TRANSCRIPTOMIC PROFILING OF CROSSBRED BULL SPERMATOZOA IN RELATION TO FERTILITY AND CAPACITATION

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL (HARYANA)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
LIVESTOCK PRODUCTION AND MANAGEMENT

BY

Dr. M. ARUL PRAKASH
(M.V.Sc.)

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ADUGODI, BENGALURU- 560030, INDIA

2020
Regd. No: 14-P-LP-02

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Dedicated to

My Beloved Family Members

&

My Guide

LIVESTOCK PRODUCTION AND MANAGEMENT
SOUTHERN REGIONAL STATION
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This is to certify that the thesis entitled **"TRANSCRIPTOMIC PROFILING OF CROSSBRED BULL SPERMATOZOA IN RELATION TO FERTILITY AND CAPACITATION"** submitted by **Dr. M. ARUL PRAKASH** in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in **LIVESTOCK PRODUCTION AND MANAGEMENT** of **NATIONAL DAIRY RESEARCH INSTITUTE (Deemed University), Karnal (Haryana),** is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Place : Bengaluru
Date : 24th October, 2019

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ACKNOWLEDGEMENT

“தினைத்துணை நன்றி செயினும் பனைத்துணையாக்
கொள்வர் பயன்தெரி வார்”.
(Though the benefits conferred be as small as a millet seed, those who know its advantage will consider it as large as a Palmyra fruit")
-Thiruvalluvar

In line with the above Thirukkural, I express my profound and deep sense of gratitude and personal indebtedness to my guide, major advisor and Chairman of the Advisory Committee and also my professional mother ***Dr. A. Kumaresan****, Principal Scientist, Theriogenology Laboratory, SRS, ICAR-NDRI, for his dynamic guidance, constant encouragement, constructive ideas & suggestions, tireless efforts, whole hearted interest and infinite help during the course of my research work. I am very much blessed and feel proud to have worked under his amazing leadership.*

I convey my profound recognition to the members of my Advisory Committee, Dr. S, Jeyakumar, Principal Scientist (ARGO), SRS - NDRI; Dr. A. Manimaran, Scientist (LPM), SRS - NDRI; Dr. S. Selvaraju, ICAR-National Fellow (ARGO), NIANP; Dr. Mukund A. Kataktalware , Senior Scientist (LPM), SRS - NDRI and Dr. K. P. Ramesha, Principal Scientist (AGB), Jt. Director Nominee and Head of the Station, SRS - NDRI whose support helped me to complete my research work within the stipulated time.

I am indebted to the officials of ***Tamil Nadu Veterinary & Animal Sciences University*** *for giving me the opportunity to pursue my Ph.D. in NDRI. My sincere thanks to the Vice-Chancellor and Registrar of TANUVAS for granting me leave to continue my doctoral research. I appreciate my senior and junior colleagues from TANUVAS, Dr. V. Boopathi, Dr. K. Rajkumar Dr. Manobavan and Dr. Hudson who tirelessly encouraged me with their valuable suggestions and their help throughout my work.*

I extend my thanks to all the teachers of LPM Division, NDRI - Karnal, scientists and of NDRI-SRS Bangalore, non-teaching staff of Dairy Production Section and education section and, staff of Reproductive Physiology Laboratory, NIANP for providing me the necessary help and amenities to carry out my research work.

I specially thank the staff of Dept. of Veterinary Gynaecology & Obstetrics, Dept. of Veterinary Pathology and Dept. of Veterinary Parasitology of ***Madras Veterinary College*** *for providing me the necessary facilities for sample processing. I also extend my thanks to* ***Mr. Udaya*** *of Perambur slaughter house, who helped me in sample collection at odd hours.*

My sincere thanks to my near and dear well-wishers from ***Tamil Nadana Sabha****, Drs. Mooventhan, Karpagam, Senthil, Rajendran, Sivakumar, Sakthivel, Dinesh Kumar, Chandrasekar for wishing me good luck in all my accomplishments.*

I thank my ***Theriogenology lab mates*** *Manish sir, Praveen sir, Ebenezer, Mohua, Kaustubh, Karuthu, Pradeep, Elango, Arun, Ankur, Vignesh, Nilendu and Deeksha for extending their timely help, exposing me to new learning experiences and making my days livelier for carrying out my research activities confidently.*

I outspread my deep gratitude and respect to my parents ***Mr. C. Mani*** *and* ***Mrs. Sumathi****; my sisters* ***Anitha*** *and* ***Prema****; all my relatives, especially my chithappa* ***Dr. C. Soundararajan,*** *Prof, TANUVAS who has been my mentor since childhood in shaping my career; my in-laws and friends Jana and Naveen for their moral support and motivation to reach yet another milestone in my profession.*

Special thanks to my lovable wife ***Dr. R. Divisha*** *for all her sacrifices, affection and mental support during the ups and downs of my research work. I also thank her pets Dolly, Mittu, Granny, Jumma, Tiger, Blacky, Julee, Piggy etc who served as my stress busters by making me happy with their company and unconditional love.*

I am thankful to ***ICAR - National Dairy Research Institute*** *for providing me the necessary facilities and financial support to complete my Ph.D. programme successfully.*

To wrap it up, I thank everyone for making my college life enjoyable and unforgettable. Several people who remain unnamed helped me at various stages of my research work and I owe my gratitude to each one of them. They were directly or indirectly involved in the successful completion of my research and thesis.

Above all, I thank ***God Almighty*** *for all the blessings bestowed upon me.*

THANK YOU ALL!

Date: 24.10.2019

(M. Arul Prakash)

ABSTRACT

Although the significance of bull infertility has been documented well, the underlying reasons for high incidence of infertility in crossbred bulls still remains elusive. The present investigation was carried out to identify fertility related sperm transcripts in HF crossbred bulls and to find out the ability of spermatozoa to undergo capacitation on exposure to oestrous oviductal fluid and its relationship with bull fertility. The present study identified a total of 13,563 transcripts in crossbred bull spermatozoa; after total hit normalization, a total of 776 transcripts were detected, of which 524 transcripts were common to both high- and low-fertile bulls, while 84 sperm transcripts were unique to high-fertile bulls and 168 transcripts were unique to low-fertile bulls. Among the co-expressed genes, 176 genes were up-regulated while 209 genes were down regulated in low-fertile bulls. Using bioinformatics analysis, we identified that the biological processes down-regulated in low fertile bull spermatozoa included multicellular organism development and spermatogenesis. Further, ribosomal pathway was upregulated whereas oxidative phosphorylation was down-regulated in low fertile bull spermatozoa. Moreover, sperm transcripts unique to high fertile bulls were involved in oxidative phosphorylation pathway and transcripts unique to low fertile bulls were involved in ribosomal pathway. Select transcripts (*TPT1*, *PFN1*, *ZNF706*, *CRISP2*, *MDB4*, *TNP2*, *ADIPOR1* and *TNP1*) were validated using qPCR and found that the transcriptional abundance of *ZNF706*, *CRISP2*, *MDB4*, *TNP2* and *TNP1* genes were significantly ($p<0.05$) higher in high-fertile bulls compared to low-fertile bulls and were positively correlated with conception rate ($p<0.01$). During incubation of spermatozoa from above- and below-average bulls with NL-ODF, it was observed that the extent of reduction in sperm viability and acrosome reaction between 0 h and 4 h of incubation was lower in above average bulls compared to below average bulls. Further, the hallmarks of sperm capacitation were significantly higher in above- compared to below-average bulls at 4 h of incubation indicating the dual role of oviductal secretions on spermatozoa. Transcriptional abundance of genes related to sperm capacitation (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2* and *IQCF1*) were upregulated in below average bulls. Conception rate was positively correlated ($p<0.01$) with sperm viability, intracellular calcium level and acrosome intactness at 4 h incubation with NL-ODF. It was therefore concluded that oxidative phosphorylation was the most affected pathway in low-fertile bull spermatozoa and the sperm transcriptional abundance of *ZNF706, CRISP2, MDB4, TNP2* and *TNP1* genes was significantly related to bull fertility and thus these genes could serve as potential candidate biomarkers for fertility in crossbred bulls. Further, the ability to undergo capacitation upon exposure to NL-ODF varied with individual bulls and also significantly related to bull fertility.

