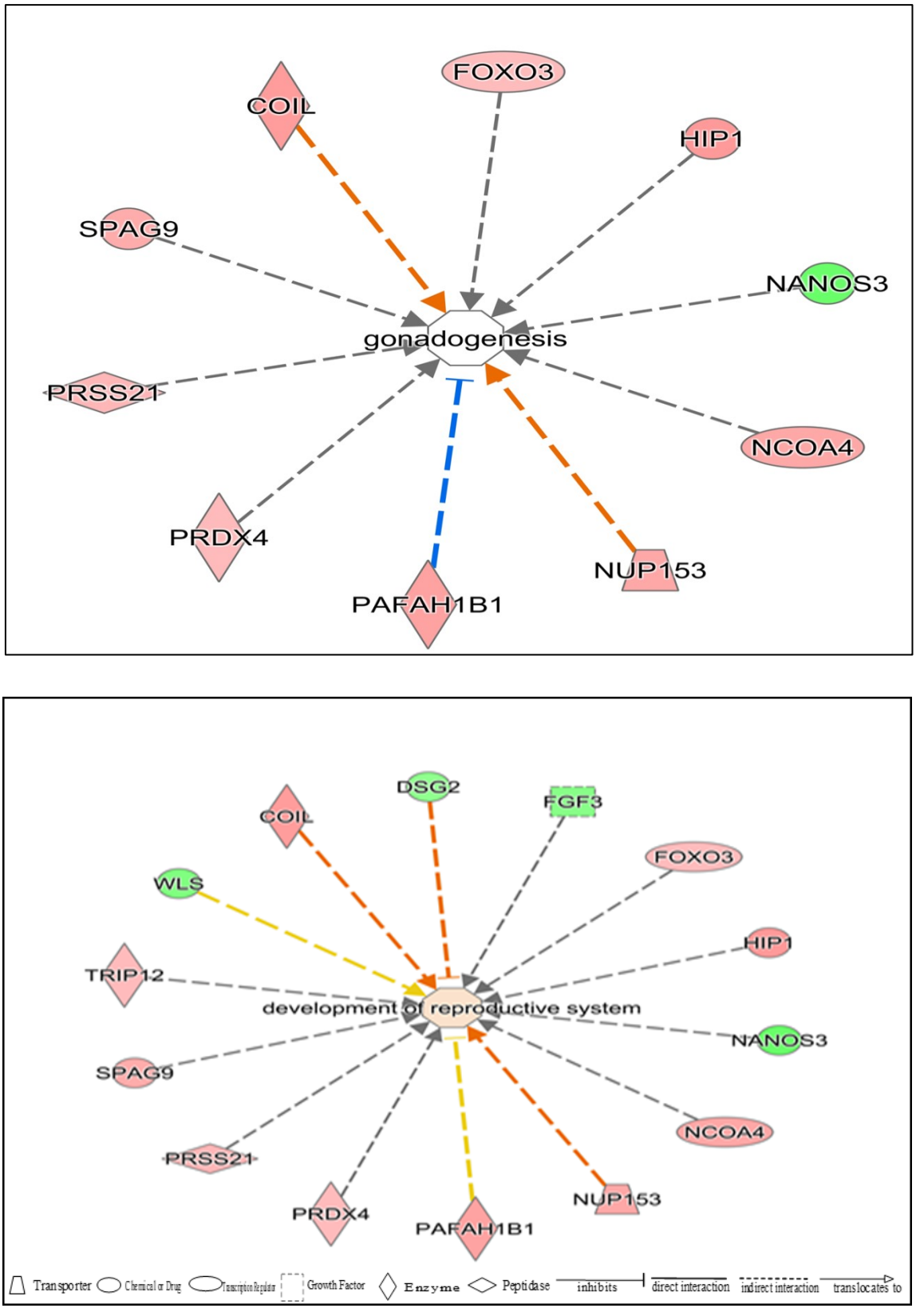

Figure 12: Graphical representation of two networks that related to biological functions of DEG genes. In this graphical representation, genes or gene products are represented as nodes and the biological relationship between two nodes is represented as a line. Nodes are displayed using various shapes that represent the functional class of the gene product

4.3 Experiment 3: Investigation of molecular processes linked to stallion fertility

4.3.1 Fertility data and semen quality of the six stallions, which were used in this experiment

The aim of this experiment was to identify molecular processes that are associated with male fertility by comparing microarray-derived sperm transcriptomes of stallions that were clearly assigned to either a fertile or subfertile group based on reproductive success and sperm characteristics. Individual measures for sperm morphology and progressive motility of each ejaculate are represented in Table 10. Furthermore, the mean of four measures ± SD of sperm morphology and progressive motility for each stallion as well as the mean of the measures ± SD of sperm morphology and progressive motility for each group were calculated. Based on motility analysis using CASA system for fertile and subfertile stallions in breeding season, there was a significant difference in progressive motility between fertile and subfertile group (70.33±2.80 vs. 43.37±4.14 p = 0.001), respectively. Furthermore, the percentage of morphologically normal spermatozoa was significantly higher in fertile group in comparison to subfertile group in breeding season (71.67±2.89 vs. 33.00±12.12 p = 0.012), respectively.

4.3.2 RNA concentration and quality assessment

As described above in experiment 2, approximately 400 - 600 ng of total RNA was isolated from each sperm pellet of 100×10^6 sperm as measured by NanoDrop spectrophotometer. The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA. All RNA samples had values between 1.5 and 1.7 for absorbance ration 260/280 but lower values were observed for the ratio of 260/230, between 1.2 - 1.5.

The purity of RNA samples was further checked by RT-PCR. The PRM2 gene was targeted to detect the presence of genomic DNA contamination in RNA samples.

All cDNA samples (Lanes1- 13) as shown in Figure 8 had a single band of 167 bp indicating good RNA qualities and absence of genomic DNA contamination in the RNA samples, which were used to prepare the microarray probes. The analysis of microarray data for each group was carried out to obtain a gene list with a significant difference between fertile and subfertile stallion groups (DE). Next, the list of significantly correlated genes was analyzed for enrichment of functional annotation groups as defined in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway using DAVID. Finally, the Ingenuity Pathways Analysis (IPA) was used to identify top biological functions and significant canonical pathways associated with the differentially expressed genes (DE).

Table 10: Fertility data and semen quality of the stallions, which were used in experiment (3)

Group	Stallion	Ejaculate	Sperm quality parameters						Seasonal pregnancy rate [%]	
			Progressive sperm motility [%]			Normal sperm morphology [%]				
			Individual	Mean of four measures ± SD	Mean of progressive motility for group ± SD	Individual	Mean of four measures ± SD	Mean of normal morphology for group ± SD	Individual	Mean ± SD
Fertile group	A	1	67.58	72.02±5.35	70.33±2.80	76	75.75±1.79	71.67±2.89	90	83.30±5.70
		2	81.11			78				
		3	70.45			76				
		4	68.93			73				
	B	1	79.98	72.59±4.87		73	69.75±6.18		80	
		2	67.58			62				
		3	68.93			78				
		4	73.86			66				
	C	1	80.92	66.38±9.32		74	69.50±2.87		80	
		2	61.79			69				
		3	55.67			66				
		4	67.13			69				
Subfertile group	E	1	40.13	48.74±8.62	43.37±4.14	39	45.25±5.80	33.00±12.12	60	50.00±10.00
		2	57.87			40				
		3	56.84			50				
		4	40.13			52				
	F	1	39.84	38.65±3.03		15	16.50±2.06		40	
		2	33.46			15				
		3	40.18			16				
		4	41.12			20				
	G	1	39.84	42.72±3.22		37	37.25±0.83		50	
		2	47.83			38				
		3	40.08			36				
		4	43.12			38				
p-value					0.001			0.012		0.007

4.3.3 Bioinformatic analysis of DE genes using software tools

4.3.3.1 Functional annotation and KEGG pathway analysis of DE genes using DAVID database

A total of 437 genes were found having significantly different abundance between fertile and subfertile stallion groups ($p \leq 0.05$, $FC \geq 1.2$). To explore and view functionally related genes together, the list of DE genes was submitted to DAVID. DE genes were assessed by GO and KEGG pathway database. DAVID software tool was used to identify gene-associated molecular functions that were overpresented in the gene list generated by the comparative gene expression analysis between fertile and subfertile stallions.

In this experiment, 437 genes were found having significantly different abundance of transcripts between the two stallion groups in response to different pregnancy rate and sperm characteristics.

DAVID assigned 216 transcripts of them to GO and according to its clustering analysis, revealing differentially expressed transcripts were categorized in five clusters with enrichment score of 1.25, 0.53, 0.44, 0.25 and 0.2.

These clusters show significant enrichment of three genes (OAS1, OAS2 and RPL5) in GO term RNA binding with a p-value of 0.05. In addition, eight genes from the submitted gene list (CSF3R, GM-CSF, IL17A, IL13, IL22RA1, CD134, CD27, SF9) were found significantly enriched in the KEGG pathway cytokine-cytokine receptor interaction with a p-value of 0.02 as summarized in Table 11.

DAVID provides in addition to GO analysis, an additional analysis module, which summarizes graphically the distribution of genes in the KEGG pathway of cytokine-cytokine receptor interaction in this experiment as shown in Figure 16 in which the KEGG pathway for cytokine-cytokine receptor interaction is presented (as generated by DAVID). The eight genes (CSF3R, GM-CSF, IL17A, IL13, IL22RA1, CD134, CD27, and SF9) that were significantly upregulated in the pathway are denoted with red star.

Table 11: Significant enrichment of DE genes according to DAVID and KEGG pathway analysis

Category	Term	Count	Genes	p-value
GOTERM-MF-ALL	RNA binding	3	**OAS1**: 2',5'-oligoadenylate synthetase 1, 40/46kDa **OAS2**: 2',5'-oligoadenylate synthetase 2, 69/71kDa **RPL5**: ribosomal protein L5	0.05
KEGG-Pathway	Cytokine-cytokine receptor interaction	8	**CSF3R**: colony stimulating factor 3 receptor (granulocyte) **GM-CSF**: granulocyte-macrophage colony-stimulating factor **IL17A**: interleukin 17A **IL22RA1**: interleukin 22 receptor, alpha 1 **IL13**: interleukin 13 **CD134** homologue: tumor necrosis factor receptor superfamily, member 4 **CD27** ligand: CD70 molecule **SF9**: tumor necrosis factor receptor superfamily, member 9	0.02

4.3.3.2 Identification of biological functions and canonical pathways associated with DE genes using IPA

IPA was applied to select the main biological functions and canonical pathways represented in DE gene list. In this experiment, a total of 437 probe-sets with low or high transcript abundance ($p \leq 0.05$, $FC \geq 1.2$) were used to identify biological functions and canonical pathways association by IPA tool.

Ingenuity pathway analysis of DE genes showed several different associated biological functions. The target genes were significantly enriched for diverse biological functions known to be fundamental for spermatogenesis and male fertility. These include cellular assembly and organization, cellular function and maintenance, cellular development, cellular growth and proliferation, tissue development, cell morphology, organismal injury and abnormalities, cardiovascular disease, reproductive system disease, cellular movement and cell cycle.

Figure 13 shows the top 20 significantly enriched IPA biological functions that were found to be significant to the target genes. The X-axis on the left indicates -log (p-value) and the orange horizontal line denotes the threshold for significant ($p = 0.05$). Each individual blue bar is a particular biological function and the functions are listed from most significant to least significant.

Further analysis was also conducted in IPA to understand the enriched and significant canonical pathways of genes identified in this dataset. The pathway analysis using IPA identifies 17 enriched canonical pathways and scores directional changes based on gene expression.

As shown in Figure 14, the top selected canonical pathways, which were found to be most significant to DE are G1/S checkpoint regulation, cyclin and cell cycle regulation, ATM signaling, netrin signaling, natural killer cell signaling, role of oct4 in mammalian embryonic stem cell pluripotency, semaphorin signaling in neurons, role of NFAT in regulation of the immune response, role of CHK proteins in cell cycle checkpoint control and tumoricidal function of hepatic natural killer cells.

The X-axis on the left in Figure 14 indicates -log (p-value). Orange line represents the ratio of genes from the DE list divided by the total number of genes that make up the pathway and the orange horizontal line denotes the threshold for significance ($p = 0.05$). Each individual colored bar is a particular pathway and gray bar means that the pathway currently ineligible for a prediction and no prediction can be made.