सारांश

यद्यपि सांडों में बांझपन का महत्व अच्छी तरह से प्रलेखित किया गया है, लेकिन क्रॉसब्रेड सांडों में बांझपन की उच्च घटनाओं के अंतर्निहित कारण अभी तक अज्ञात हैं। वर्तमान अध्ययन का उद्देश्य क्रॉसब्रेड सांडों में प्रजनन संबंधी शुक्राणु ट्रांसक्रिप्ट्स की पहचान करना तथा शुक्राणुओं की एस्ट्रस ओवीडक्टल द्रव्य की उपस्थिति में कपसिटेशन की क्षमता एवं इसका सांडों की प्रजनन क्षमता के साथ सम्बन्ध ज्ञात करना था। वर्तमान अध्ययन ने क्रॉसब्रेड सांडों के शुक्राणुओं में कुल १३,५६३ ट्रांसक्रिप्ट्स की पहचान की; कुल हिट सामान्यीकरण के बाद, कुल ७७६ ट्रांसक्रिप्ट्स का पता चला, जिनमें से ५२४ ट्रांसक्रिप्ट्स उच्च और निम्न-प्रजनन क्षमता वाले सांडों के लिए सामान्य थे, जबकि ८४ शुक्राणु ट्रांसक्रिप्ट्स उच्च-प्रजनन क्षमता वाले सांडों के लिए अद्वितीय थे तथा १६८ ट्रांसक्रिप्ट्स निम्न-प्रजनन क्षमता वाले सांडों के लिए अद्वितीय थे। सह-अभिव्यक्त जीनों में से, १७६ जीनों की अभिव्यक्ति निम्न-प्रजनन क्षमता वाले सांडों में उच्च प्राप्त हुई जबकि २०९ जीनों की अभिव्यक्ति क्रमश: निम्न थी। जैव सूचना विज्ञान विश्लेषण का उपयोग करते हुए हमें यह ज्ञात हुआ कि निम्न-प्रजनन क्षमता वाले सांडों के शुक्राणुओं में निम्न अभिव्यक्ति वाली जैविक प्रक्रियाओं में बहुकोशिकीय जीव विकास और शुक्राणुजनन शामिल थीं। इसी क्रम में निम्न-प्रजनन क्षमता वाले सांडों के शुक्राणुओं में राइबोसोमल पाथवे की उच्च-अभिव्यक्ति जबकि ऑक्सीडेटिव फॉस्फोराइलेशन की निम्न-अभिव्यक्ति प्राप्त हुई। इसके अलावा, उच्च-प्रजनन क्षमता वाले सांडों के लिए अद्वितीय शुक्राणु ट्रांसक्रिप्ट्स ऑक्सीडेटिव फास्फोरिलीकरण जबकि निम्न-प्रजनन क्षमता वाले सांडों के शुक्राणुओं के अद्वितीय ट्रांसक्रिप्ट्स रिबोसोमल पाथवे में शामिल थे। क्यू-पी.सी.आर. तकनीक द्वारा चुनिंदा ट्रांसक्रिप्ट्स (*TPT1*, *PFN1*, *ZNF703*, *CRISP2*, *MDB4*, *TNP2*, *ADIPOR1* और *TNP1*) को सत्यापित किया गया और पाया गया कि *ZNF706*, *CRISP2*, *MDB4*, *TNP2* और *TNP1* जीन की ट्रांसक्रिप्शनल अधिकता उच्च-प्रजनन क्षमता वाले सांडों में निम्न-प्रजनन क्षमता वाले सांडों की तुलना में सार्थक रूप से (पी<०.०५)अधिक थी तथा यह गर्भाधान दर के साथ सकारात्मक रूप से सहसंबद्ध थी (पी<०.०१)। उच्च और निम्न-प्रजनन क्षमता वाले सांडों के शुक्राणुओं को एनएलओडीएफ के साथ उपचारित करने पर यह देखा गया कि ० h और ४ h के बीच शुक्राणु जीवन-क्षमता और एक्रोसोम में कमी की मात्रा निम्न-प्रजनन क्षमता वाले सांडों की तुलना में अधिक थी। इसके अतिरिक्त, ४ h उपचार के पश्चात शुक्राणु कपसिटशन के हॉलमार्क उच्च प्रजनन क्षमता वाले सांडों में निम्न-क्षमता वाले सांडों की तुलना में सार्थक रूप से अधिक प्राप्त हुए जो डिंबवाहिनी संबंधी स्राव की शुक्राणुओं पर दोहरी भूमिका को इंगित करता है। शुक्राणु कैपेसिटेशन से संबंधित जीनों (*CATSPER1*, *PEBP1*, *CRISP2*, *ADIPOR1*, *ADIPOR2* और *IQCF1*) की ट्रांसक्रिप्शनल बहुतायत निम्न-प्रजनन क्षमता वाले सांडों में अधिक प्राप्त हुई। EODF के साथ ४ h उपचार करने पर गर्भाधान दर शुक्राणु जीवन क्षमता, इंट्रासेल्युलर कैल्शियम के स्तर और आक्रोसोम अक्षुण्णता EODF के साथसकारात्मक रूप से सहसंबद्ध (पी <०.०१) थी। अंततः यह निष्कर्ष निकाला गया कि निम्न-प्रजनन क्षमता वाले सांडों के शुक्राणुओं में ऑक्सीडेटिव फास्फारिलीकरण सर्वाधिक प्रभावित पाथवे था तथा *ZNF706*, *CRISP2*, *MDB4*, *TNB2* और *TNP1* जीनों की शुक्राणुओं में ट्रांस्क्रिप्शनल बहुतायत सांडों की प्रजनन क्षमता से सार्थक रूप से जुडी थी अतः ये जीन क्रॉसब्रेड सांडों में प्रजनन क्षमता के लिए संभावित उम्मीदवार बायोमार्कर के रूप में काम कर सकते हैं। इसके अतिरिक्त ईओडीएफ के संपर्क में कपसिटशन की क्षमता सांडों में व्यक्तिगत रूप से भिन्न तथा सांडों की प्रजनन क्षमता से सार्थक रूप से जुडी होती है।

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ABBREVIATIONS

α	: Alpha
AI	: Artificial insemination
ANOVA	: Analysis of variance
β	: Beta
bp	: base pair
BSA	: Bovine Serum Albumin
CT	: Cycle threshold
CL	: *Corpus luteum*
cDNA	: Complementary DNA
δ	: Delta
et al.	: Co-workers
fg	: Femto gram
FPKM	: Fragments per kilobase million
γ	: Gamma
g	: Relative centrifugal force
HF	: High fertile
h	: Hour
HISAT	: Hierarchical indexing for spliced alignment of transcripts
KEGG	: Kyoto Encyclopedia of Genes and Genomes
LF	: Low fertile
ml	: Milliliter
μl	: Microlitre

mg : Milligram
mM : Millimolar
min : Minute
MSP : Minimum standard protocol
n : Number of observation
ng : Nanogram
nm : Nanometer
OD : Optical density
pH : Potential of Hydrogen
pg : Pico gram
pmol : Picomoles
$P<0.01\%$: 99% of significance
$P<0.05\%$: 95% of significance
rpm : revolutions per minute
RNA : Ribonucleic acid
RNA-Seq : RNA Sequencing
sec : Seconds
SD : Standard deviation
SEM : Standard error of the mean
SDS : Sodium dodecyl sulfate
U : Units
% : Percentage

CHAPTER - 1

Introduction

1. INTRODUCTION

Infertility is a serious condition that affects not only humans but also animals (Capra *et al*., 2017). In the last 5 decades, even with several achievements and developments in the milk production, the efficiency of reproduction and fertility of individual dairy cattle has reduced in due to intensive genetic selection for milk production (Lucy, 2001). Worldwide, the reduction of reproductive performance and economic sustainability in dairy cattle is of serious concern to the farmers and the dairy industry (Lone *et al*., 2017; Pryce *et al*., 2004). Though artificial insemination (AI) is a successful reproductive tool, the prevalence of infertility in dairy animals leads to a major challenge in cattle breeding industry (Kaya and Memili, 2016). Most studies on cattle reproduction have focused only on the cow, with little consideration on male fertility (Kropp *et al*., 2017; Penagaricano *et al.,* 2013). Studies have reported that a significant proportion of reproductive failure in dairy cattle is attributed to male infertility or subfertility and approximately half of the conception failure cases are a consequential to this (DeJarnette *et al*., 2004; Watson, 2000). In cattle breeding industry, male infertility or sub-fertility is a major problem because it still remains a non-predictable trait causing great economic losses for livestock breeders. Since, only a single bull is used to impregnate several thousands of females, the use of semen from sub-fertile or infertile males can have dreadful effects in the dairy industry.

Fertility of an individual bull varies significantly and among breeds, the male progenies that have resulted from crossing of *Bos taurus* and *Bos indicus* suffer from serious infertility/sub-fertility problems (Aslam *et al*., 2015). Moreover, a high incidence of sub-fertility and poor semen quality in crossbred bulls compared to indigenous breeds leads to a disintegration of breeding system. Recently, it has been reported that about 50% of the ejaculates from crossbred bulls are of inferior quality (Vijetha *et al*., 2014). A successful conception is linked to the maintenance of both structural and functional characters of spermatozoa. Conventional semen analysis tests focus more on the structural characters (motility & membrane related parameters) rather than functional characters that have more importance in conception (Kumaresan *et al*., 2012a), hence they are not always indicative of the actual fertility of bulls (Rodriguez-Martinez, 2013;

2006; Garrido *et al*., 2009). Advanced sperm function-based tests, although superior than the conventional semen analysis, also have a limited value for bull fertility prediction (Ferraz *et al*., 2013; Rodriguez-Martinez and Barth, 2007; Rodriguez-Martinez, 2006; Januskauskas *et al*., 2001; 2003; Saacke *et al*., 2000). Efficient cellular and molecular methods to evaluate semen are necessary to predict the fertility potential of a bull with high reproductive efficiency (Jodar *et al*., 2013).

The gene expression profiling of mammalian sperm is a novel non-invasive tool to evaluate male fertility (Ganguly *et al*., 2016). A series of high throughput technologies emerging from omics (genomics, transcriptomics, proteomics, and metabolomics) when combined, could possibly develop the resolution of non-invasive male fertility diagnostic tools (Simon and Carrell, 2018; Selvaraju *et al*., 2017; Jodar *et al*., 2015). Among the high throughput technologies, the RNA fingerprints of sperm could be used as an important tool to evaluate semen quality and predict bull fertility. Transcriptomic profiling of spermatozoa is significant as it conveys facts about spermatogenesis, sperm function, and paternal roles in post-fertilization events related to sire fertility (Selvaraju *et al*., 2018). Global sperm transcriptome studies using RNA sequencing have been done in several species like human (Sendler *et al*., 2013), bulls (Raval *et al*., 2019; Singh *et al*., 2019; Selvaraju *et al*., 2017; Card *et al*., 2017; 2013), buffalo (Vijayalakshmy, 2017), boars (Wasilewska-Sakowska *et al*., 2019; Yang *et al*., 2009; Kempisty *et al*., 2008), stallion (Das *et al*., 2013) and laboratory animals (Margolin *et al*., 2014; Kawano *et al*., 2012). The differential transcriptome profiling was used to correlate with process like spermatogenesis (Yatsenko *et al*., 2006), past spermatogemnic events (Selvaraju *et al*., 2017), sperm functional parameters (Ganguly *et al*., 2013), fertilization, early embryonic development (Ostermeier *et al*., 2004) and idiopathic cases of infertility (Bissonnette *et al*., 2009) and to evaluate bull fertility potential (Arangasamy *et al*., 2011; Bissonnette *et al*., 2009; Lalancette *et al*., 2008b). The absolute expression level of sperm transcripts may be used as a valuable marker to identify male infertility (Selvaraju *et al*., 2018).

Before interaction of oocyte in the female reproductive tract, the ejaculated spermatozoa must undergo a series of physio-chemical transformations known as capacitation to fertilize the egg (Bernecic *et al*., 2019). Oviduct its secretion and lining of epithelium were involved in sperm maturation, capacitation, protein phosphorylation acrosome reaction, fertilization and early embryo development. Spermatozoa are devoid of cytoplasmic ribosome and therefore cannot translate mRNA however, reports been available mentioning the translation activity occur in sperm mitochondria (Baker, 2011). The translation of nuclear encoded mRNA in mitochondria occurs in spermatozoa. The possible role of sperm transcripts in capacitation and its influence on fertility of sperm has been reported (Brukman *et al*., 2016; Fang *et al*., 2015; Killan, 2012; Lu *et al*., 2006).