IPA was also used to generate metabolic pathways that were most significant to the target genes (DE). The most significant metabolic pathways are D-myo-inositol $(1,4,5,6)$-titrakisphosphate biosynthesis, D-myo-inositol $(3,4,5,6)$-titrakisphosphate biosynthesis, 3-phosphoinositide degradation, D-myo-inositol-5-phosphate metabolism and 3-phosphoinositide biosynthesis as presented in Figure 15 in which each individual bar is a particular metabolic pathway and the pathways are listed from most significant to least significant. The X-axis indicates –log (p-value) and the orange horizontal line denotes the threshold for significance ($p=0.05$). The ratio (orange dots connected by a line) represents the ratio of genes from the submitted gene list divided by the total number of genes that make up this pathway.

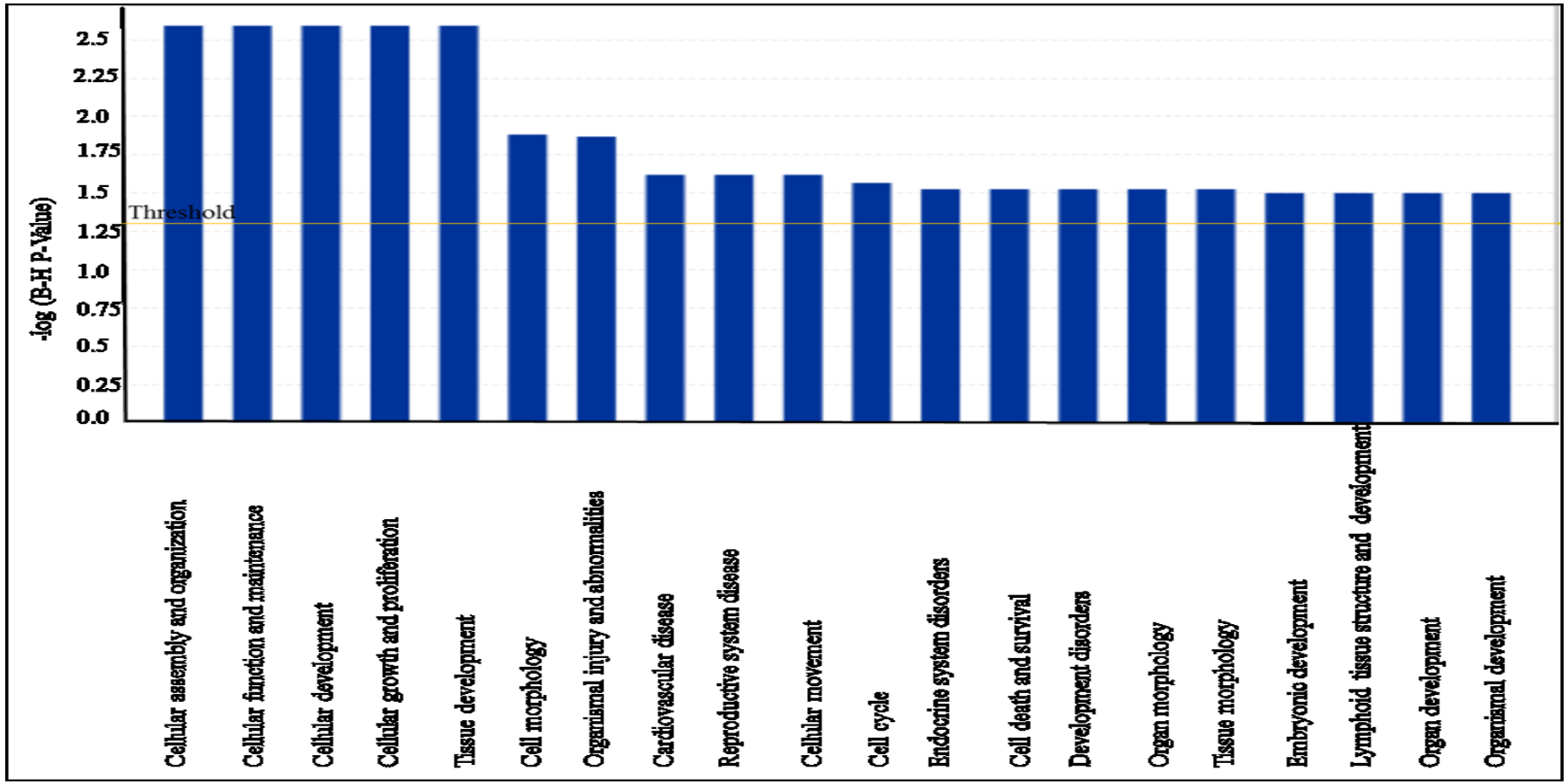

Figure 13: IPA analysis of biological functions associated with DE genes. The IPA blue bars display biological functions along the X-axis. The Y-axis displays the (log) significance. Functions are listed from most significant to least significant. The orange horizontal line denotes the threshold for significant (p= 0.05)

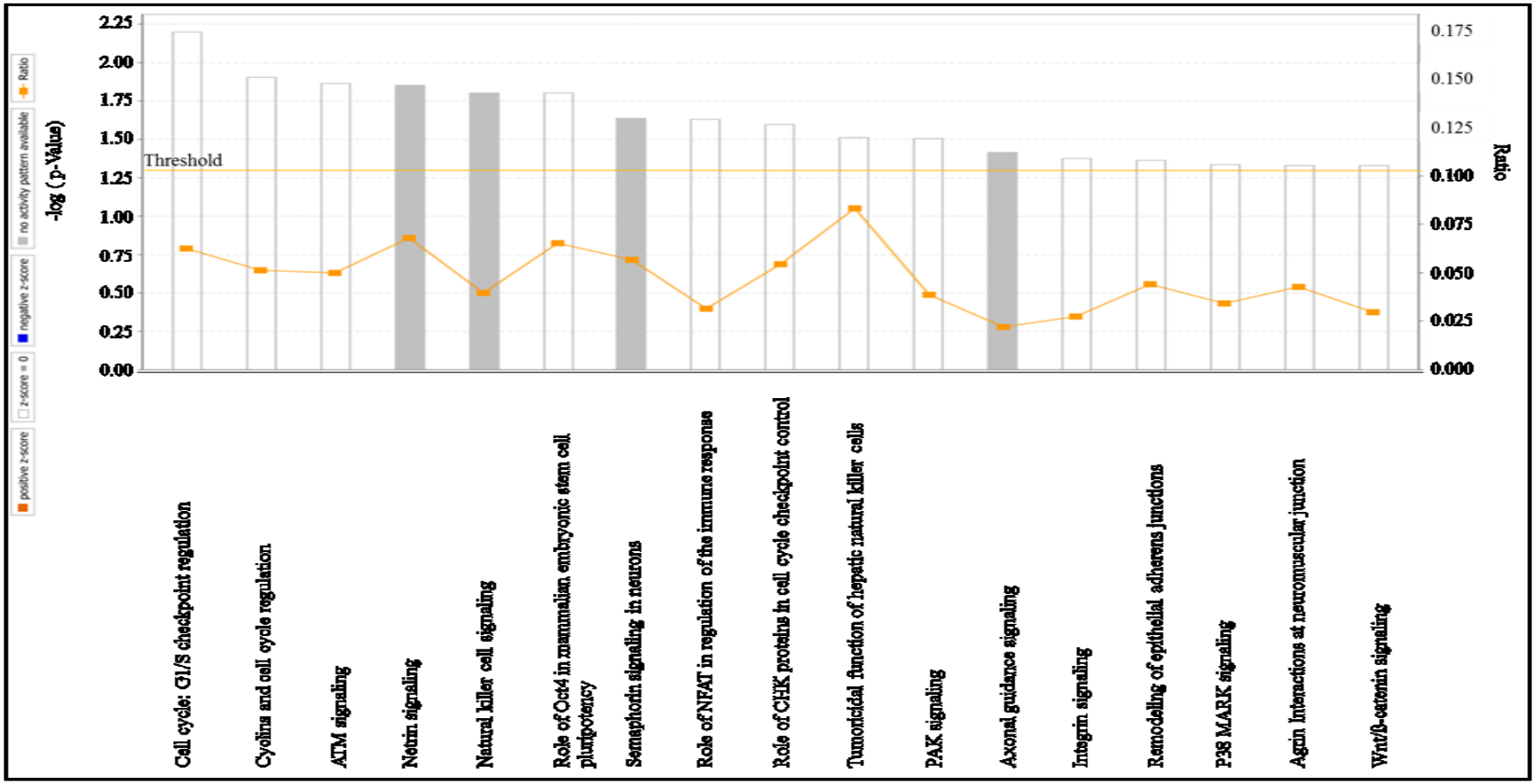

Figure 14: Significantly canonical pathways associated with DE genes using Benjamin-Hochberg multiple testing correction [-log (B-H P-value)] as generated by IPA. Orange line represents the ratio of genes from the DE list divided by the total number of genes that make up the pathway and the orange horizontal line denotes the threshold for significant (p= 0.05)

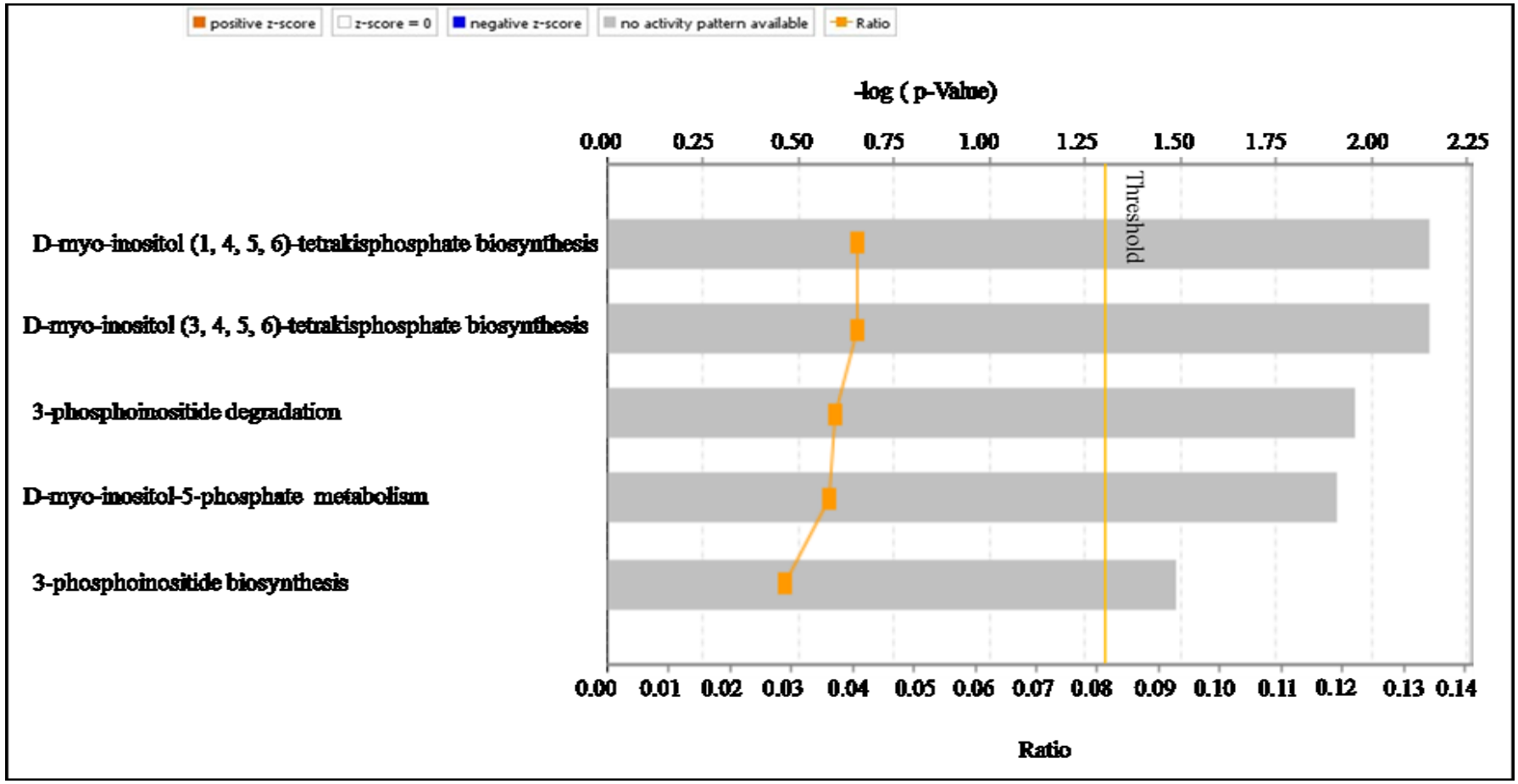

Figure 15: Significantly enriched metabolic pathways associated with DE genes as generated by IPA. Orange line represents the ratio of genes from the submitted gene list divided by the total number of genes that make up the pathway and the orange horizontal line denotes the threshold for significant (p= 0.05)