In recent past, several attempts have been made to identify male fertility markers. However, there is no single or combined sperm function tests/assay or technology for accurate fertility prediction in bulls is available (Gillan *et al.,* 2008; Rodríguez-Martínez, 2003). While the significance of male infertility is well documented, the underlying reason for such high incidence of infertility in crossbred bulls still remains unclear. Earlier studies that tried to identify the underlying causes for infertility in bulls, either studied a particular protein or few genes and assessed their association with bull fertility. However, a thorough understanding of global molecular differences between high- and low-fertile spermatozoa is the need of hour for developing of bull fertility prediction tools.

With this backdrop, the present study was carried out to understand the differential gene expression between spermatozoa from high- and low-fertile crossbred bulls using high throughput sequencing techniques. The aim of the study was to identify the most relevant panel of transcripts for bull fertility prediction. The specific objectives of the study includes

1. Differential transcriptome profile between high- and low-fertile crossbred bulls
2. Ability of spermatozoa to undergo capacitation upon exposure to oviductal fluid and its relationship with bull fertility

CHAPTER - 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 Spermatogenesis

Spermatogenesis is a precisely controlled process of male reproduction which results in the formation of highly differentiated transcriptionally inactive haploid sperm cells that are capable of fertilization (Griswold, 2015). The whole process occurs in the seminiferous tubules of adult testes wherein a complex program of mitotic proliferation, meiosis and post-meiotic differentiation take place (Griswold, 2015). A series of mitotic divisions cause the diploid spermatogonial cells to develop into primary spermatocytes which further undergo meiosis to produce haploid secondary spermatocytes (O' Donnell, 2014). Post-meiotic events include morphological and developmental changes undergone by spermatids to form spermatozoa (Miller and Ostermeier, 2006). During spermiogenesis, the spermatid specific H_2B variants are specially synthesized to replace the conical histones, thus leading to the formation of unstable nucleosomes which facilitate histone acetylation. Also, these hypercalated histones are gradually replaced by transitional proteins (TP). Finally, the TP are replaced by protamines (Dadoune, 2003). In many species, the histones are removed when spermatogenesis ceases and DNA is condensed by protamines resulting in the formation of highly dense nucleoprotamine complexes (Balhorn, 2007). About 5-10% of histones are reserved and are believed to be involved in the zygotic and early embryonic gene expression. Hence, the common features of spermiogenesis are believed to be the termination of transcription at a defined point of cell differentiation, translational regression and storage of mRNAs for terminal stages of spermatogenic differentiation (Steger, 2001).

2.2 Spermatozoal RNA

The sperm is a small, highly specialized compact cell that carries the male genetic material to the next generation. Unlike other cell, spermatozoa cannot synthesize any protein on their own because spermatozoa is transcriptionally and translationally, inert in their activity (Lalancette *et al*., 2008a). The spermatozoa are thought to serve the function of only delivering the DNA and that they do not have any traces of RNA due to the loss of cytoplasm during the complex process of spermiogenesis (Bansal *et al*., 2015). In contrast to somatic cells and embryos, the sperm cells lack 18s and 28s ribosomes due to the absence of translational activity (Kaya and Memili, 2016). Though transcription ends during the spermatid stage of spermatogenesis, terminally-differentiated spermatozoa contain a complex variety of RNAs, which are assumed to be originated from the trapped cytoplasmic content that remains after spermiogenesis (Gilbert *et al*., 2007; Dadoune *et al*., 2005).

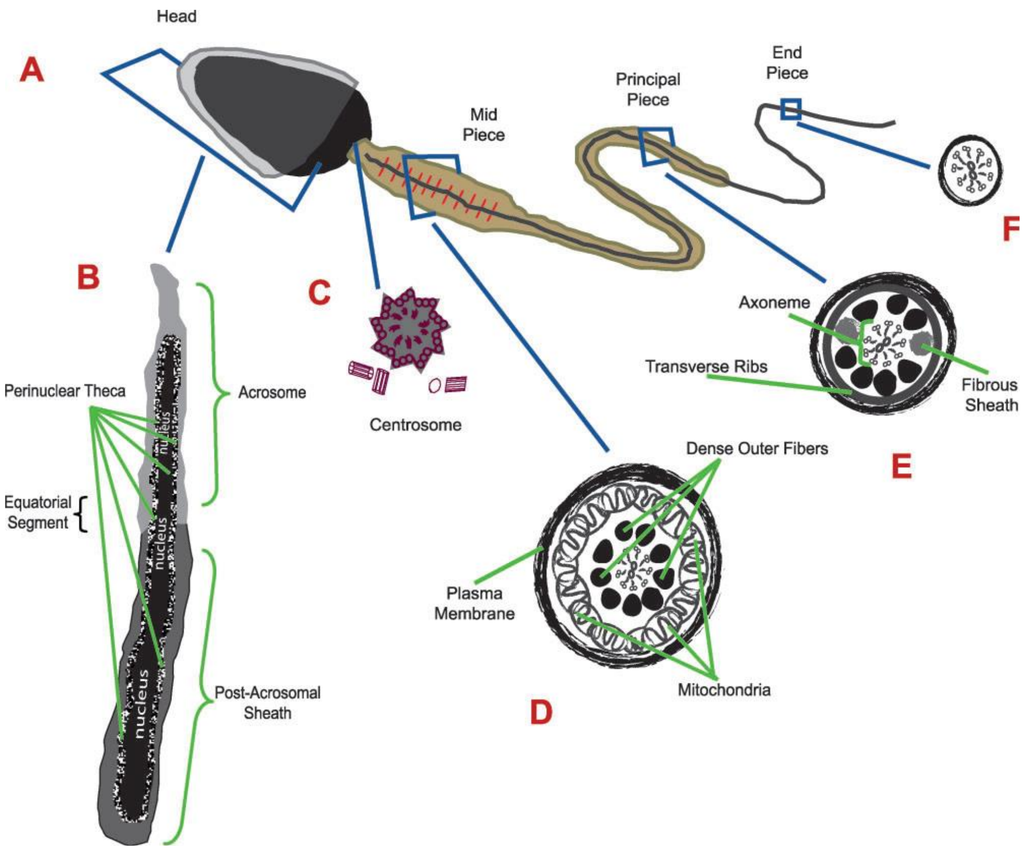


Fig. 2.1 The possible areas for spermatozoal RNA repositories in nucleus and mid-piece (**A**) Highlights the four main segments of a mature spermatozoon (**B**) longitudinal section of the sperm head. Nucleus, the perinuclear theca and the post-acrosomal sheath are highlighted in grey indicating the possible areas for spermatozoal RNA repositories (**C**) and (**D**) centrosome and the mitochondria (**E**) and (**F**) show the cross-sectional diagrams of the principal piece and end piece Again, the fibrous sheath and axoneme are highlighted in grey, indicating that they too may carry spermatozoal RNA. (Image adopted from Miller and Ostermeier, 2006)

Earlier reports have claimed the existence of RNA in mouse (Betlach and Erickson, 1973), bull (Paul and Duerksen, 1975), rat and human spermatozoa (Pessot *et al*., 1989). But of late, the presence of spermatozoal RNA has been confirmed in many mammals such as human (Goodrich *et al*., 2007; Ostermeier *et al.,* 2002), cattle

(D'Amours *et al*., 2010; Feugang *et al.,* 2010; Bissonnette *et al.,* 2009; Gilbert *et al.,* 2007), boar (Yang *et al*., 2009; Kempisty *et al.,* 2008) stallion (Das *et al*., 2013) and poultry (Shafeeque *et al*., 2014) using several techniques such as reverse transcription-polymerase chain reaction (RT-PCR) (Parthipan *et al*., 2017; Kasimanickam *et al*., 2013; Arangasamy *et al*., 2011; Yatsenko *et al*., 2006; Lambard *et al*., 2004; Miller *et al*., 1999), in situ hybridization (Wykes *et al*., 1997), targeted microarrays (Suliman *et al*., 2018; Paul, 2018; Yathish *et al*., 2017; Singh *et al*., 2015; Liu *et al*., 2012; Feugang *et al.,* 2010; Bissonnette *et al.,* 2009; Gilbert *et al.,* 2007), differential display method (Geisinger *et al*., 1996), subtractive hybridization experiments (Chen *et al*., 2014; Lalancette *et al*. 2008b) and RNA Sequencing (Raval *et al*., 2019; Singh *et al*., 2019; Card *et al*., 2017; Selvaraju *et al*., 2017; Fang *et al*., 2015; Card *et al*., 2013; Das *et al*., 2013; Sendler *et al*., 2013).

Around 3000-13000 different kinds of spermatozoal mRNAs have been reported so far (Selvaraju *et al*., 2018; Bansal *et al*., 2015; Card *et al*., 2013; Johnson *et al.,* 2011; Ostermeier *et al*., 2002) with a heterogeneous population of coding and non-coding RNAs (Das *et al*., 2013; Jodar *et al*., 2013; Hamatani, 2012; Krawetz *et al*., 2011; Ostermeier *et al*., 2002). Sperm contain very low amounts of RNA (10-20 fg) compared to somatic cells (10-30 pg) and oocytes (0.5-1.5ng) (Selvaraju *et al*., 2017; Card *et al*., 2013; Hamatani, 2012; Das *et al.*, 2013; Krawetz, 2005). During fertilization, apart from the paternal genome, the spermatozoa also transport coding and non-coding RNAs, transcription factors, and cell signaling molecules into the oocyte (Capra *et al*., 2017; Jodar *et al*., 2013; Krawetz, 2005). The concentration of spermatozoal RNA yield in different species was reviewed by Vijayalakshmy *et al*. (2018) **(Table 2.1)**.