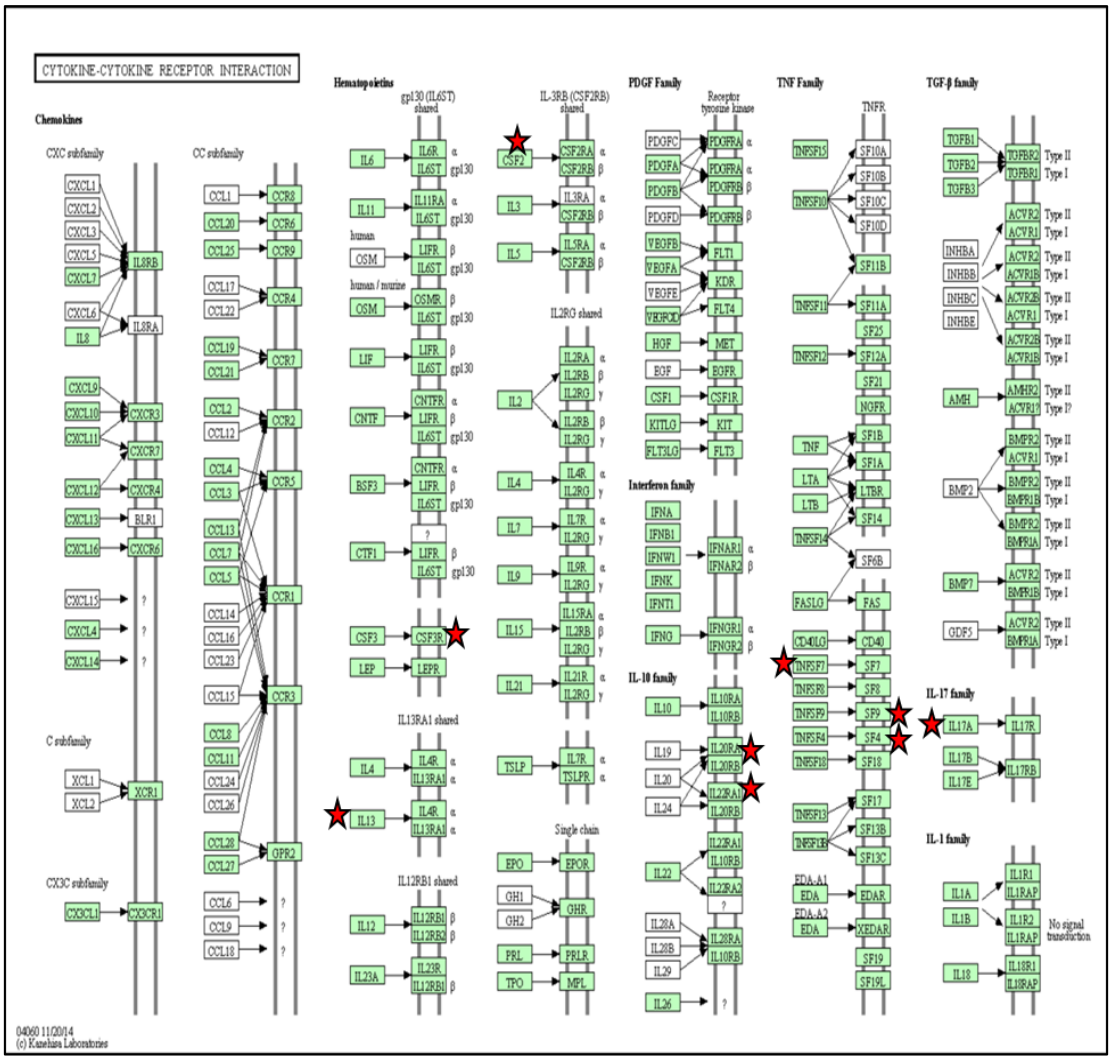

Figure 16: KEGG Pathway for cytokine-cytokine receptor interaction (horse) as generated by DAVID. Genes that were significantly upregulated in the pathway are denoted with red star

4.3.4 Quantitative RT-PCR validation of microarray results

To validate the microarray results, four genes from DE submitted gene list (OAS1, OAS2, IL13 and IL22RA1) that displayed differential expression in microarray date were selected to perform RT-PCR assay.

These genes were chosen according to their biological function and all of them were validated as having significantly different abundance transcript between fertile and subfertile stallion groups.

As presented in Table 12, the expression of the genes OAS1, OAS2, IL13 and IL22RA1 using RT-PCR showed significant differences in transcript abundance between fertile and subfertile stallions with P value of (0.035, 0.017, 0.031 and 0.033), respectively, which agree with the results obtained from microarray analysis.

Table 12: Validation of microarray results using RT-PCR for the selected genes and all of them were validated as having significantly different transcript abundance between fertile and subfertile stallion groups. T-test was used to compare the gene expression between fertile and subfertile stallions using the normalized Ct value for each stallion in both groups. Differences in gene expression between fertile and subfertile stallions were considered significant at $p \leq 0.05$

Group	Stallion	ΔCt *OAS1*	ΔCt *OAS2*	ΔCt *IL13*	ΔCt *IL22RA1*
Fertile group	A	-5.5	-3.9	-4.7	-2.0
	B	-5.5	-3.2	-4.8	-4.5
	C	-6.6	-3.0	-4.9	-1.4
Subfertile group	E	-2.5	0.2	-1.0	1.1
	F	-4.7	-0.4	-3.7	-0.1
	G	-3.5	-1.9	-2.0	2.1
p-value (RT-PCR)	-	0.035	0.017	0.031	0.033
p-value (Microarray)	-	0.023	0.00071	0.004	0.0026

The efficiency of the PCR should as close to 100 % as possible, which means that the number of target molecules doubles with every PCR cycle. As showed in Table 13, the efficiency of PCR for the target and reference genes ranged from 90 to 110 % indicating that the number of target molecules doubled with every PCR cycle.

Table 13: RT-PCR efficiency for the target and reference genes used for the validation of microarray results. An optimized PCR will result in a standard curve with a slope between -3.2 and -3.5

Gene	RPL32	HPRT	OAS1	OAS2	IL13	IL22RA1
Slope	-3.383	-3.218	-3.344	-3.461	-3.293	-3.217
RT-PCR efficiency [%]	97.69	104.64	99.09	94.54	101.73	104.64

During the PCR, the Ct values from the serial dilutions are plotted against the logarithm of the concentration of the sample and the standard curve is then constructed automatically as shown in Figure 17.

Ct value is the intersection between an amplification curve and a threshold line and recorded during the early exponential phase when the fluorescence reaches above the background level of the fluorescence. This value is crucial because it is used to calculate the concentration of transcript in the sample and to compare the gene expression between two samples. Therefore the Ct value serves as the basis for quantification.

In addition to PCR efficiency, the use of reference genes for normalization plays an important role in the success of PCR process. The use of reference genes relies on the fact that the ideal reference gene should be expressed at a constant level in all tissues or cell types, at all developmental stages and in all experimental conditions.

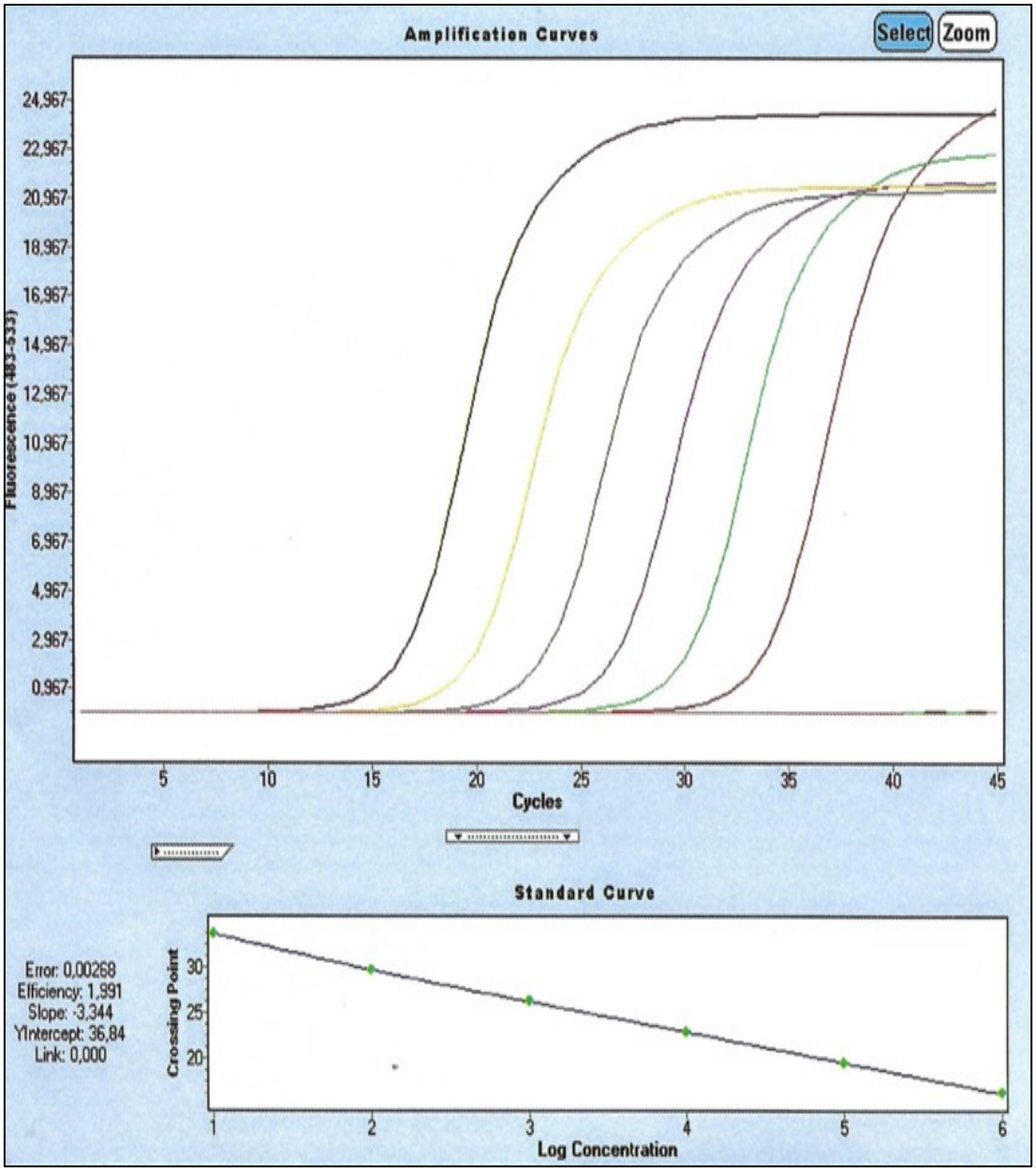

Figure 17: standard curve for the target gene OAS1 generated using a RT-PCR amplicon and serially diluted to produce the six reference points of the calibration curve (10^1-10^6 copies)

5 Discussion

5.1 Investigation of semen quality parameters associated with high fertility in stallion and the seasonal variations of these parameters between breeding and non-breeding seasons

5.1.1 Investigation of semen quality parameters associated with high fertility in stallion as measured by pregnancy rate

In the first part of this experiment, quantitative and qualitative sperm characteristics of fertile and subfertile stallions were compared to investigate what semen quality parameters associated with high fertility of stallion as measured by pregnancy rate.

Regarding semen volume, sperm concentration and total sperm number, the results of this study showed no significant differences between fertile and subfertile stallions in both breeding and non-breeding seasons. On the other hand, a significant difference was observed between the fertile and subfertile group in breeding season for the proportion of morphologically normal spermatozoa (67.1±4.9 vs. 37.7±4.9; $p < 0.05$), respectively. However, no significant differences were found between fertile and subfertile stallions for acrosome defect, head defect, detached sperm head, midpiece defect, proximal plasma droplet, distal plasma droplet, tail defect, and duplications.

Compared to previous studies in which sperm morphology was also assessed between fertile and subfertile stallions, a significant increase of morphologically normal spermatozoa was observed in fertile stallions when compared to either subfertile or infertile stallions (Pesch et al., 2006; Neild et al., 2000). The results of the present study agree with these reports but the observations in this study were detected only in breeding season while in non-breeding season no significant differences were observed in all morphology parameters between fertile and subfertile stallions.