Table 2.1: Spermatozol RNA yield in different species

Sl. No	**Species**	**RNA yield (fg/spermatozoon)**	**References**
1.	Human	10-20	Ostermeier *et al*., 2004
2.	Human	50-100	Goodrich *et al*., 2007
3.	Cattle	20-31	Selvaraju *et al*., 2017; Parthipan *et al*., 2015; Card *et al*., 2013

4.	Cattle	180	Gilbert *et al*., 2007
5.	Horse	20	Das *et al*., 2013
6.	Pig	5-10	Boerke *et al*., 2007
7.	Rat	100	Pessot *et al*., 1989
8.	Goat	38.6 ng/µL	Das, 2016

Transcriptionally inert mature spermatozoa contain assorted populations of both small and large RNAs (Yuan *et al*., 2016; Jodar *et al*., 2013; Sendler *et al*., 2013; Krawetz *et al*., 2011; Wu and Chu, 2008) such as messenger RNAs (mRNA), interference RNA, antisense RNA and non-coding RNAs (ncRNA) (Hosken and Hodgson 2014; Dadoune, 2009) including microRNAs (miRNA) (Ostermeier *et al*., 2004), mitochondrial (mtRNA), ribosomal RNA (rRNAs), long non-coding RNA (lncRNA), small noncoding RNA (sncRNA), small interfering RNA (siRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), piwi-interacting RNA (piwiRNAs) (Krawetz *et al*., 2011) and transfer RNA (tsRNAs) (Selvaraju *et al*., 2017; Peng *et al*., 2012) that are usually isolated from spermatozoa of several species including human, mouse, cattle, horse, pig and poultry (Singh *et al*., 2015; Card *et al*., 2013; Das *et al*., 2013; Sendler *et al*., 2013; Kawano *et al*., 2012; Yang *et al*., 2009; Kempisty *et al*., 2008; Gilbert *et al.,* 2007; Goodrich *et al*., 2007).

2.3 Next Generation Sequencing and Sperm Transcriptomics

Earlier, several researches have been conducted to predict the sperm fertility through profiling of sperm transcripts with techniques such as cDNA cloning (Miller *et al*., 1999), RT-PCR (Parthipan *et al*., 2017; Kasimanickam *et al*., 2012; Arangasamy *et al*., 2011) suppression subtractive (Lalancette *et al*., 2008b) and microarrays (Bansal *et al*., 2015; Bissonnette *et al*., 2009; Lalancette *et al*., 2008a; Gilbert *et al*., 2007). Though these techiniques are advantageous, they have failed to provide a complete picture of the spermatozoal RNA compostion and their actual expression level.

Currently, next generation sequencing (NGS) technique on ribonucleic acid sequencing (RNA-Seq) is the gold-standard technology for analyzing transcriptome profiles to recognize the molecular basis of individual (Wang *et al*., 2009). It has given a detailed and complete picture of various spermatozoal RNA and has improved the identification, quantification, and characterization of several coding and non-coding RNAs (Selvaraju *et al*., 2018). This technique also provides information on complete transcripts, transcript variants, novel transcripts and single nucleotide polymorphisms (SNP) and (Jodar *et al*., 2013). Moreover, transcriptome profiling identifies messengers based on their presence or absence in mature spermatozoa; this could serve as potential markers of fertility (Lalancette *et al*., 2008b).

So far, the global sperm transcriptome studies using RNA sequencing have been done in several species like human (Sendler *et al*., 2013; Krawetz *et al*., 2011), bulls (Raval *et al*., 2019; Singh *et al*., 2019; Card *et al*., 2017; 2013; Selvaraju *et al*., 2017), buffalo (Vijayalakshmy, 2017), boars (Yang *et al*., 2009; Kempisty *et al*., 2008), stallion (Das *et al*., 2013) and laboratory animals (Margolin *et al*., 2014; Kawano *et al*., 2012). NGS transcript profiling is a novel non-invasive approach to analyze the genetic defects of spermatogenesis (Yatsenko *et al*., 2006), to assess idiopathic cases of infertility (Bissonnette *et al*., 2009) and to evaluate bull fertility (Arangasamy *et al*., 2011; Bissonnette *et al*., 2009; Lalancette *et al*., 2008b). The expression level of sperm transcripts can serve as a potential marker to identify male infertility (Selvaraju *et al*., 2018). In livestock species. The first report on spermatozoa transcriptome on Human (Sendler *et al*., 2013), bovine (Card *et al*., 2013) and equine (Das *et al*., 2013) was published in 2013.

2.4 Sperm RNA and its roles and functions

Spermatozoa carry information in the form of RNA and proteins which are related to spermatogenesis, sperm functions and other reproductive processes (Selvaraju *et al*., 2018). Sperm RNA transcripts have a wide variety of coding and noncoding RNA molecules which may have vital roles in sperm development (Ostermeier *et al*., 2002), chromatin repackaging (Hamatani, 2012; Krawetz, 2005), genomic imprinting, zygote development (Das *et al*., 2013; Lalancette *et al*., 2008b), post fertilization events and integral part of many processes like genome recognition, consolidation-confrontation, early embryonic development (Sendler *et al*., 2013), epigenetic transgeneration inherence (Rando, 2016; Jodar *et al*., 2013) and placental development (Selvaraju *et* *al*., 2018). The heterogeneous RNA content of a sperm can be used to assess the semen quality traits and male fertility potential in human (Jodar *et al*., 2013) and livestock species (Das *et al*., 2013; Arangasamy *et al*., 2011; Curry *et al*., 2011; Bissonnette *et al*., 2009). The expression level of some of the sperm mRNA transcripts are correlated with spermatogenesis, past spermatogenic events, sperm functional parameters, fertilization or early embryonic development and fertility potential **(Table 2.2** and **2.3)**.

Table 2.2: Expression levels of sperm transcripts in relation to sperm physiology and fertility

Involvement of sperm transcripts in	**References**
Spermatogenesis	Card *et al*., 2017; Hamatani, 2012; Kasimanickam, 2011; Feugang *et al*., 2010; Lalancette *et al*., 2009; Rousseaux *et al*., 2009; Platts *et al*., 2007; Ostermeier *et al*., 2002; Miller *et al*., 1999
Past spermatogenic events	Selvaraju *et al*., 2017; Parthipan *et al*., 2015; Card *et al*., 2013; Arangasamy *et al*., 2011; Lalancette *et al*., 2008b
Sperm functional parameters	Selvaraju *et al*., 2018; Bianchi *et al*., 2018; Selvaraju *et al*., 2017; Jodar *et al*., 2013; 2015; Ganguly *et al*., 2013; Arangasamy *et al*., 2011; Garcia-Herrero *et al*., 2010; Bissonnette *et al*., 2009; Garrido *et al*., 2009; Lalancette *et al*., 2009; Tiwari *et al*., 2008; Lambard *et al*., 2004; Wang *et al*., 2004
Fertilization or Early embryonic development	Card *et al*., 2017; Selvaraju *et al*., 2017; Singh *et al*., 2015; Parthipan *et al*., 2015; Ganguly *et al*., 2013; Sendler *et al*., 2013; Hamatani, 2012; Arangasamy *et al*., 2011; Lalancette *et al*., 2009; Rousseaux *et al*., 2009; Platts *et al*., 2007; Ostermeier *et al*., 2004; Christians *et al*., 2003; Eddy, 1999
Male fertility	Bianchi *et al*., 2018; Selvaraju *et al*., 2018; Bansal *et al.* 2015;

	Card *et al*., 2013; Das *et al*., 2013; Jodar *et al*., 2013; Ganguly *et al*., 2013; Hamatani, 2012; Arangasamy *et al*., 2011; Kasimanickam, 2011; Feugang *et al*., 2010; Garcia-Herrero *et al*., 2010; Garrido *et al*., 2009; Lalancette *et al*., 2009; Kempisty *et al*., 2008; Lalancette *et al*., 2008b, 2009; Zhao *et al.,* 2006; Krawetz, 2005; Lambard *et al*., 2004; Wang *et al*., 2004

Table 2.3: Genes and their relevance to sperm functions and fertility

Function	Genes involved	References
Spermatogenesis	*BSP3, SPATA18, CALM, BMP2* encoding aromatase- P450, *LMNA, TSPY1, SYNJ2, ZPBP, DAZ1, NYD-SP16 (SPATA9), DAZ2, DAZL, IDE, RFX2, NFYA, SALLA, SPOLL4, SPO11, HSPA2, SPATA33, STRA8, PRM1, PRM2, TNP1, TNP2, SPANXA1, BRWD1, SPATA31A3, SPATA31D1, SPATA31D4, SPATA32, TAF7l, ZPBP, KIF3A, ODF1, BRD2, PRM1, CHMP5, WHAZ, DNAJC2, PICK1, PFN1, FBP1, TRAP1, BBS2, ARFIP2, CAPZA2, WASH1, SPATA18/ACO2, BSP3, CALM, HSPD1, MYCBP, ODF2, PPP1CC, SERPINA*	Selvaraju *et al*., 2018; Parthipan *et al*., 2017; El-Fekih *et al*., 2017; Montjean *et al*., 2012; Tiwari *et al*., 2008; Fox *et al*., 2003; Cheng *et al*., 2003; Lu *et al*., 2006
Sperm maturation	*CUL3, PRM1, HSPCD35, TPX-1, PPARGC1A, FLCN, ADPGK, ATP5F1, PPARGC1A, FLCN, BBS2, BBS4, ATP5G1, ATP5G3/EZR, GSN, SUCLG2, VCP, SOD, RNASE4*	Selvaraju *et al*., 2018; Hamatani, 2012

Sperm function	*eNOS, nNOS, c-myc, P450 aromatase, TSSK6, ADAM5P, NYD-SP 16 (SPATA9), TPX-1, LDHC transcript variant- 1, ODF2, AKAP4, LDHC, CLGN, AKAP4, CABYR, IQCF1, CLGN, CALR3, LDHC, CATSPER3, PLCB1, FYN, NCK1, NCK2, PTK2, CCL2, CCKAR, CHRNA7/AKAP4, AKAP3, EGFR, ETFDH, ATP5B*	Selvaraju *et al*., 2018; El-Fekih *et al*., 2017; Bissonnette *et al*., 2009; Lu *et al*., 2006; Wang *et al*., 2004; Lambard *et al*., 2004; Cheng *et al*., 2003
Fertilization	*CALM, CLU, PRM1, PRM2, DDX3Y, PLCz, PLCZ1, CRISP2, AKAP4, PRSS37, HSPA1L, CCT2, ZP2, ZP3, CCT7/CALM, HSPA1L*	Selvaraju *et al*., 2018; El-Fekih *et al*., 2017; Card *et al*., 2013; Kempisty *et al*., 2008; Swann *et al*., 2006; Ostermeier *et al*., 2004
Oocyte genome activation	*PRSS37, HSPA1L, PLCZ1, WBP2NL, ZP2, PLCB1/ATP5A1, CALM, HSPA1L*	Selvaraju *et al*., 2018
Embryogenesis	*WNT5A, HSBP1L1, BCL2L11, BRCA1, ZP3, FOXG1, PADI6, BCL2L11, BRCA1, HNF1B, CDH1, CDX2, HMGCR, ADAM10, LOC512953, TXNRD1, BCL10, ACVR2A/ATP5A1, PRKACA*	Selvaraju *et al*., 2018; Selvaraju *et al*., 2017; El-Fekih *et al*., 2017; Fang *et al*., 2015; Das *et al*., 2013
Placental development	*THSD4, PAG5, PAG7, PAG10, HNF1A, NANOG, SMAD1, ARNT, CDX2, EPOR, GJB3, CITED2, SP1/PLET1*	Selvaraju *et al*., 2018; Selvaraju *et al*., 2017
Male fertility	*BIK, ACVR2A, DMRT1, FSHB, HSD17B4, LHCGR, GNRHR, INHBA, INHBB*	García-Herrero *et al*., 2010; Lin *et al*., 2006

Both fertilization and embryo development	*AKAP4, WNT5A, CLU, FOXG1, WNT4, HSBP1L1, INTS1, IGF2, STAT4, TDGF1, NANOG, SOX2, POU5F1*	El-Fekih *et al*., 2017

2.5. Role of spermatozoal transcripts on various processes

Spermatogenesis and past spermatogenic events

Spermatogenesis is a highly synchronized process with many genes closely regulating the different maturation steps (La and Hobbs, 2019); it plays a key role in fertility potential of the male (Jodar *et al*., 2015). Several transcripts having a specific and indirect function on spermatogenesis are listed above. Of these, transcripts like Deleted in azoospermia 1 (*DAZ1*) has a role in germ cell formation and differentiation, Spalt-like transcription factor 4 (*SALL4*) function as spermatogonia proliferation, *SPO11* is involved in meiosis, *BRWD1* is involved in spermatid differentiation, *SPATA31A3* is involved in acrosome formation and *TAF7L* is involved in the regulation of spermiogenesis.

Chromatin repackaging and regulation

BSP3 and *SPATA18* are the most translated bovine transcripts in abundance. They are involved in regulating germ cell development and maintaining chromatin integrity during spermatogenesis. The proteins of sperm transcripts protamine 1 (*PRM1*), Casein beta (*CSN2*) and Amelogenin X/Y (*AMELX* and *AMELY*) are involved in chromatin condensation, transport and signal transduction, respectively (Feugang *et al.,* 2010).

Oocyte activation and early embryogenesis

The sperm not only carries genomic DNA to oocyte but also delivers a complex reserve of remnant sperm RNA from spermatogenesis which are either totally or partially transmitted to the oocyte (Hamatani, 2012; Feugang *et al*., 2010; Lalancette *et al*., 2008b; Gur and Breitbart, 2006; Miller and Ostermeier, 2006; Miller *et al*., 2005; Ostermeier *et al*., 2002; 2004). Spermatozoa-derived mRNAs of some genes, viz.,

PRM1, PRM2, PSG-1, CLU, HLA-E, DBY, PLCZ1 are delivered to the zygote exclusively by the sperm (Kempisty *et al*., 2008; Ostermeier *et al*., 2004). Similarly, transcripts *AKAP4, CRIPS2, ADAM2* (Fertilin BETA), *PLCZI* present in the sperm are expressed only in cleaved and blastocyst stage but absent in immature and mature oocytes. It is revealed that these transcripts are sperm specific and are transmitted to the embryo (Vijayalakshmy, 2017). Sperm transcripts, *PLCZ1* is translated in the oocyte; it initiates oocyte activation at fertilization (Swann *et al.*, 2006). Sperm specific phospholipase C-tau is transferred to the oocyte, which is responsible for the activation of embryonic development (Saunders *et al*., 2002; 2007). Transcripts protamine 2 and clusterin may have a role in oocytes during fertilization and early embryonic development (Bukowska *et al.,* 2013; Carrell, 2008; Lalancette *et al.,* 2008a; Boerke *et al.,* 2007). Transcript *BMP2* present in the oocyte is crucial for post fertilization events, mainly during early embryonic development (Lee *et al*., 2014). Transcripts like *AKAP4* is involved in post fertilization events, *WNT5A* involved in embryogenesis and *HSBP1L1* involved in embryonic growth, *ZPBP* is involved in sperm- oocyte interaction (El-Fekih *et al*., 2017) and *TFB2M* is involved in embryonic development (Kropp *et al*., 2017).

Epigenetic modification

Epigenetics is defined as the changes that occur in gene expression without altering the DNA sequence (Lone *et al*., 2017). Key epigenetic dynamics that influence the sperm fertility are DNA methylation and changes in chromatin structure (Kaya and Memili, 2016). Sperm specific mRNAs may influence fertilization, early embryonic development and may also epigenetically modify the phenotype of offspring (Jodar *et al*., 2013). Besides coding RNAs, spermatozoal non-coding RNAs too form the components of transgenerational epigenetic mechanism which are known to regulate DNA methylation and chromatin structure (Rinn and Chang, 2012). The epigenetic transgenerational inheritance studies of sperm RNA were first done in mice by injecting total sperm RNAs from a mentally stressed male to normal female zygotes resulting in offsprings with same behavioral and metabolic effects as that of the father. This has proven the basis of inheritance of paternal phenotypes and their behavior in offspring's (Gapp *et al*., 2014). Similarly, when total sperm RNAs from high fat diet mouse model were introduced in normal zygotes, the resulting offspring developed same metabolic disorders suggesting the role of RNA as an epigenetic modifier (Chen *et al*., 2016a).

Sperm functions and fertility

Initially the spermatozoal RNAs were thought to have no functions of their own and were simply considered as residues of spermatogenesis, reflecting the events that occur during their formation in the testes (Krawetz, 2005). Around 5,000 different mRNA transcripts have been reported in the sperm of which, 25% of them encode for proteins associated with transcription and its regulation (Miller and Ostermeier, 2006). The expression level of sperm transcripts can serve as a potential marker to identify male infertility (Selvaraju *et al*., 2018; Parthipan *et al*., 2017). The diverse RNA content of a sperm can be used for assessing semen quality and male fertility potential (Kasimanickam, 2011). Several spermatozoal mRNA transcripts as fertility markers studied between different populations are listed in **Table 2.4**

Table 2.4: Various spermatozoal transcripts reported to be associated with sperm quality and fertility

Gene	Expression	Condition	Species	References
FOS, EAF2, CPT2, CCDC85A, DTD2	Up	Poor semen freezability boars	Porcine	Wasilewska-Sakowska *et al*., 2019
ND6	Down	Good semen freezability boars		
AKAP4, PRM1, ATP2B4, TRIM71, SLC9B2	Up	Good vs poor quality semen	Bovine	Singh *et al*., 2019
SEMG1	Up	Astheno-zoospermia (AZS) vs normal	Human	Heidary *et al*., 2019
CRISP2	Down			
ORAI3, YBX1, TFAP2C	Up	Low fertile vs high fertile	Buffalo	Paul, 2018

PEBP4	Up	High fertile vs low fertile	Bovine	Selvaraju *et al*., 2018
AQP7	Up	High SCR vs low SCR	Bovine	Kasimanickam *et al*., 2017
BMP2	Up	Good vs poor quality semen producers	Bovine	Parthipan *et al*., 2017
CASP3, UBE2D3, TRADD, HSFY2	Down			
FABP3, CYB5R4, CCT5, GUK1, PACSIN3	Up	Poor vs good motility	Bovine	Yathish *et al*., 2017
RPL31, PRKCE, PAPSS2, PLP1, R1G7	Up	Frozen thawed semen vs fresh semen	Bovine	Chen *et al*., 2015
RPL24, RPL4, RPL9, RPL18, RPL11, RPL28, RPL35, RPS16, CANX, NONO, RHOA, OAZ1, FAU, SLC25A3, HNRNPM, C1D, PRPF8, HTN3, CERCAM, GDI2, PARK7	Up	Asthenozoospermic group with normal group	Human	Bansal *et al*., 2015
RPS13, RPL27, RPS24, RPS11, RPS5, RPS27A, RPL30, RPL34, RPS25, DAD1, ILF2, SRSF9,	Down			

HSP90AB1, EIF4G2, HNRNPC, SMARCAD1, HINT1, KIFAP3				
RPS25, RPS11, RPS13, RPL30, RPL34, RPL27, RPS5, HINT1, HSP90AB1, SRSF9, EIF4G2, ILF2	Up	Normozoospermic vs asthenozoospermic	Human	Bansal *et al*., 2015
CAPNS1, FAM153C, ARF1, CFL1, RPL19, USP22	Up	Normozoospermic group vs normal group	Human	Bansal *et al*., 2015
ZNF90, SMNDC1, c14orf126, HNRNPK	Down			
TNP2	Up	Teratozoospermic group vs normal	Human	Savadi-shiraz *et al*. 2015
PRM1, PRM2	Down			
PRM1	Up	Good vs poor semen producers	Bovine	Ganguly *et al*., 2013
AdipoR1, AdipoR2	Up	High fertile to avg. and low fertile	Bovine	Kasimanickam *et al*., 2013
MYC, CYP19, ADAM2, PRM1, PRM2	Up	High cleavage embryo group vs low cleavage group	Pig	Hwang *et al*., 2013

ADH4, HSD1B7, CYGB, NXNL1, LMNA	Up	Oligozoospermic vs normal	Human	Montjean *et al*., 2012
TPD52L3; PRM2, JMJD1A, NIPBL	Down			
EPS15, TPT1, SPZ1, PRM2, DDX4, NME5, HOOK1, TSGA10, SPATA4, CREM, MEA1, AKAP4, UCP2, NIPBL, PARK7, DDX3X, JMJDIA, TAF10, SAP130	Down	Oligozoospermic vs normal	Human	Montjean *et al*., 2012
PRM1	Up	Low vs high motility	Human	Li and Zhou, 2012
CRISP2, PEBP1	Up	High SCR vs low SCR	Bovine	Arangasamy *et al*., 2011
CCT8	Down			
PSG1, HLA-E	Up	Fertile vs infertile	Human	Avendano *et al*., 2009
LMNA	Up	Oligozoospermic vs normal	Human	Lalancette *et al*., 2009
UBAP2, TPD52L3, PRM2, AKAP14, MYL6B, DNAJB8, TXNDC17, RFTN1	Down			
FCER1G, PYCARD, CASP1, CX3CR1, TNFSF13B, IL-32, IRAK1	Up	Impaired spermatogenesis	Human	Spiess *et al*., 2007

PRM1	Up	Low motile vs high motile sperm	Human	Lambard *et al*., 2004
eNOS, nNOS	Up	Low vs high motility	Human	Rosselli *et al*., 1995
COX7C	Down	Low vs high SCR	Bovine	Card *et al*., 2017
AKAP4, UBQLN3, CAPN11, GGN, SPACA4, SPATA3, FAM71F1	Down	Non-obstructive azoospermia vs normal spermatogenesis	Human	Malcher *et al*., 2013
MYC	Down	Capacitated	Pig	Hwang *et al*., 2013
CD36	Down	Low vs high fertile	Bovine	Feugang *et al*., 2010
PRM1, PRM2	Down	Asthenozoospermic vs normal	Human	Kempisty *et al*., 2008

2.6 Global transcriptomic studies in relation to sire fertility

RT-PCR

Arangasamy *et al*. (2011) studied selected transcripts in cryopreserved semen of *Holstein* bulls with sire conception rate (SCR) ranging from -4 to 4. The mRNA expression of *CRISP2* (r=0.88) and *PEBP1* (r=0.83) was positively correlated whereas the expression of *CCT8* was negatively correlated (r=-0.87). *CRISP2, CCT8* and *PEBP1* proteins participate in sperm capacitation and sperm-egg fusion. This study revealed that bulls with greater *CRISP2*, *PEBP1* and lower *CCT8* mRNA expression in sperm had higher chances of siring.