Recently, Whitesell et al. (2019) compare in their study the outcome of the traditional breeding soundness examination of stallion between the traditional and modern semen evaluation methods, suggesting that the use of modern techniques such as CASA for evaluating sperm motility and differential interference contrast microscopy to evaluate sperm morphology, may give more accurate results about the fertility state of the stallion. They found that the use of modern evaluation techniques yielded in better estimates of sperm motility and morphology. They also found that the proportion of morphologically normal sperm as determined using differential interference contrast microscopy was the only single parameter, which found to be significantly correlated to stallion fertility.

In several previous studies in which correlations between male fertility and sperm morphology were studied, very conflicting results were observed.

Thus Jasko et al. (1990) reported a positive correlation between stallion fertility and normal sperm morphology whereas (Dowsett and Pattie, 1982; Voss et al., 1981) did not find any relationship between sperm morphology and stallion fertility. Moreover, Kavak et al., (2004) reported that the presence of sperm morphological abnormalities in an ejaculate belongs to normal stallion fertility.

Regarding sperm motility in this experiment, significant differences between fertile and subfertile group in breeding season for a part of sperm movement characteristics derived from CASA were also investigated including total motility (80.97±3.6 vs. 61.5±3.6; $p < 0.05$), progressive motility (68.2±3.7 vs. 46.5±3.7; $p < 0.05$), VAP (77.4±2.3 vs. 65.8±2.3; $p < 0.05$), and VCL (129.2±4.1 vs. 111.2±4.1; $p < 0.05$). In this work, CASA system was used to evaluate sperm motility characteristics. CASA system is the most popular method used to assess sperm motility and it is able to determine a series of variables, which cannot be determined by the human eye (Gil et al., 2009; Quintero-Moreno et al., 2003).

Progressive sperm motility is considered to be one of the most important parameters, which has been found to be related to stallion fertility (Hurtgen, 1992; Jasko et al., 1992) and the percentage of progressively motile spermatozoa shown to be significantly higher in fertile stallion in comparison to subfertile stallions (Neild et al., 2000).

Using frozen stallion spermatozoa, Krik et al. (2005) found that the progressive motility and straightness of the spermatozoa were significantly correlated to first cycle fertility. However, Pesch et al. (2006) did not find any significant correlation between total and progressive motility and fertility in stallion.

Also the results of the present study suggested that the pattern of motility and velocities of sperm movement were differed between fertile and subfertile stallions only in breeding season. Detailed analysis by CASA system demonstrated a significantly higher percentage of VAP and VCL in fertile group when compared with subfertile stallions.

In the study of Broekhuijse et al. (2011), CASA system was used to investigate the relationship between CASA parameters and boar fertility. Their results demonstrated that VAP was the only CASA parameter that showed a positive relationship with boar fertility. However, VSL showed a negative relationship to total number of piglets born.

In contrast, Liu et al. (1991) found a positive correlation between VSL and human fertility, and an increase of VSL enable sperm to better fertilize the oocyte. Furthermore, Holt et al. (1985) found that the swimming speed (VCL) of ejaculated human spermatozoa was strongly correlated with in vitro fertilization rates. These conflicting

data obtained from several studies could be related to different factors such as stallion, season, food quality, semen dilution and CASA settings (Sieme et al., 2004; Davis and Katz, 1992; Jasko et al., 1991).

5.1.2 Seasonal variations in quantitative and qualitative sperm characteristics of fertile and subfertile stallion groups

In the next part of this experiment, the seasonal variations in quantitative and qualitative semen parameters for each stallions group were investigated. Significant seasonal variations on ejaculate volume, sperm concentration and total sperm number were not obderved in this study between breeding and non-breeding season for fertile and subfertile stallions. In contrast to the results of the present study, Janett et al. (2003) reported that the gel free volume of stallion semen was highest in spring and summer and lowest in winter. Regarding sperm concentration, they also reported that the highest sperm concentration obtained in autumn and the lowest in summer.

However, in agreement to the investigations of the present study, Magistrini et al. (1987) suggested that there were no clear seasonal differences on stallion semen quality parameters.

Variations in seminal characteristics have been found to be influenced by ejaculate frequency and seasons, so that the increase in ejaculation frequency has been found to be associated with a decrease in sperm concentration and total sperm number as reported by Pickett et al. (1976).

In the present study, morphological examination of spermatozoa in fresh stallion semen revealed that the percentage of normal spermatozoa was significantly higher in breeding season in fertile stallion group (67.1±4.9 vs. 47.3±6.02; $p < 0.05$) in comparison to non-breeding season. In contrast, proportions of defect spermatozoa in fresh stallion semen including acrosome defect, head defect, detached sperm head, midpiece defect, proximal and distal plasma droplets, tail defect and duplications showed no significant differences between breeding and non-breeding seasons.

In subfertile stallions a great morphological similarity between the breeding and non-breeding season was observed, where no significant differences in proportion of morphologically normal spermatozoa as well as percentage of defect spermatozoa were observed. Results from routine semen quality analysis in Holland showed more sperm abnormalities during the non-breeding season than in breeding season in stallion (Van der Holst, 1975). In addition, Blottner et al. (2001) compared quality and freezability of stallion semen during breeding and non-breeding seasons and found that higher values of

morphological intact spermatozoa were detected in non-breeding season. These findings are in contrast to the results of the present study, where higher values of morphologically normal spermatozoa were observed in breeding season and only in fertile stallions.

Another important parameter for semen quality evaluation is the sperm motility.

For this purpose, CASA system was used, which allows obtaining detailed and repeatable evaluations of sperm motility and velocity. In the present study, significant seasonal variations on a part of CASA derived sperm movement characteristics in both fertile and subfertile stallions were observed in which the same parameters (VAP, VSL, and BCF) were influenced by season and tended to be significantly lower in non-breeding season in fertile and subfertile stallions. In addition, no significant differences were found in total and progressive motility as well as VCL, STR, LIN, WOB and ALH between breeding and non-breeding season in fertile and subfertile stallions.

Conflicting opinions concerning the effect of season on stallion sperm motility reported by Warnke et al. (2001) where the percentage of motile spermatozoa was higher during non-breeding season in winter compared to its percentage during the breeding season.

Furthermore, they found that the parameter BCF was significantly reduced in non-breeding season and mean sperm velocities were significantly lower in non-breeding season when compared with breeding season, which agree with the findings in the present study.

Although, there is clear evidence that seasons have effects on sperm production and semen quality in stallion despite some conflicting results (Janett et al., 2003; Jasko et al., 1991; Magistrini et al., 1987; Pickett et al., 1975). In the present study, the seasonal variations in quantitative and qualitative sperm characteristics in fertile and subfertile stallions were investigated, where fertile stallions seemed to be more influenced by seasons than subfertile stallions. In fertile stallions, the percentage of morphologic intact spermatozoa was significantly higher in breeding season than in non-breeding season and this percentage was significantly higher in fertile stallions than in subfertile stallions only in breeding seasons.

In the study of Roser and Hughes (1992), it has been demonstrated that the basal secretion of testosterone, LH and FSH in ferile stallions, increased significantly in breeding season. In contrast, basal secretion of these hormones in subfertile stallions did not significantly change between breeding and non-breeding seasons. This lack of seasonal differences in basal levels of testosterone, LH and FSH in subfertile stallions may reflect the lack of seasonal regulation of the synthesis of gonadotropins (Roser and Hughes, 1992).

That could interpret the results of the present study in which subfertile stallions showed no significant differences between breeding and non-breeding season in the most of semen quality parameters studied. Furthermore, the significant increase of basal hormones concentration in fertile stallions in breeding season compared to non-breeding season, may be responsible for initiating and controlling processes, which lead to the increases in the proportion of morphologic intact spermatozoa in breeding season.

In summary, this analysis of multiple semen quality parameters in fertile and subfertile stallions indicated that the proportion of morphologic intact spermatozoa, total and progressive sperm motility as well as VAP and VCL associated with high fertility in stallion as measured by pregnancy rate. In addition, the effect of season on semen quality parameters differed between fertile and subfertile stallions, where the fertile stallions were more affected. Moreover, a part of CASA derived parameters seemed to be significantly lower in non-breeding season than in breeding season in both fertile and subfertile stallions.

5.2 Investigation of cellular processes, functional networks and biological functions in stallion in response to different seasonal conditions

Seasonal changes such as temperature and day length have effects on reproduction in many species. The change in day length is the main reason, which is responsible for the circannual rhythm of reproductive activity. Horses are seasonal polyestrous breeders with a natural breeding season begins in the spring and extends to mid-summer in the northern hemisphere.

The most fertile period is from April to June associated with increase in daylight, temperature and availability of food. The seasonal reproductive features of stallions are characterized by an increase in testicular size and weight, sperm production and libido during the spring and summer (Pickett, 1993; Johnson and Thompson, 1983; Thompson et al., 1977). Moreover, spermatogenesis and reproductive tract function seem to be optimal during the breeding season.

Several previous studies attempted to characterize seasonal variations in equine semen quality. Unlike the majority of previous studies, attempts in this study were performed to investigate cellular processes, functional networks and biological functions in stallion in response to different seasonal conditions, which associated with increase of daylight and temperature during the breeding season.

For this experiment, eight warmblood stallions with known fertility were used. Stallions were divided in two groups in each one four animals with different fertility. Semen samples were collected during the breeding season (April-September) for the first group and during the non-breeding season for the second group (October-March). Using microarray approach a gene list with significant difference abundance between the groups (fold change> 1.5 und p< 0.05) was obtained. Gene list (DEG) was then submitted to DAVID and IPA to identify GO terms, canonical pathways and biological functions associated with these genes. The results of the present study identified 582 significantly different transcripts between breeding and non-breeding seasons.

These transcripts associated with different biological functions and molecular processes know to be fundamental for spermatogenesis, sperm function, regulation of stress response and energy metabolism.

5.2.1 Functional annotation analysis of DEG genes using DAVID database

DAVID was used for the functional Gene Ontology (GO) analysis of the differentially expressed genes. DAVID database showed significant enrichment of five genes in GO term acetylation with p= 0.0024 as presented in Table 9.

Acetylation

Spermatogenesis refers to the development of male germ cells from the spermatogonial stem cells to mature spermatozoa. This process occurs in the seminiferous tubule of the testis (Pang and Rennert, 2013). The differentiation of spermatogonia to mature spermatozoa involves extreme cellular, functional, genetic and chromatin changes. In the first stage of spermatogenesis, spermatogonia are amplified in numbers by mitotic division. Then spermatogonia undergo meiosis I to become first primary spermatocytes and by the end of the first meiotic division secondary spermatocytes. Spermiogenesis is the final stage of spermatogenesis, which refers to the dramatic differentiation and maturation of spermatids into mature sperm.

During the spermiogenic phase, spermatids undergo a chromatin-condensation process before they become mature spermatozoa. The purposes of such chromatin-condensation process are to facilitate the transport of spermatozoa through the female genital tract and to save the paternal genetic information. Chromatin-condensation process is achieved in elongated spermatids by the replacement of histones by transition proteins and subsequently by sperm-specific-protamines (Griffin et al., 2019; Jha et al.,2017; Balhorn, 2007; Balhorn et al., 1984).

After the fertilization process, these protamines must be removed from the DNA of zygote and replaced by histones in order to decondense and activate the chromatin of the developing embryo (Schagdarsurengin et al., 2012).

Male germ cells contain testis-specific histones in addition to the somatic histones, which are replaced by smaller protamines during spermatogenesis. Interestingly, in the last stage of spermatogenesis and prior to the removal and replacement of histones by transition proteins in elongated spermatids, the histones become highly acetylated (Gaucher et al., 2010). Protein acetylation is critical for correct DNA packaging and chromatin condensation. This process has long been observed in elongated spermatids in many species (Griffin et al., 2019; Ketchum et al., 2018). Acetylation is one of the most important modifications among over 200 others, which occur in mammalian cells.

Phosphorylation, acetylation, methylation, sumoylation and ubiquitination are the most common post-translational modifications involved in the regulation of protein function and structure of chromatin (Yu et al., 2015; Sheng et al., 2014; Vigodner, 2011; Nottke et al., 2009; Polevoda and Sherman, 2002; Sassone-Corsi, 2002).