Kasimanickam *et al*. (2013) studied the mRNA expression of adiponectin and its receptors in cryopreserved semen of *Holstein* bulls with varying conception rates classified as low (≥ -4 to ≤ -2), medium (≥ -2 to ≤ 2) and high fertile (≥ 2 to ≤ 4). The mRNA expression of adiponectin and its receptors, *AdipoR1* and *AdipoR2* were greater in high-fertile bulls than medium- or low-fertile bulls. Adiponectin and its receptors were found to have a role in steroidogenesis, sperm capacitation, sperm-egg fusion and fertilization.

Ganguly *et al*. (2013) studied *PRM1* expression in fresh bull semen and revealed that bulls with good quality semen had a significantly ($P<0.05$) higher expression of PRM1 compared to poor-quality semen producers.

Ferraz *et al*. (2013) studied mRNA gene expression in bovine post-pubertal testes and revealed that *PRM1* expression was relatively higher than other genes *PRM2, TNP1, TNP2* and *PRM3.*

Hwang *et al*. (2013) studied the transcriptome profile of Duroc semen and revealed that *MYC, CYP19, ADAM2, PRM1* and *PRM2* were significantly ($P<0.05$) greater in high cleavage embryo group than low cleavage embryo group. Transcript *MYC* was significantly ($P<0.05$) downregulated in capacitated spermatozoa than in non-capacitated spermatozoa.

Parthipan *et al*. (2017) studied the transcriptome profile of good- and poor-quality frozen semen of *HF* bulls and revealed that the fold expression levels of *BMP2* was significantly ($p<0.01$) higher in good-quality semen than poor-quality semen. The regulation of genes *UBE2D3* and *CASP3* involved in apoptosis and *HSFY2* involved in homeostasis were significantly negatively correlated with the percentage of post-thaw motility. The expression levels of apoptotic/homeostasis associated genes such as *CASP3, TRADD, UBE2D3* and *HSFY2* had significant negative correlation with SCR. The expression level of *TRADD* was negatively correlated with mitochondrial membrane potential of semen samples and SCR. The *BMP2* and *TRADD* expression levels could be used to predict the semen quality and *TRADD* could be used to assess the SCR of the bull. The study concluded that, to attain more than 40% SCR, spermatozoal mRNAs like *UBE2D3, CASP3* and *HSFY2* should be absent or at very low levels.

Microarray

Gilbert *et al*. (2007) used Microarray Hybridization to study the sperm and spermatids of *HF* bulls. The array analysis revealed positive sequences of 2583 and 1117 for spermatids and spermatozoa, respectively. Among these, 996 were found in both cell types. Transcripts *MEA, SSP411, SPAG4, KLHDC3, TEGT, NSEP1, PPIH, FLOT1* were present in both sperm and spermatids, whereas, transcripts *H2AFZ, STRBP, EIF2B2* were present exclusively in spermatids.

Bissonnette *et al*. (2009) revealed that spermatozoal transcripts *TSSK6* and *ADAM5P* are associated with high-motility status (P<0.001) of bovine fresh semen; the same was also confirmed by qPCR.

Feugang *et al*. (2010) used DNA microarray on bull frozen semen of low (10.8 ± 4.1%) and high (5.1 ± 0.8%) fertility status. 24,000 transcripts were revealed out of which, 415 transcripts were detected in bull spermatozoa of varied fertility (fold change ≥2.0; P<0.01); 211 transcripts were upregulated and 204 transcripts were down regulated in high-fertile bull spermatozoa. Transcripts *CSN2, KRT8, PRM1, AMELX* or *AMELY* were fully annotated and found in abundance irrespective of the fertility status. High-fertile bull spermatozoa contained more transcripts for membrane and extracellular proteins than low-fertile bull spermatozoa. On validation, *CD36* displayed a low concentration in low-fertile bulls which could be used as a marker for bull fertility status. The top 10 transcripts with highest mRNA concentration in high- and low-fertile bull spermatozoa is listed in **Table 2.5** and **2.6**.

Table 2.5: Transcripts with highest mRNA concentration in high fertile bull

Gene name	Accession number	Fold change
Heat shock 70 kDa protein 3	NM_174344	18.30
Solute carrier family 2, (facilitated glucose transporter) membrane 8	NM_201528	10.64
Calcium channel, voltage-dependent, beta 2 sub unit	NM_175789	8.08
F-box and leucine-rich repeat protein 6	XM_867842	7.91
Claudin 1	NM_001001854	7.55

Citrate synthase	NM_001044721	5.60
Erythrocyte membrane protein band 4.9 (dematin)	NM_001034431	4.95
Pleckstrin homology domain containing, family Q member	NM_001076283	4.61
Biphenyl hydrolase-like (serine hydrolase; breast epithelial mucin associated antigen	NM_001045918	4.51
UDP-N-acetyl-aplha-d-galactosamine	NM_001017943	4.04

Table 2.6: Transcripts with highest mRNA concentration in low fertile bull

Gene name	**Accession number**	**Fold change**
Zinc finger, MYND domain containing 11	NM_001045940	10.34
Small unbiquitin-like modifier 1	NM_001035458	7.55
15 kDa selenoprotein	NM_001034759	6.08
Mitochondrial fission regulator 1	NM_001035412	5.92
Galactokinase 1	XM_869646	4.69
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	NM_001034231	4.17
Zinc finger protein 54	BC108220	3.79
Mucosal address in cellular adhesion molecule-1	NM_001037821	3.32
Metal response element binding transcription factor 2	NM_001046556	3.19
Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit (45 kDa)	NM_174663	3.06

Singh *et al*. (2015) used microarray technique to study the expression of transcripts in chicken semen and testes and revealed the expression of 17,542 and 21639 transcripts in sperm and testes, respectively. Transcripts unique to sperm and testes were 3142 and 7239, respectively. Sperm and testes shared 87.3% of the transcripts, while 12.7% transcripts were detected only in the sperm. Functional annotation of up-regulated sperm transcripts revealed signal transduction, embryonic development and cell structure. Microarray results validated with RT-PCR showed that transcripts *PRM, PLK2, ENS1* were up-regulated and transcripts *PLCZ1, PABPC1* and *RPL26L1* were down-regulated in sperm compared to testes.

Chen *et al*. (2015) used SSH and microarray hybridization on fresh and frozen-thawed semen of *Holstein* bulls. Microarray data analysis revealed 15 differentially expressed unique genes; 12 transcripts were upregulated in frozen–thawed sperm and three transcripts were up-regulated in fresh sperm. The upregulated transcripts (*RPL31, PRKCE, PAPSS2, PLP1 and R1G7)* validated by RT-PCR showed that *RPL31* expression was significantly ($P<0.05$) higher in frozen-thawed semen than fresh semen.

Yathish *et al*. (2017) used Microarray on fresh semen of Vrindavani crossbred cattle showing good and poor motility status. Analyses revealed a total of 19,454 genes; about 305 genes were differentially ($P<0.01$) expressed (160 - upregulated, 145 - down regulated) in bulls with poor sperm motility. Certain transcripts like *FABP3, CYB5R4, CCT5, GUK1* and *PACSIN3* were upregulated ($P<0.01$ and $P<0.05$) with a 16 to 4 (fold change) in poor sperm motility compared to bulls with good sperm motility.

Paul (2018) used microarray hybridization on frozen semen of buffalo bulls with varying fertility rates. A total of 51,284 transcripts were detected in buffalo spermatozoa; among which 950 were up-regulated and 3100 were down-regulated in low-fertile buffalo bulls. With a cut off of fold change >2, a total of 32 transcripts were up-regulated while 489 transcripts were down-regulated in spermatozoa of low-fertile bulls. Among the most differentially expressed genes, 10 were most relevant in terms of sperm functions and fertility including *YBX1, RPL39, PGAM1, CASP4, TFAP2C, H3F3B, ZAR1, CHRNA3, MAP2K6* and *ORAI3*. Validation of few genes inferred from microarray, using qPCR indicated that transcriptional abundance of *ORAI3, YBX1* and *TFAP2C* genes were 138.22, 18.43, and 10.8 times, respectively higher in LF bull spermatozoa compared to HF bull spermatozoa.

Suliman *et al*. (2018) used microarray on high- and sub-fertile spermatozoa of stallion. 437 genes were differentially expressed (DE) between high- and sub-fertile groups ($FC \geq 1.2$). Functional annotation of three differentially expressed genes *OAS1, OAS2, RPL5* were associated with RNA binding and genes like *CSF3R, GM-CSF, IL17A, IL13, IL22RA1, CD134, CD27* and *SF9* were found to be significantly ($P=0.02$) enriched in the cytokine-cytokine receptor interaction pathway.

Elango (2019) analyzed the testes of *Bos indicus* and *Bos taurus* by microarray and revealed 14,419 transcripts. Among the detected transcripts, 1466 were differentially expressed between crossbred and indigenous bulls, out of which 1038 transcripts were up-regulated and 428 transcripts were down-regulated in crossbred testes compared to testes of indigenous bulls. Around 12,953 transcripts were co-expressed between crossbred and indigenous bull testes. *PI4KB* and *SPATA7* genes were significantly down-regulated in crossbred bull testes.