Protein acetylation can be defined as the catalytic transfer of an acetyl moiety from acetyl CoA to a free amino-group of the target protein (Pang and Rennert, 2013). The addition of acetyl group to the free amino-group located at the end of histone decreases the affinity of histones to DNA allowing the protamines to interact with the DNA, which clarifies the important role of protein acetylation for the replacement of histones by protamines during the last stage of spermatogenesis (Oliva et al., 1987).

Protein acetylation is essential for sperm function and the increased number of acetylated protein during sperm capacitation might be required for the subsequent sperm-oocyte recognition and fertilization process (Yu et al., 2015; Kim et al., 2014). Previous studies showed that the inhibition of histone acetylation causes spermatid apoptosis in mice and an abruption in translation process (Yu et al., 2015; Xia et al., 2012).

Furthermore, Yu et al. (2015) suggested that the ejaculated spermatozoa might require dynamic acetylation to support sperm capacitation, sperm-oocyte recognition, fusion of sperm-oocyte plasma, and fertilization.

According to DAVID, in the present study five genes from the DEG list were involved in GO term ‘Acetylation’ (with $p = 0.0024$). This result highlights the importance of acetylation of protein in the regulation of spermatogenesis and male fertility.

5.2.2 Identification of biological functions and canonical pathways associated with DEG genes using IPA

The differentially regulated genes were then imported into IPA for biological functions, canonical pathways and network analysis. Most of the DEG were associated with biological functions related to cellular assembly and organization, cellular function and maintenance, cellular compromise, carbohydrate metabolism, lipid metabolism, organ morphology, organismal development and small molecule biochemistry.

Further analysis also conducted in IPA was to understand the enriched and significant canonical pathways of DEG identified in this dataset. In all, 27 significantly enriched canonical pathways were identified, using threshold values of $p < 0.05$, $FC > 1.5$.

Of these pathways, the significance was higher (based on p-values) for pathways associated with FAK signaling, clathrin-mediated endocytosis signaling, regulation of actin-based motility by Rho, VEGF signaling and actin cytoskeleton signaling.

As briefly summarized above, spermatogenesis is a regulated process by which spermatogonial stem cells divide and develop to form highly specialized mature spermatozoa. Spermatogenesis occurs in the epithelium of seminiferous tubules.

The seminiferous tubule is the functional unit in the mammalian testis, which produces millions of spermatozoa each day since puberty.

The seminiferous tubule is anatomically divided into two distinct compartments, the basal compartment in which spermatogonia and early spermatocytes are found, and the apical compartment containing more developed spermatocytes as well as spermatids and spermatozoa. These two compartments are separated by the blood-testis barrier (BTB) (Li et al., 2012). During the process of spermatogenesis, the renewal of spermatogonia and their differentiation up to fully developed spermatocytes take place in the basal compartment of the seminiferous tubule, whereas the first and second meiosis of spermatocytes to form spermatids occur in the apical compartment of the seminiferous tubule. Thus, the transition of developing sperm across the epithelium need a wide junction restructuring at the sertoli- sertoli and sertoli- germ cell interface (Gungor-Ordueri et al., 2014; Li et al., 2012). The mechanism, which governs and controls all these cell- cell junctions during spermatogenesis have been studied and explored in which the focal adhesion kinase (FAK) plays an important role. FAK is crucial regulator, which regulates and modulates the junction restructuring at the sertoli-sertoli interface at the BTB as well as at the sertoli-spermatid interface (Cheng et al., 2013; Li et al., 2012).

FAK is a non-receptor protein tyrosine kinase, which has been found to be expressed in mammalian tissues including brain (Andre and Beckerandre, 1993), lymphocytes (Whitney et al., 1993) and testis (Mulholland et al., 2001; Wine and Chapin, 1999).
FAK is found in all mammalian cells and/or tissues (Broussard et al., 2008).
FAK involved in a wide of cellular events such as cell adhesion and cell migration in mammalian tissues. It is mostly used by motile cells such as fibroblasts, lymphocytes and metastatic cancer cells for their movement over basal lamina under physiological or pathophysiological conditions. As briefly shown above, FAK involved in the events of sertoli- sertoli and sertoli-germ cell adhesion and germ cell transport across the epithelium during spermatogenesis because of the active restructuring at the cell junctions so that the spermatocytes can enter the apical compartment of the seminiferous epithelium for their further development processes. This adhesion between sertoli cells and germ cells in the seminiferous epithelium is very essential for the process of sperm formation.
Also one of the most significant enriched canonical pathways using IPA is clathrin-mediated endocytosis. Clathrin mediated endocytosis is a cellular pathway, which allows cells to internalize essential nutrients such as ion channel, receptors, iron, cholesterol and extracellular molecules. In addition, this cellular pathway allows cells to remove activated receptors from the cell surface (Twyman, 2009). During this cellular pathway, clathrin mediates the internalization of various receptors and ligands, bringing them to the cell within a protein-coated vesicle (Twyman, 2009). In addition to the role of clathrin-mediated endocytosis as a coat to mediate the bringing of nutrients into cells, this cellular pathway has a range of functions such as control the density of receptors on the cell surface, regulating the surface expression of proteins and controlling the activation of signaling pathways (McMahon and Boucrot, 2011). Furthermore, protein endocytosis initiates the process of endosomal signaling, which regulates signaling function in mammalian cells in response to changes in environmental conditions such as the presence of toxicant, heat and ions during growth, development and other cellular processes such as spermatogenesis. In addition, the endosomal signaling affects multiple cellular events such as migration, cell division, metabolism, survival and proliferation (Xiao et al., 2014).
During spermatogenesis in the testis, developing spermatocytes migrate from the basal to the apical compartment and cross the BTB of the seminiferous epithelium cycle for further development (Gao and Lui, 2013; Cheng and Mruk, 2002). This movement of spermatozoa involves extensive junction restructuring at the BTB, which allows

progressive movement of developing germ cells along the seminiferous epithelium and provides physical support of developing germ cells (Gao and Lui, 2013).

Yan et al. (2008) assessed the kinetics of endocytosis and recycling of BTB-associated integral membrane proteins, and demonstrated that these proteins were continuously endocytosed and recycled back to the sertoli cell surface via the clathrin-mediated endocytosis pathway.

In addition, results derived from other studies using specific inhibitor to clathrin-mediated endocytosis showed that the blocking of this pathway also lead to block in micropinocytosis, phagocytosis and protein tyrosine phosphatase (Xia et al., 2009; Gerhard et al., 2003; Massol et al., 1998; Retta et al., 1996; Frost et al., 1989).

Based on IPA in the present study, FAK signaling and clathrin-mediated endocytosis signaling are the most highly significantly enriched canonical pathways identified for the DEG. These pathways are known to be the fundamental regulatory elements involved in cell-cell junctions during spermatogenesis and in coordinating cellular events that occur across the seminiferous epithelium during the process of spermatogenesis.

Furthermore, IPA identified significantly enriched metabolic pathways that were related to inositol hexakisphosphate and triphosphate biosynthesis and metabolism.

Inositol is a chemical compound, which considered as a number of vitamin B complex. Inositol is involved in several biological processes such as control of the intracellular Ca^{2+} concentration, in the cytoskeleton assembly and in gene expression. Furthermore, inositol is an important component of structural lipids (Calogero et al., 2015; Condorelli et al., 2011). The most widespread form of inositol in nature is myoinositol, which plays an important role in the male reproductive system (Calogero et al., 2015). In addition, myoinositol have been reported to mediate different events involved in fertilization process, which include spermatozoa migration from the vagina to the fallopian tubes, penetration the cumulus oophorus of the oocytes, binding to the zona pellucida and acrosome reaction (Calogero et al., 2015).

Oliva et al. (2016) investigated in their study the effect of dietary supplement of andrositol, which contains myoinositol as principal compound, selenium and L-arginine, in patients affected by reduced sperm motility with metabolic syndrome. They found that andrositol normalized the metabolic profile of these patients improving their insulin sensitivity. In addition to that, testosterone levels were increased and the semen quality such as sperm motility and morphology as well as sperm concentration were also highly

improved. These findings support the identification of myoinositol within the top metabolic pathways in the present study.

Network analysis using IPA

In this study, IPA also creates networks, where the uploaded differently expressed genes between breeding and non-breeding season can be connected in either direct or indirect interactions using the Ingenuity Knowledge Base. The most significant ten networks produced by IPA have significant scores of at least 10 (ranged between 10 and 44); indicating that each of these networks is linked to biological process, which are affected when the seasonal conditions are changed between breeding and non-breeding seasons. The most highly significant two networks produced by IPA (score = 44) clustered together molecules linked to cardiovascular disease, organismal injury and abnormalities, skeletal and muscular disorders, embryonic development, tissue morphology and cellular development. Furthermore, two networks were found to be enriched for biological functions, which related to development of reproductive system ($p=0.018$) and gonadogenesis ($p= 0.033$). Many of genes in both networks encode proteins, which are known to be important for sperm formation, cell cycle, apoptosis and regulation of stress response. One of these genes is Forkhead box O3 (FOXO3), which is upregulated in both networks. The FOXO belong to the O subclass of the forkhead box transcription factor (Hosaka et al., 2004; Arden and Biggs, 2002). There are four types of FOXO transcription factors FOXO1, FOXO3, FOXO4 and FOXO6 (Choi et al., 2015). These transcription factors are involved in a number of physiological and pathological processes including apoptosis, control of the cell cycle, aging and cancer. In addition, FOXO proteins regulate stress response, insulin sensitivity and ontogenesis (Choi et al., 2015; Huang and Tindall, 2007; Lee et al., 1999). In the study of Choi et al. (2015), the FOXO3 expression and location were investigated from mouse embryonic stage to 12 weeks. They found that FOXO3 plays an important role in the activity and regulation of leydig cells in the testis. Another gene, which has been found to be upregulated in both biological networks, is nuclear receptor activator A4 (NcoA4). NcoA4 belongs to the nuclear receptor coregulator family, which consists of nuclear receptor coactivators and nuclear receptor corepressors. The nuclear receptor coactivators accomplish reactions required for activation of transcription whereas the nuclear receptor corepressors suppress transcription (Bulynko and O'Malley, 2011; Tetel, 2009). Nuclear receptors contain a large family of transcription factors, which bind to the DNA and regulate the expression of target genes in response to steroid hormones and other ligands (Bulynko and O'Malley,

2011; McKenna and O'Malley, 2002). Nuclear receptor coregulators have emerged as the principal modulators of the function of nuclear receptors and other transcription factors (Dasgupta et al., 2014). Nuclear receptor coactivators have been shown to be essential for efficient transcriptional activity of steroid receptors (Molenda et al., 2003), which act in brain and throughout the body and have profound effects on development, reproduction, homeostasis and behavior (Tetel, 2009).

NcoA4 is an unique nuclear receptor coactivator, which is expressed in various cell lines during early development, particularly in cardiac, hepatic, and lung tissue (Kollara and Brown, 2010) and is altered in the case of breast, ovarian and prostate cancers (Kollara and Brown, 2010; Mestayer et al., 2003; Magklara et al., 2002). NcoA4 is expressed in moderate amount in different adult rodent tissues such as heart, lung, kidney, stomach, brain and prostate (Kollara and Brown, 2010; Siriett et al., 2006; Alen et al., 1999).

A high level of NcoA4 expression was reported in the testis (Kollara and Brown, 2010; Alen et al., 1999).

NcoA4 interacts with and amplifies the activity of several ligand-activated nuclear transcription factors such as the androgen receptor, estrogen, progesterone, glucocorticoid and vitamin D (Kollara and Brown, 2010; Kollara and Brown, 2006; Lanzino et al., 2005; Ting et al., 2005; Heinlein and Chang, 2003; Yeh and Chang, 1996).

Kollara and Brown (2010) investigated in their study the significant role of NcoA4 during normal mouse development and reported that NcoA4 may exert important regulatory effects on the actions during development and adulthood. They also investigated the important role of NcoA4 on the development process by altering nuclear receptor activity.

There is many literature about the role and function of FOXO proteins and nuclear receptor coactivators in the regulation of development and reproduction. In the present study, two networks were found to be enriched for biological functions, which related to development of reproductive system and gonadogenesis. FOXO3 and NcoA4 were found to be upregulated in both functional networks. These proteins are known to be the principal regulatory elements involved in a number of physiological processes, which are responsible for the reproduction process.

In this study and unlike to the majority of previous studies, attempts to investigate molecular processes in response to different seasonal conditions associated with increase of daylight and temperature during the breeding season, were performed. Therefore, it is difficult to compare this study with other studies. Furthermore, there is not much literature about the molecular processes in stallion, which are different between breeding and non-

breeding seasons. In addition to that, there is only a few experiments, which were achieved in the field of gene expression in stallion. Therefore, I have tried to discuss the different roles and functions of many biological functions, pathways and networks, which were found to be significantly different between the breeding and non-breeding seasons.

5.3 Investigation of molecular processes linked to stallion fertility

Research into human fertility as well as that of other mammals has advanced considerably in recent years. However, research into the molecular mechanisms affecting stallion fertility remains in the beginning. Genetic markers associated with stallion fertility may be useful in selection and breeding management. Analysis of stallion sperm transcriptome by microarray analysis in the present study allowed comparison of transcript abundance between fertile and subfertile stallions.

Analysis of the sperm transcriptome by gene expression microarray is typically affected by the RNA isolation technique used. RNA degradation is one of the most crucial problems in the RNA-extraction process. The concentration of RNA in spermatozoa is lower than in somatic cells. Therefore, it is essential to avoid any RNA degradation during the extraction process. Furthermore, global microarray analysis of the low quantity of RNA in samples obtained from spermatozoa compared with the quantity of RNA obtained from somatic cells, has traditionally required either one or more amplification steps or mixing of samples depending on the RNA concentration to obtain sufficient nucleic acid material for subsequent detection. In the present study, the RNA samples for each stallion were pooled, glycogen was also used as a coprecipitant for the small amounts of RNA and the purity of the RNA samples was checked using RT-PCR and a set of primers specific to protamine 2.

In several previous studies, many genes and proteins were analyzed for their association with fertility in stallion (Zaabal and Ahmed, 2010; Hamann et al., 2005; Schambony et al., 1998). For example, equine cysteine-rich secretory protein (CRISP) genes were found to be significantly associated with male fertility (Schambony et al., 1998).

The CRISP proteins represent the major equine fraction of seminal plasma proteins and they were found to be involved in sperm–oocyte fusion (Töpfer-Petersen et al., 2005). Furthermore, Giesecke et al. (2011) chose three genes, namely angiotensin-converting enzyme (ACE), sperm autoantigenic protein 17 (SPA17) and FSH beta subunit (FSHB), to test as candidates for determining Hanoverian stallion fertility, finding that haplotypes

of all three genes significantly contributed to the paternal and embryonic fertility components of the pregnancy rate per oestrus.

In addition, Gamboa and Ramalho-Santos (2005) investigated the presence of soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins in equine spermatozoa and found that stallions with fertility problems had the worst sperm and acrosomal membrane quality as well as fewer sperm cells that stained positive for SNAREs and caveolin1 compared with spermatozoa from fertile donors.

In the present study, the microarray approach was used to identify molecular pathways relevant to stallion fertility. Six stallions with different fertility were chosen, as evaluated in terms of semen profile (progressive motility and percentage of spermatozoa with normal morphology) and percentage of pregnancies per season calculated by dividing the number of all pregnant mares (for each stallion individually) by the number of mares bred during the year.

Recently, many developments have been achieved in the field of gene expression analysis, providing new molecular detection tools to aid the understanding of stallion fertility (Novak et al., 2010; Bright et al., 2009).

RNA in the spermatozoa could be used in genomic analysis to evaluate male fertility. Currently, several studies have emerged focusing on the analysis of transcripts or proteins in spermatozoa that could be associated with male fertility.

Feugang et al. (2010) identified 415 different expressed transcripts from high- and low-fertility Holstein bulls. These transcripts were associated with different cellular functions and biological processes. Kropp et al. (2017) focused in their study on bull fertility and demonstrated that bulls with different fertility status delivered embryos with different transcriptome profiles despite similar morphology of these embryos and adequate development to blastocyst stage.

In addition, the identification of paternal components, which are delivered into the oocyte during fertilization, could lead to a better understanding of male fertility and suitable selection of breeding sires.

Bansal et al. (2015) identified several genes and molecular pathways involved in spermatogenesis and male fertility. In that study, analysis of 2081 transcripts that were differentially expressed between three groups of men with known fertility, showed that some of these transcripts were related to heat shock proteins, testis-specific genes and Y chromosome genes, which suggested that sperm RNA has considerable potential as a marker in the evaluation of fertility.

Proteomics has also been used to identify putative biomarkers associated with male fertility. In particular, Ashrafzadeh et al. (2013) identified several proteins that differed in abundance between two groups of fertile and subfertile zebu cattle using frozen semen samples. These proteins were involved in energy metabolism, glycolysis, sperm motility and male fertility.

Similarly, in the present study, the RNA profile of spermatozoa obtained from fertile and subfertile stallions were compared using affymetrix microarray technology. The analysis identified 437 transcripts that differed significantly between fertile and subfertile stallions. These transcripts were associated with different biological functions and molecular processes known to be fundamental for spermatogenesis and male fertility.

5.3.1 Functional annotation and KEGG pathway analysis of DE using DAVID database

DAVID software tool was used to identify gene-associated molecular functions that were overpresented in the gene list generated by the comparative gene expression analysis between fertile and subfertile stallions.

DAVID database showed significant enrichment of three genes (OAS1, OAS2 and RPL5) in GO term RNA binding with a p-value of 0.05. In addition, eight genes from the submitted gene list (CSF3R, GM-CSF, IL17A, IL13, IL22RA1, CD134, CD27, SF9) were found significantly enriched in the KEGG pathway cytokine-cytokine receptor interaction with (p= 0.02) as summarized in Table 11.

RNA-binding proteins

RNA-binding proteins (RBPs) are a class of proteins that are expressed in the nucleus and are defined by their ability to bind RNA. RBPs play important roles in the organism.

They are involved in many cellular processes that occur during tissue development. Moreover, these proteins regulate immune response, formation of dendrites and the differentiation of embryonic stem cells (Idler and Yan, 2012; Colegrove-Otero et al., 2005).

RBPs are highly expressed during spermatogenesis and play an essential role during all stages of germ cell development. During mammalian spermatogenesis, chromatin structure is markedly modified, resulting in the shutting down of nuclear gene expression in mature spermatozoa (Miller and Ostermeier, 2006).

In elongating and condensing spermatids, the histones are largely removed and replaced first by the transition proteins, which are subsequently displaced with sperm-specific protamines (Balhorn et al., 1984). The resulting chromatin is highly condensed and the

sperm nucleus is transcriptionally inert and does not contain sufficient rRNA to support translation (Miller et al., 1999).

In post-meiotic spermatozoa, de novo transcription is silenced and the synthesis and storage of sufficient mRNA is very important to compensate for the lack of mRNAs during the subsequent transcriptionally inactive stage of spermatogenesis (Sassone-Corsi, 2002). Germ cells have been shown to express high levels of RBPs throughout spermatogenesis, which are very essential to post-transcriptional events during all stages of spermatogenesis (Idler and Yan, 2012). According to DAVID, in the present study three genes from the DE genes were involved in GO term 'RNA binding'.

This result highlights the importance of RBPs in the regulation of spermatogenesis and male fertility (Suliman et al., 2018).

Bettegowda and Wilkinson (2010) reported the role of four RBPs, namely gonadotrophin-regulated testicular RNA helicase (GRTH), Src-associated in mitosis 68 kDa (SAM68), mouse Y-box protein 2 (MSY2) and deleted in azoospermia associated protein-1 (DAZAP1), which were shown to be critical for spermatogenesis by promoting the translation of a subset of germ cell mRNAs, indicating the importance of translation control for normal spermatogenesis. Furthermore, Idler and Yan (2012) reported that in mice, the inactivation of many of the RBPs studied caused infertility and spermatogenic arrest at various steps of germ cell differentiation.

Cytokine–cytokine receptor interaction

The results of the present study also indicated that eight genes from the gene list created (i.e. CSF3R, GM-CSF, IL17A, IL13, IL22RA1, CD134, CD27 and SF9) were significantly enriched in the KEGG pathway cytokine–cytokine receptor interaction (Suliman et al., 2018).

Cytokines are a broadly defined group of regulatory proteins that have a wide range of biological activities in addition to their original functions in the immune system (Ihsan, 2014; Hales et al., 1999). Several cytokines have direct effects on testicular cell function, and several of these are involved in normal reproductive physiology and fertility regulation (Hedger and Meinhardt, 2003; Hales et al., 1999).

Cytokines act as growth and differentiation factors during pathological states, as well as under normal physiological conditions (Hales et al., 1999). Various immunological factors, cytokines, chemokines and growth factors have been documented in human semen (Poltich et al., 2007), as well as that from rodents (Ingman and Rebecca, 2008) and other livestock species (Paulesu et al., 2010). These molecules in semen affect vaginal

immunology and female fertility following insemination and play an important role in regulating the proliferation, viability and differentiation of blastomeres in embryos (Kane et al., 1997). However, the presence of cytokines in stallion spermatozoa were investigated, whose quantitative expression levels were significantly different between fertile and subfertile stallions (Suliman et al., 2018).

It has been reported that cytokines and growth factors play an essential role in both implantation and development of embryos (Sharkey, 1998).

Robertson et al. (2001) reported the important role of GM-CSF in murine preimplantation embryos by promoting blastocyst formation and increasing the number of viable blastomeres by reducing the incidence of apoptosis and increasing the uptake of glucose. In addition, the in vitro culture of human embryos with GM-CSF showed that GM-CSF has a positive effect on the development of embryos to the blastocyst stage by increasing the number of cells in the inner cell mass and in the trophectoderm (Sjöblom et al., 1999). Many other cytokines have been studied extensively in mice and other mammalian species including IL-1, IL-2, IL-6, IL-8, IL-10, colony-stimulating factor 1 (CSF1) and anti-inflammatory cytokines of the transforming growth factor b family, where some of them are directly involved in the production of male and female gametes, embryo implantation and development (Robertson et al., 2007; Hedger and Meinhardt, 2003; Sharkey, 1998; Simo´n et al. 1998).

5.3.2 Identification of biological functions and canonical pathways associated with DE genes using IPA

IPA was used to obtain a deeper understanding of the significantly altered biological functions and canonical pathways associated with the DE genes that were identified in the microarray analysis. IPA of DE genes showed several different associated biological functions. The target genes were significantly enriched for diverse cellular functions and biological processes know to be fundamental for spermatogenesis and male fertility including cellular assembly and organization, cellular function and maintenance, cellular development, cellular growth and proliferation, tissue development, cell morphology and organismal injury and abnormalities.

Further analysis was also conducted in IPA to understand the enriched and significant canonical pathways of DE genes identified in this dataset. In all, 17 significantly enriched canonical pathways were identified, using threshold values of $p \leq 0.05$ and $FC \geq 1.2$.

Of these pathways, significance was higher (based on p-values) for pathways associated with G1/S checkpoint regulation, cyclin and cell cycle regulation, ataxia-telangiectasia mutated (ATM) signaling, netrin signaling and natural killer cell signaling.

Sperm formation refers to the differentiation of diploid spermatogonia into mature spermatozoa. During this transformation, spermatogonia grow in the first gap phase (G1), synthesize DNA in the synthesis phase (S), prepare for mitosis in the second gap phase (G2) and undergo mitosis and meiosis in the mitosis phase (M) (Ruwanpura et al., 2010). The G1 phase is the longest phase in the cell cycle during which the cell grows continuously and undergoes most of its physiological activity.

The next phase of the cell cycle is the S phase during which DNA replication occurs. Therefore, passing the G1 phase is tightly controlled by both cell cycle machinery and checkpoint pathways (Neganova and Lako, 2008). The G1/S checkpoint is essential to ensure that all conditions of cell division are successfully met and to guarantee the possibility of replicating the DNA and producing healthy daughter cells.

This transition is signaled and controlled by cyclins and cyclin-dependent kinases (CDKs) (Wolgemuth et al., 2013; Neganova and Lako, 2008). Cyclins are the principal cell cycle regulatory proteins and have been identified in simple as well as higher-order organisms (Wolgemuth et al., 2002). Cyclins can be divided into eight classes (cyclin A-H) according to their amino acid similarity and the timing of their appearance during the cell cycle (Wolgemuth et al., 2002).

Although the function of cyclins during mitosis and meiosis has been studied in somatic cells, their function during mitosis and meiosis in the germ line is poorly understood. Wolgemuth et al. (2013) reported that cyclin A2 is highly expressed in differentiated type A and B spermatogonia and plays an important role during both meiosis and mitosis in the germ cell line.

Based on the IPA in the present study, G1/S checkpoint regulation and cyclin and cell cycle regulation are the most highly significantly enriched canonical pathway identified for the DE genes. These pathways are known to be the principal regulatory elements involved in cell cycle regulation and therefore play an important role during sperm proliferation and male fertility (Suliman et al., 2018).