RNA Seq

Card *et al*. (2013) studied the transcriptome profile of cryopreserved semen of *HF* bulls (n=3) with varying conception rate (-2.9 to 3.5) using Illumina (HiSeq 2000). Totally, 6166 sperm transcripts were observed. Translation was the most predominant biological process expressed with FPKM >100. Among the transcripts *PRM1*, *HMGB4*, and mitochondrial-encoded transcripts were highly expressed. The top 10 transcripts observed in bovine spermatozoa are listed in **Table 2.7**.

Table 2.7: Spermatozoal transcripts expressed in global transcriptomic profiling

Gene Symbol	Gene name	Accession no.	FPKM
PRM1	Protamine 1	BC108207; M14559	20667; 12461
LOC783058	Hypothetical protein	BC126791	10290
HMGB4	High mobility group box 4	BC109790	6022
LOC404073	Histone 2B variant PT15	BC108210; AF315690	3048; 2158
CHMP5	Chromatin modifying protein 5	BC103182	2778
TMSB4X	Thymosin beta 4 X-linked	FJ795030	2487
LOC281370	Polyubiquitin	AB099044	2426
GSTM3	Glutathione S-transferase mu 3	BC112491	2374
N/A	cDNA clone; IMAGE:7944277	BC134702	2050
KIF5C	Kinesin family member 5C	BC151732	1862

Das *et al*. (2013) studied the transcriptomic profile of fresh semen and testes of stallion, using Microarray and RNA-Seq. RNA-Seq analysis showed totally 19,257 transcripts while microarray analysis showed totally 6761 transcripts (sperm) and 11,112 transcripts (testes). 165 transcripts were sperm-enriched and 155 transcripts were differentially expressed between the sperm and testes. In DGE, 60 were upregulated (fold change >2) and 95 were downregulated in sperm (fold change< -2) compared to testes. Gene ontology analysis of upregulated genes expressed cell motility and cytoskeleton functions; the down regulated genes were associated with translation and ribosome. Sperm transcripts detected by microarray analyses shared 60-70% with RNA-seq data. Microarray results were validated with RT-PCR and its results revealed that genes *PADI6, DNAJC168, DCDC2, CTTN, REEP6, ARID5B* and *ATC12* were significantly ($p<0.05$) upregulated and genes *GSTA1, DYNTL1, SPA17, PRPSAP1* were significantly ($p<0.05$) down-regulated in the sperm than the testes.

Card *et al*. (2017) studied the transcriptome profile of high- and low-fertile *HF* bulls (n=4) with CR ranging from 1.8-3.5, using Illumina (HiSeq 2000). Totally, 3227 and 5336 number of transcripts were observed in high- and low-fertile bulls, respectively. Among these, 2422 transcripts were common, 805 were unique to high- and 2944 were unique to low-fertile population. On validation of 27 transcripts in 9 bulls, only *PRM1* was present in all the bulls with different CR. Transcripts, *PRM2* and *TNP1*, were expressed in eight out of nine sires. Transcripts *CKS2, COX7C, CRISP2, EEF1G, G2E3, GSTM3, GTSF1, PRM1, PRM2, PSMA6, TNP1* were expressed in five or more sires while transcripts *AKAP4, APOABP1, ATPIF1, ATP5J, BRP, CCT8, CETN2, CLGN, CMYC, COX7A2, DNAJB7, EIF1, GPX4, HEMGN, PCNA, SPATA6* were present in 50% of the sires. *COX7C* was the only differentially expressed transcript between high and low fertile populations and it was present in six of the nine bulls. Certain previously reported transcripts (*TSSK6, ADAM5P, CD36, CENPA, AKI, ITGB5, DOPPEL, LDC1*, *ADIPO)* related to sire fertility were not observed. RNA-Seq with validation on different fertility bulls revealed that *COX7C* transcript was negatively correlated with sire fertility. The top 10 transcripts unique to high- and low-fertile bulls are listed in **Table 2.8** and **2.9**.

Table 2.8: Transcripts unique to high fertile bull spermatozoa

Gene Symbol	Gene name	Accession no.	FPKM
MIR708	microRNA 708	n/a	200
C4H7orf55	chromosome 4 open reading fram, human C7orf55	NM 001076997	122
VSNL1	visinin-like 1	NM 174490	106
AKR1B1	aldo-keto reductase family 1,	NM 001012519	95

	member B1 (aldose reductase)		
APOA1BP	apolipoprotein A-I binding protein	NM 205796	91
SQRDL	sulfide quinone reductase-like (yeast)	NM 001040511	91
CD28	CD28 molecule	NM 181004	87
MPI	mannose phosphate isomerase	NM 001035284	65
RNGTT	RNA guanylyltransferase and 5-phosphatase	NM 001046085	64
FCF1	FCF1 small subunit (SSU) processome component homolog	NM 001037452	63

Table 2.9: Transcripts unique to low fertile bull spermatozoa

Gene Symbol	Gene name	Accession no.	FPKM
RN5-8S1	5.8s ribosomal RNA	NR 036643	348
VDAC3	voltage-dependent anion channel 3	NM 174729	300
DEFB122A	beta-defensin 122a	NM 001102339	247
ATP6V1D	ATPase, H+ transporting, lysosomal 34 kDa, V1 subunit D	NM 001075141	228
LOC526524	FK506 binding protein 1A, 12 kDa	NM 001035456	203
UQCR10	ubiquinol-cytochrome C reductase complex 7.2 kDa protein	NM 001113723	195
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1	NM 001035310	171
INSL6	insulin-like 6	NM 001077521	162
C10H14orf166	chromosome 14 open reading frame 166 ortholog	NM 001035280	161
METAP2	methionyl aminopeptidase 2	NM 001040493	157

Selvaraju *et al*. (2017) studied the transcriptome profile of bull fresh semen (n=3), using Illumina (NextSeq-500) and Ion Proton. Totally 13,833 sperm transcripts were observed with TPM>10. Ion Proton and Illumina platform analyses revealed 12,307 and 12,470 genes (>10 TPM). *PRM1*, *CHMP5* and *YWHAZ* were the most predominant transcripts involved in spermatogenesis and sperm functions. The top 10 transcripts observed in two different RNA-Seq platform are listed in **Table 2.10**.

Table 2.10: Transcripts observed in two different RNA-Seq platform

Gene ID	Gene Name	TPM (Transcripts per million)
PRM1	Protamine 1	8659
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	3050
FABP1	Fatty acid binding protein 1	2923
SCP2D1	Sterol-binding domain containing 1	2726
THSD4	Thrombospondin type 1 domain containing 4	1961
CHMP5	Charged multivesicular body protein 5	1693
NR2E3	Nuclear receptor subfamily 2 group E member 3	1610
SV2C	Synaptic vesicle glycoprotein 2 C	1518
MGC137055	Det1 and ddb1 associated 1	1434
GTSF1L	Gametocyte specific factor 1-like	1416

Vijayalakshmy (2017) studied the transcriptome profile of cryopreserved semen of high- and sub-fertile buffaloes (n=10) with CR ranging from <40% to >50%. A total of 33,715 and 21,788 transcripts were expressed in sub-fertile and high-fertile spermatozoal RNA profiling. While 15,895 transcripts were upregulated and 17,820 transcripts were downregulated in sub-fertile group, 10,126 transcripts were upregulated and 11,662 transcripts were down regulated in high-fertile group. Transcripts were annotated with buffalo RNA-Seq; 1248 and 802 transcripts were unique to high- and sub-fertile populations, respectively. Among this, 2527 transcripts were common to both the populations. On validation of four genes *AKAP4, CRIPS2, ADAM2* (Fertilin BETA), *PLCZI*) were upregulated in HF buffalo bulls compared to sub-fertile buffalo bulls. The top 10 transcripts unique to high- and low-fertile bulls are listed in **Table 2.11** and **2.12**

Table 2.11: Transcripts unique to high fertile bull spermatozoa

Gene name	Function
Cysteine rich secretory protein	Protein folding and stabilization of tertiary structure
Putataive V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like	Inhibiting co-receptors in B cell activation
Phosphate carrier protein, mitochondrial isoform X1	Mitochondrial permeability transition pre

A-kinase anchoring protein	Regulation sperm function
Molybdenum cofactor biosynthesis protein 1 isoform X1	Molybdenum cofactor biosynthesis
Eukaryotic intimation factor delta isoform X2	RNA metabolism
Casein Kinase 1 isoform delta isoform X2	Cellular growth and survival processes, including DNA circadian rhythms
Multidrug resistance protein	Resisting multidrug resistance
Testis specific gene 10 protein isoform X1	Sperm tail fibrous sheath formation
Histone H2A type 2-B-like	Transcription regulation, Unrepair, DNA replication and Ch stability

Table 2.12: Transcripts unique to high fertile bull spermatozoa

Gene name	Function
Nucleolar GTP-binding protein 1 isoform X1	Biogenesis of the 60s ribosomal subunit
60KDa heat shock protein, mitochondrial isoform X1	Prevent misfolding and promote the refolding
Protamine 2	Condensing chromatin required to induce the quality of potent spermatozoa
3-hydroxybutyrate dehydrogenase type 2 isoform X2	Iron homeostasis and transport
Elongation factor Tu, mitochondrial	Delivers amino-acylated tRNAs to the ribosome during the elongation step translation
Pro-cathepsin H	Overall degradation of protein in lysosomes
Glutamine synthetase isoform X1	Regulation of liver
Vesicle transport through interaction with t-SNAREs homolog 1A isoform X3	Intracellular trafficking
Hydroxyacid oxidase 1 like isoform X2	Redox signalling and lipid homeostasis
S-adenosylmethionine synthase isoform type-2	Catalyzes the formation of sS-adenosylmethionine from methionine and ATP

Capra *et al*. (2017) studied the transcriptome profile of cryopreserved semen of buffalo bulls (n=4) with low- and high-motile fractions using Illumina (TrueSeq). 83 miRNAs and 79 putative piRNAs were identified as differentially expressed in high- and low-motile fractions, respectively. Dysregulated *miR-17-5p, miR-26a-5p, miR-486-5p,* *miR-122-5p, miR-184* and *miR-20a-5p* were involved in *PTEN, PI3K/AKT* and *STAT* pathways.