Furthermore, IPA identified significantly enriched metabolic pathways that were related to phosphatase and D-myoinositol phosphate metabolism. Inositol is a cyclic polyol that is considered as a member of vitamin B complex. Myoinositol is the most available form

of inositol in nature. Myoinositol is distributed in the tissues of many organisms and plays an essential role in the signal transduction system in cells (Condorelli et al., 2011).

In addition, inositol is involved in several activities and pathways such as mRNA transcription, cytoskeleton and chromatin remodeling and tumor protein (P53) activity (Bizzarri et al., 2016). In conclusion, myoinositol plays a key role in the male reproductive system by regulating the osmolality of the seminal plasma as well as sperm motility and acrosome reaction (Condorelli et al., 2011).

Condorelli et al. (2012) suggested that myoinositol significantly increased the percentage of spermatozoa with progressive motility based on 2 h incubation of human spermatozoa with 2 mg ml^{-1} myoinositol.

The increase in progressive motility of spermatozoa was associated with a significant increase in the percentage of spermatozoa with high mitochondrial membrane potential. In addition, inositols have been reported to affect different processes involved in oocyte fertilization, which improve the penetration of the ovum cumulus oophorus, binding with the zona pellucida and the acrosome reaction (Calogero et al., 2015).

Furthermore, high concentrations of myoinositol have been found in the fluid of the seminiferous tubule in many mammals (Chauvin and Griswold, 2004; Hinton et al., 1980; Setchell et al., 1968). Myoinositol is produced by two enzymatic steps from glucose-6-phosphate and its synthesis is regulated by myoinositol-1-phosphate synthase and myoinositol monophosphatase-1. This activity of these two enzymes is high in the testis (Chauvin and Griswold, 2004).

These findings support the identification of myoinositol within the top metabolic pathways in the present study (Suliman et al., 2018).

In summary, prediction of stallion fertility is one of the most important problems in horse breeding and the analysis of semen characteristics has limited capacity for predicting fertility. In addition, there are only a few genetic studies on stallion fertility, which may provide possibilities and new tools that would help in predicting male fertility.

In the present study, comparison of transcript abundance between fertile and subfertile stallions provided a glimpse of the various functional pathways involved in stallion fertility. Furthermore, this analysis confirmed the involvement of the immune response in stallion fertility. These results identify molecular processes in spermatozoa that are associated with stallion fertility.

6 Summary

Fertility of stallion is of increasing importance in the horse industry with a complex environmental and genetic background. Horses have the lowest reproductive rate among farm animals. This relatively low fertility may be influenced with breeding and care, which is different between the animals used for sport and those that are exclusively used in breeding. Furthermore, selection of equines for breeding is based on their pedigree, athletic prowess and conformation characteristics with a neglected attention on the fertility potential during selection.

The aim of the first part of this study was to evaluate different semen quality parameters in groups of fertile and subfertile stallions during the breeding season and non-breeding season in order to investigate semen quality parameters associated with high stallion fertility as measured by pregnancy rate and to characterize seasonal variations in equine semen quality, namely monitoring ejaculate volume, sperm concentration, total sperm count, sperm motility and sperm morphology. To achieve this purpose, eight stallions with different fertility were collected during the breeding and non-breeding season. CASA system was used to determine sperm motility and eosin-nigrosin stain to evaluate sperm morphology. The results of this part indicated that differences between fertile and subfertile stallion were observed only in breeding season and a few of CASA derived parameters seemed to be significantly lower during non-breeding season than during the breeding season in both fertile and subfertile stallions.

In the second part of this study and unlike to the majority of previous studies, attempts to investigate molecular processes in response to different seasonal conditions associated with increase of daylight and temperature during the breeding season, were performed by comparing the microarray-derived sperm transcriptomes of stallions between breeding and non-breeding seasons.

For this experiment, eight warmblood stallions with known fertility were used. Stallions were divided in two groups in each one four animals with different fertility. Using microarray approach a gene list with significant different abundance between the seasons (fold change >1.5 und $p < 0.05$) was obtained. In this experiment, 581 genes were found having significantly different abundance of transcripts between breeding and non-breeding seasons. These transcripts associated with different biological functions and molecular processes know to be fundamental for spermatogenesis, sperm function, regulation of stress response and energy metabolism.

The aim of the last part was to identify molecular processes that are associated with male fertility by comparing microarray-derived sperm transcriptomes of stallions that were clearly assigned to either a fertile or subfertile group based on reproductive success and sperm characteristics. For this experiment, six warm blood stallions were used. Stallions were divided into two groups that were different in pregnancy rates and proportions of normal and progressive motile sperm. Using microarray approach a gene list with significant different abundance between the groups (fold change ≥ 1.2 und $p \leq 0.05$) was obtained. In all, there were 437 differentially expressed genes between fertile and subfertile stallion groups. These transcripts associated with different biological functions and molecular processes know to be fundamental for spermatogenesis and male fertility.

In summary, prediction of stallion fertility is one of the most important problems in horse breeding and the analysis of semen characteristics has limited capacity for predicting fertility. In addition, there are only a few genetic studies on stallion fertility, which may provide possibilities and new tools that would help in predicting male fertility.

The comparison of transcript abundance between fertile and subfertile stallions and between breeding and non-breeding seasons provided a glimpse of the various functional pathways involved in stallion fertility. The results of the present study may help predict stallion fertility as well as in the selection of future breeding stallions.

A follow-up of this study would be to confirm the results in a large sample of stallions and to optimize the sperm RNA isolation procedure by finding specific primers for somatic cells and for sperm cells in order to isolate pure sperm RNA.

Furthermore, analyses are necessary to find the relationship between the immune system and male fertility and to identify the role of different molecular processes in the regulation of spermatogenesis during the breeding and non-breeding seasons.

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8 Appendix

Appendix I

Table A1: individual assessment of fresh semen for 64 ejaculates collected from eight stallions in breeding and non-breeding seasons

Stallion	Group	Season	Ejaculate	Ejaculate volume [ml]	Sperm concentration [10^6 ml^{-1}]	Total sperm number [10^9]
1	Fertile group	Breeding season	1	60	210	12.600
1			2	35	190	6.650
1			3	80	180	14.400
1			4	50	210	10.500
1	Fertile group	Non-breeding season	1	40	217	8.680
1			2	36	309	11.124
1			3	60	160	9.600
1			4	35	245	8.575
2	Fertile group	Breeding season	1	50	438	21.900
2			2	30	360	10.800
2			3	64	419	26.816
2			4	46	250	11.500
2	Fertile group	Non-breeding season	1	35	420	14.700
2			2	30	430	12.900
2			3	34	357	12.138
2			4	34	425	14.450
3	Fertile group	Breeding season	1	35	386	13.510
3			2	35	335	11.725
3			3	25	332	8.300
3			4	30	465	13.950

3	Fertile group	Non-breeding season	1	10	537	5.370
3			2	35	448	15.680
3			3	22	473	10.406
3			4	20	424	8.480
4	Fertile group	Breeding season	1	50	183	9.150
4			2	45	159	7.155
4			3	55	120	6.600
4			4	40	168	6.720
4	Fertile group	Non-breeding season	1	20	378	7.560
4			2	20	360	7.200
4			3	37	407	15.059
4			4	20	383	7.660
5	Subfertile group	Breeding season	1	32	305	9.760
5			2	56	238	13.328
5			3	26	443	11.518
5			4	26	381	9.906
5	Subfertile group	Non-breeding season	1	20	561	11.220
5			2	26	415	10.790
5			3	30	410	12.300
5			4	20	460	9.200
6	Subfertile group	Breeding season	1	70	160	11.200
6			2	55	290	15.950
6			3	60	170	10.200
6			4	50	220	11.000
6	Subfertile group	Non-breeding season	1	30	459	13.770
6			2	30	385	11.550
6			3	40	360	14.400
6			4	35	430	15.050

7	Subfertile group	Breeding season	1	50	173	8.650
7			2	25	153	3.825
7			3	55	117	6.435
7			4	50	172	8.600
7	Subfertile group	Non-breeding season	1	28	177	4.956
7			2	45	226	10.170
7			3	20	207	4.140
7			4	15	189	2.835
8	Subfertile group	Breeding season	1	30	226	6.780
8			2	40	187	7.480
8			3	42	234	9.828
8			4	30	262	7.860
8	Subfertile group	Non-breeding season	1	20	364	7.280
8			2	30	293	8.790
8			3	20	371	7.420
8			4	30	246	7.380

Table A2: individual assessment of sperm morphology for 64 ejaculates collected from eight stallions in breeding and non-breeding seasons

Stallion	Group	Season	Ejaculate	Normal sperm [%]	Acrosome defect [%]	Head defect [%]	Detached sperm head [%]	Midpiece defect [%]	Proximal plasma droplet [%]	Distal plasma droplet [%]	Tail defect [%]	Duplications [%]
1	Fertile group	Breeding season	1	76	0	5	1	8	2	5	3	0
1			2	78	7	0	2	9	2	0	2	0
1			3	76	3	2	1	10	1	4	3	0
1			4	73	3	0	3	15	0	3	3	0
1	Fertile group	Non-breeding season	1	59	0	5	5	20	0	0	11	0
1			2	60	1	0	2	27	2	1	7	0
1			3	57	5	2	2	23	1	2	8	0
1			4	61	4	3	2	19	3	4	4	0
2	Fertile group	Breeding season	1	55	3	7	2	20	9	0	3	1
2			2	56	8	5	2	19	8	0	2	0
2			3	50	6	8	3	26	6	0	1	0
2			4	52	5	8	2	21	9	0	3	0
2	Fertile group	Non-breeding season	1	40	3	4	0	33	17	0	3	0
2			2	44	7	6	1	33	4	0	5	0
2			3	29	3	4	0	36	21	1	6	0
2			4	32	10	8	3	32	9	0	6	0
3	Fertile group	Breeding season	1	73	2	2	2	16	2	1	1	1
3			2	62	2	5	2	20	6	1	0	2
3			3	78	1	2	1	12	2	1	2	1
3			4	66	2	9	2	17	3	0	0	1
3	Fertile group	Non-breeding season	1	49	0	5	8	32	3	0	2	1
3			2	37	12	7	6	36	0	0	2	0
3			3	59	3	1	2	28	1	0	6	0
3			4	66	2	0	1	24	3	0	4	0

4	Fertile group	Breeding season	1	74	2	3	2	16	1	1	1	0
4			2	69	2	2	1	22	0	2	1	1
4			3	66	6	3	0	19	1	2	3	0
4			4	69	4	4	0	19	1	1	2	0
4	Fertile group	Non-breeding season	1	40	3	5	2	39	8	1	2	0
4			2	43	1	0	2	41	7	4	0	2
4			3	41	3	3	2	35	6	6	3	1
4			4	40	6	5	2	40	1	2	2	2
5	Subfertile group	Breeding season	1	50	0	8	2	18	6	8	8	0
5			2	49	1	6	4	21	5	8	6	0
5			3	51	3	7	4	24	2	5	4	0
5			4	57	0	3	2	16	9	5	8	0
5	Subfertile group	Non-breeding season	1	36	1	7	0	39	0	0	17	0
5			2	38	4	6	2	42	2	3	3	0
5			3	38	8	3	4	26	15	0	5	1
5			4	35	1	8	1	40	3	9	3	0
6	Subfertile group	Breeding season	1	39	18	1	1	30	4	3	3	1
6			2	40	6	3	0	21	9	21	0	0
6			3	50	9	1	0	19	15	5	0	1
6			4	52	5	2	2	18	12	4	4	1
6	Subfertile group	Non-breeding season	1	35	9	5	0	34	11	6	0	0
6			2	38	5	1	1	45	6	3	1	0
6			3	40	8	2	4	30	4	7	3	2
6			4	37	8	2	1	29	9	12	2	0
7	Subfertile group	Breeding season	1	15	4	10	6	56	5	1	3	0
7			2	15	6	11	6	61	0	0	1	0
7			3	16	11	12	10	44	1	1	4	1
7			4	20	3	6	6	61	0	0	2	2

7	Subfertile group	Non-breeding season	1	10	1	17	17	53	1	0	1	0
7			2	11	2	12	7	66	1	0	0	1
7			3	17	1	13	6	61	0	0	2	0
7			4	14	2	12	9	59	2	0	1	1
8	Subfertile group	Breeding season	1	37	0	3	3	46	7	1	3	0
8			2	38	14	6	6	32	0	0	3	1
8			3	36	11	4	4	34	1	0	9	1
8			4	38	12	5	4	35	1	2	1	2
8	Subfertile group	Non-breeding season	1	37	7	4	10	37	2	1	2	0
8			2	26	5	4	1	57	1	3	2	1
8			3	31	6	1	5	49	1	2	4	1
8			4	40	6	4	2	42	1	4	1	0

Table A3: individual assessment of sperm motility for 64 ejaculates collected from eight stallions in breeding and non-breeding seasons

Stallion	Group	Season	Ejaculate	Total motility [%]	Progressive motility [%]	VAP [µm s^{-1}]	VCL [µm s^{-1}]	VSL [µm s^{-1}]	STR [%]	LIN [%]	WOB [%]	ALH [µm]	BCF [Hz]
1	Fertile group	Breeding season	1	80.13	67.58	78.38	133.03	56.93	72.00	42.00	58.00	3.76	31.23
1			2	89.30	81.11	84.12	139.11	62.54	74.00	44.00	60.00	3.74	32.90
1			3	82.86	70.45	67.36	112.49	53.17	78.00	47.00	59.00	3.27	32.77
1			4	82.38	68.93	83.61	142.90	60.00	71.00	41.00	58.00	3.90	30.88
1	Fertile group	Non-breeding season	1	63.77	47.83	68.47	115.55	49.51	72.00	42.00	59.00	3.62	31.63
1			2	64.45	49.16	79.43	129.82	57.84	72.00	44.00	61.00	3.83	31.24
1			3	70.51	61.79	89.52	148.59	70.32	78.00	47.00	60.00	3.55	34.61
1			4	61.34	50.19	41.26	70.08	23.48	56.00	33.00	58.00	3.07	25.61
2	Fertile group	Breeding season	1	78.78	60.89	75.39	121.42	49.89	66.00	41.00	62.00	3.59	34.08
2			2	81.57	67.19	68.17	110.86	47.87	70.00	43.00	61.00	3.52	33.77
2			3	79.86	58.63	66.35	111.92	42.50	64.00	37.00	59.00	3.89	30.10
2			4	75.00	58.59	70.93	114.25	49.09	69.00	42.00	62.00	3.55	33.93
2	Fertile group	Non-breeding season	1	50.32	40.08	58.73	105.18	40.93	69.00	38.00	55.00	3.20	32.83
2			2	49.44	38.35	63.75	114.29	40.64	63.00	35.00	55.00	3.54	29.47
2			3	70.16	54.55	56.33	117.24	41.11	72.00	35.00	48.00	3.75	30.33
2			4	65.39	48.86	51.29	90.29	30.07	58.00	33.00	56.00	3.74	27.33
3	Fertile group	Breeding season	1	89.30	79.98	84.12	139.11	62.54	47.00	44.00	60.00	3.74	32.90
3			2	80.13	67.58	78.38	133.03	56.93	72.00	42.00	58.00	3.76	31.23
3			3	82.38	68.93	83.61	142.9	60.00	71.00	41.00	58.00	3.90	30.88
3			4	80.50	73.86	81.96	139.06	60.01	62.00	43.00	58.00	3.32	32.63
3	Fertile group	Non-breeding season	1	79.13	47.84	45.32	73.26	27.72	61.00	37.00	61.00	3.29	26.77
3			2	77.84	48.61	45.58	75.95	27.47	60.00	36.00	60.00	3.20	26.97
3			3	70.68	57.34	44.25	72.19	29.61	66.00	41.00	61.00	2.60	28.50
3			4	72.31	49.96	44.34	72.90	29.50	60.00	37.00	61.00	3.12	26.50

4	Fertile group	Breeding season	1	89.30	80.92	84.12	139.11	62.54	47.00	44.00	60.00	3.74	32.90
4			2	70.51	61.79	89.52	148.59	70.32	78.00	47.00	60.00	3.55	34.61
4			3	77.48	55.67	60.01	104.29	36.31	60.00	34.00	57.00	3.78	28.63
4			4	75.96	67.13	82.17	135.26	63.59	63.00	46.00	60.00	3.56	33.01
4	Fertile group	Non-breeding season	1	51.67	35.41	37.24	65.82	28.36	76.00	43.00	56.00	3.73	32.19
4			2	63.77	47.83	68.47	115.55	49.51	72.00	42.00	59.00	3.62	31.63
4			3	64.45	49.16	79.43	129.82	57.84	72.00	44.00	61.00	3.83	31.24
4			4	55.36	43.95	45.23	95.86	35.15	71.00	42.00	57.00	3.06	31.63
5	Subfertile group	Breeding season	1	80.35	61.25	62.51	110.02	37.21	59.00	33.00	56.00	4.09	29.46
5			2	85.94	69.29	70.91	122.73	44.09	62.00	35.00	57.00	4.06	31.24
5			3	77.96	53.76	58.14	101.57	35.76	61.00	35.00	57.00	3.94	29.49
5			4	52.17	39.38	66.20	112.58	45.23	68.00	40.00	58.00	3.41	33.96
5	Subfertile group	Non-breeding season	1	40.39	20.95	51.29	90.29	30.07	58.00	33.00	56.00	3.74	27.33
5			2	30.47	14.36	44.28	74.05	27.80	62.00	37.00	59.00	3.43	24.90
5			3	68.01	55.74	63.63	111.27	39.60	62.00	33.00	57.00	3.84	30.36
5			4	70.01	54.11	43.35	91.19	31.93	56.00	32.00	58.00	3.93	29.24
6	Subfertile group	Breeding season	1	53.06	40.13	74.41	115.94	57.22	76.00	49.00	64.00	3.47	33.86
6			2	70.67	57.87	68.23	120.23	48.66	71.00	40.00	56.00	3.46	33.28
6			3	73.66	56.84	65.46	112.71	46.54	71.00	41.00	58.00	3.68	31.50
6			4	55.13	40.13	67.93	111.21	51.23	70.00	42.00	59.00	3.65	32.05
6	Subfertile group	Non-breeding season	1	53.39	29.40	53.21	95.53	36.26	68.00	37.00	55.00	3.42	29.33
6			2	58.11	33.46	55.01	93.31	38.15	69.00	40.00	58.00	3.36	31.83
6			3	40.39	24.85	51.29	90.29	31.07	59.00	33.00	55.00	3.69	27.39
6			4	46.09	36.15	52.29	100.12	32.96	60.00	39.00	55.00	3.56	28.91
7	Subfertile group	Breeding season	1	53.06	39.84	74.41	115.94	57.22	76.00	49.00	64.00	3.47	33.86
7			2	58.11	33.46	55.01	93.31	38.15	69.00	40.00	58.00	3.36	31.83
7			3	50.32	40.18	58.73	105.18	40.93	69.00	38.00	55.00	3.20	32.83
7			4	52.11	41.12	60.76	110.29	46.91	72.00	43.00	57.00	3.37	32.81

7	Subfertile group	Non-breeding season	1	53.39	29.40	53.21	95.53	36.26	68.00	37.00	55.00	3.42	29.33
7			2	20.95	8.36	40.55	67.07	26.67	65.00	39.00	60.00	2.68	27.98
7			3	32.07	13.74	41.26	70.08	23.48	56.00	33.00	58.00	3.07	25.61
7			4	36.12	20.14	43.09	76.56	28.80	59.00	38.00	56.00	2.92	26.83
8	Subfertile group	Breeding season	1	53.06	39.84	74.41	115.94	57.22	76.00	49.00	64.00	3.47	33.86
8			2	63.77	47.83	68.47	115.55	49.51	72.00	42.00	59.00	3.62	31.63
8			3	50.32	40.08	58.73	105.18	40.93	69.00	38.00	55.00	3.20	32.83
8			4	54.54	43.12	68.59	111.19	52.11	69.00	45.00	57.00	3.69	32.78
8	Subfertile group	Non-breeding season	1	34.33	25.02	51.39	83.61	35.60	69.00	42.00	61.00	1.78	30.74
8			2	48.89	24.72	41.66	72.02	27.57	66.00	38.00	57.00	2.91	28.16
8			3	53.39	29.40	53.21	95.53	36.26	68.00	37.00	55.00	3.42	29.33
8			4	46.11	26.91	49.11	75.55	30.28	67.00	42.00	55.00	2.98	29.54

Eidesstattliche Erklärung

Hiermit erkläre ich durch eigenhändige Unterschrift, die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die aus den Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Dissertation ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

Rostock, den 23.10.2019 Unterschrift

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- Suliman, Y., Becker, F., Tuchscherer, A., Wimmers, K. (2020): Seasonal variations in quantitative and qualitative sperm characteristics in fertile and subfertile stallions. Archives Animal Breeding, 63: 145-154.
- Suliman, Y., Becker, F., Wimmers, K. (2018): Implication of transcriptome profiling of spermatozoa for stallion fertility. Reproduction, Fertility and Development, 30(8): 1087-1098.

Tagungen und Fachvorträge

- Suliman, Y., Wimmers, K., Becker F. (2016): Relationship between fertility and spermatozoal transcriptome characteristics in stallion. Abstract, 10-12. Februar 2016, 49th Annual Conference of Physiology & Pathology of Reproduction, Leipzig.
- Suliman, Y. K. Wimmers K., Becker F. (2015): Relationship between fertility and spermatozoal transcriptome characteristics in stallion. Poster, Forschungscamp 2015, 26.11.15 DFG-Fördermöglichkeiten für Postdocs.
- Suliman, Y., Alm, H., Torner, H., Janowski D. (2011): Establishment of an in vitro method for single bovine oocyte and embryo culture. Poster 258, ESDAR, Türkei, 2011.

Theses of the doctoral thesis

1. The semen quality profiles of stallions differ depending on their fertility as measured by pregnancy rat and varies between the breeding and non-breeding season

to test this thesis eight stallions with known fertility were divided into fertile and subfertile groups based on their pregnancy rate. Semen samples were collected from each stallion in breeding and non-breeding seasons. After that, semen samples were assessed individually for motility and for morphology and the spermatozoal qualitative and quantitative parameter were compared between the two groups of stallion in both breeding and non-breeding seasons.

The results showed no significant differences in ejaculate parameters including ejaculate volume, sperm concentration and total sperm number between fertile and subfertile stallion group in both breeding and non-breeding seasons. In addition, a significant increase of morphologically normal spermatozoa was observed in fertile stallions when compared to subfertile stallions. However, the observations in this study were detected only in breeding season while in non-breeding season no significant differences were observed in all morphology parameters between fertile and subfertile stallions. Furthermore, significant differences between fertile and subfertile group in breeding season for a part of sperm movement characteristics derived from CASA were also investigated including total motility, progressive motility, VAP and VCL. In non-breeding season, no significant differences were found between fertile and subfertile stallions in all CASA derived sperm movement characteristics.

2. Different gene-associated molecular functions are overrepresented in stallion spermatozoa in response to different seasonal conditions

The aim of this expression analysis was to investigate cellular processes, functional networks and biological functions in stallion in response to different seasonal conditions associated with increase of daylight and temperature during the breeding season. This approach was performed by comparing the microarray-derived sperm transcriptomes of stallions in breeding and in non-breeding season.

The results of this study identified 582 significantly different transcripts between breeding and non-breeding seasons (fold change >1.5 und $p< 0.05$). These transcripts associated with different biological functions and molecular processes know to be fundamental for spermatogenesis, sperm function, regulation of stress response and energy metabolism such as protein acetylation, FAK signaling, clathrin-mediated endocytosis signaling as well as

metabolic pathways that were related to inositol hexakisphosphate and triphosphate biosynthesis and metabolism.

3. Different gene-associated molecular processes are linked to stallion fertility

the aim of this experiment was to identify molecular processes that are associated with male fertility by comparing microarray-derived sperm transcriptomes of stallions that were clearly assigned to either a fertile or subfertile group based on reproductive success and sperm characteristics.

To test this thesis, six warm blood stallions were used. Stallions were divided into two groups that were different in pregnancy rates and proportions of normal and progressive motile sperm. In this experiment, 437 genes were found having significantly different abundance of transcripts (fold change ≥ 1.2 und $p \leq 0.05$) between fertile and subfertile stallion groups.

These transcripts were associated with different biological functions and molecular processes known to be fundamental for spermatogenesis and male fertility such as cytokine–cytokine receptor interaction, RNA binding proteins, G1/S checkpoint regulation, cyclin and cell cycle regulation as well as metabolic pathways that were related to phosphatase and D-myoinositol phosphate metabolism. These results identify molecular processes in spermatozoa that are associated with stallion fertility

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