Van Son *et al*. (2017) studied the transcriptome profile of Landrace and Duroc testes based on DNA fragmentation index (DFI), using Illumina (HiSeq 2000). RNA Seq revealed expression of 308 (high DFI) and 374 (low DFI) genes; 71 genes were differentially expressed in both in Landrace and Duroc testes. In Landrace, *NXPH2* was the most upregulated gene and *ACTA1* was the most down-regulated gene in high DFI group; *DCT* was the most down-regulated gene and *ADAMTS4* was the most upregulated gene in high DFI group. Increased expression of differentially expressed genes were observed in the high DFI group compared to the low DFI group. Extracellular matrix, extracellular region and calcium ion binding were the common GO terms observed between the two breeds.

Kropp *et al*. (2017) studied the transcriptome profile of bovine embryos (n=12) in its blastocyst stage with high- (3.4 to 4.1) and low-SCR (-2.3 to 7.5), using RNA Seq. A total of 16,710 genes were revealed out of which, 98 genes were DE between high- and low-SCR. On validation with RT-PCR, *CYCS, TFB2M, MEPCE* were significantly expressed in HF sire-derived embryos and *EEA1, SLC16A7* were highly expressed in LF sire-derived embryos.

Li *et al*. (2018) studied the transcriptome profile of fresh and capacitated semen in Landrace boars by high throughput RNA-Seq. Totally 5342 mRNAs and 204 miRNAs, differentially expressed between fresh and capacitated semen, were observed. Twelve miRNAs and their differentially expressed target mRNAs were found to participate in sperm capacitation-related *PI3K-Akt, MAPK, cAMP-PKA* and Ca2+ signaling pathways.

Raval *et al*. (2019) studied the transcriptome profile in fresh semen of *Bos indicus* (Gir bull) using Illumina (MiSeq Platform). 14,306 transcripts with FPKM>0 and 405 transcripts with FPKM>5 were identified. Transcripts with FPKM>5 were linked to biological processes like translation and ribosomal assembly (small- and large-subunits). *RN7SL1* (high abundant) and *ZFP280B* (low abundant) genes were validated with RT-PCR. The top 10 transcripts observed in bovine spermatozoa is listed in **Table 2.13**.

Table 2.13: Transcripts observed in bovine spermatozoa

Gene ID	Gene Name	FPKM
LOC101904117	uncharacterized LOC101904117	2942
LOC107131417	uncharacterized LOC107131417	1864
TRNAQ-CUG	transfer RNA glutamine (anticodon CUG)	1543
RN7SL1	RNA, 7SL, cytoplasmic 1	653
LOC100848009	uncharacterized LOC100848009	368
LOC101908506	uncharacterized LOC101908506	311
LOC107132266	uncharacterized LOC107132266	302
LOC529686	F-box only protein 27	296
LOC107132430	uncharacterized LOC107132430	259
LOC101903977	F-box only protein 27-like	218

Singh *et al*. (2019) studied the transcriptome profile of frozen semen of varying quality obtained from Frieswal bull, using Ion torrent personal genome machine. Deep sequencing of crossbred bull spermatozoa expressed totally 3,510 and 6,759 transcripts between good- and poor-quality semen. Most of the differentially expressed transcripts were associated with sperm function, embryo development and fertilization. Transcripts *AKAP4, PRM1, ATP2B4, TRIM71, SLC9B2* were expressed significantly ($p<0.05$) between samples of differed quality.

Wasilewska-Sakowska *et al*. (2019) studied the transcriptome profiles of polish large white boars (n=6) using RNA-Seq (NextSeq 500). The profile was analyzed based on the sperm freezability condition as good and poor. Three packages (DESeq2, DESeq EdgeR) were used to detect varying numbers of DE genes in which, 28 DE gene transcripts commonly detected and they were up-regulated ($\log2FC > 1$; $q\text{-value} < 0.05$) in spermatozoa from boars with poor semen freezability. The expression of 6 DE gene transcripts, *FOS, EAF2, CPT2, CCDC85A, DTD2* and *ND6* was verified by qRT-PCR. Analysis confirmed that expression of *FOS, EAF2, CPT2, CCDC85A* and *DTD2* were upregulated in the poor semen freezability boars, whereas the expression of *ND6* was down regulated in the good semen freezability boars.

2.7 Sperm-oviduct interaction

Spermatozoa undergo a sequence of complex changes (biochemical, metabolic and structural) in the oviduct before attaining the fertilizing capacity of oocytes, this process is termed as capacitation (Austin, 1952) and it is one of the hall mark event of sexual reproduction. Initially, it was studied in a rabbit model by Austin (1951) and Chang (1951).

Fallopian tube is a multifaceted organ which is involved in various processes like sperm selection, interaction of gametes, functional sperm reservoir (Harper, 1994), gamete maturation, fertilization and early embryonic development (Aviles *et al*., 2010; Suarez, 2008). It secrets a complex and dynamic fluid from the epithelial cells, blood plasma and compounds of the pre-ovulatory follicular fluid (Aviles *et al*., 2010; Leese *et al*., 2007). It contains a variety of components like intra-cellular and extra-cellular ions, carbohydrates, proteins, lipids, steroids, growth factors, hormones, sugars, amino acids and electrolytes (Hugentobler *et al*., 2010; Aviles *et al*., 2010; Killian, 2004; Gadella and Harrison, 2000). Some of the more specific components like bicarbonate (Rodriguez-Martinez, 2007), glycosaminoglycans (Bergqvist and Rodriguez-Martinez, 2006; Parrish *et al*., 1989), calcium (Visconti *et al*., 2002) and norepinephrine (Way and Killian, 2002) are considered as agents of sperm-capacitation

The oviductal secretions play a role in modulating sperm functions, including capacitation (Rodriguez-Martinez, 2007). It varies with region (isthmus or ampulla) (Killian, 2004; Hunter and Rodriguez-Martinez, 2004), stage of estrous cycle (luteal or non-luteal/ pre-ovulation or post ovulation) (Lamy *et al*., 2016; Kumaresan *et al*., 2012a; Rodriguez-Martinez, 2007; Tienthai *et al*., 2004) and state of spermatozoa (Fresh and Frozen) (Kumaresan *et al*., 2019; Kadirvel *et al*., 2009). Based on these differences, the oviductal fluid secretions differ in its functions too (Way *et al*., 1997). A recent report by Kodithuwakku *et al*. (2007) revealed that, oviductal secretion could be modified by the presence of gametes.

A series of biochemical changes happened during sperm capacitation leads to a cholesterol efflux from the sperm membrane resulting in increased fluidity in the membrane (Visconti *et al*., 1999); increased permeability to $HCO3^-$ and Ca^{2+} ions, causing membrane hyperpolarization (Hernandez-Gonzalez *et al*., 2006), alterations in protein phosphorylation (Urner and Sakkas, 2003; Visconti *et al*., 1995), increases protein kinase activity (Visconti, 2009; Arcelay *et al*., 2008; Baldi *et al*., 2000), increased $HCO3^-$ concentration, intracellular pH, Ca^{2+} and cAMP levels thereby leading to hyper activation (Visconti *et al*., 2002) and acrosome reaction (Naz and Rajesh, 2004). Increased intra-cellular calcium concentration and protein tyrosine phosphorylation are the two well-recognized events in sperm capacitation (Kumaresan *et al*., 2017, Zumoffen *et al*., 2010; Visconti *et al*., 2002).

Similar to *in-vivo* capacitation, cryopreservation induces changes similar to capacitation (Kumaresan *et al*., 2012ab; Thomas *et al*., 2006; Cormier and Bailey, 2003; Bailey *et al*., 2000; Watson, 2000) this is termed as cryo-capacitation (Bailey *et al*., 2000). Cryopreservation alters the membrane fluidity thereby increasing the intra-cellular calcium and promotes protein kinase A to phosphorylate some proteins (Urner and Sakkas, 2003); these changes are similar to true sperm capacitation (Green and Watson, 2001; Bailey *et al*., 2000). It is proved that tyrosine phosphorylation of proteins was observed in frozen-thawed bull spermatozoa (Kadirvel *et al*., 2009; Bailey *et al*., 2000) and boar (Kumaresan *et al*., 2012ab; Tardif *et al*., 2001). Hence, the review is focused on the effect or modulation of oviductal fluid (different region and different stage) in fresh and frozen-thawed spermatozoa on sperm function and fertility.

2.8 Effect of oviductal fluid on sperm function and fertility

Parrish *et al*. (1989) successfully induced *in vitro* capacitation and acrosome reaction in bovine spermatozoa within 4 h of incubation with oviduct fluid collected at the time of estrus

McNutt and Killian, (1991) found that spermatozoa incubated on non-luteal ODF were hyper activated and capacitated more rapidly than the luteal ODF.

Zhu *et al*. (1994) compared the effect of incubating semen in human oviductal fluid and follicular fluid for 9 h. Spermatozoa incubated in ODF showed a significantly higher motility than the sperm incubated in follicular fluid (FF) or the control medium

Grippo *et al*. (1995) reported that the region of oviduct and the stage of the estrous cycle alters the effect of ODF on sperm functions

Oliveria *et al*. (1999) found that on adding different dilutions of oviductal fluid to mouse spermatozoa, the sperm velocity improved and induced chemotactic responses.

Taft *et al*. (1992) studied the rate of capacitation on different fertility bulls with non-luteal oviductal fluid showing that the population of dead acrosome reacted (DAR) spermatozoa was high in LF bulls compare to HF bulls.

Kadirvel *et al*. (2009) studied the cholesterol level, membrane fluidity and intracellular calcium of fresh and frozen-thawed buffalo spermatozoa in relation to capacitation. Frozen-thawed semen showed a significant ($P<0.01$) high membrane fluidity (53.62% vs 25.67%), high intracellular Ca^{2+} (43.68% vs 11.72%) and high capacitation of spermatozoa (42.21% vs 14.32%) than fresh semen.

Coy *et al*. (2010) reported that porcine oviductal fluid collected during estrus phase when incubated with boar spermatozoa *in-vitro*, showed decreased membrane fluidity, increased sperm viability, acrosomoal integrity and zona pellucida binding.

Kumaresan *et al*. (2012a) studied the effect of pre- and post-ovulatory ODF on boar spermatozoa frozen different (conventional freezing (CF) and simplified freezing (SF) methods. Results indicated that these freezing methods significantly ($P<0.05$) influenced the effect of ODF on post-thaw sperm motility, velocity and global tyrosine phosphorylation during incubation. Moreover, spermatozoa frozen by CF method showed significantly ($P<0.001$) higher proportion of tyrosine phosphorylation on exposureto pre-ovulatory isthmic ODF than post-ovulatory ODF during incubation.

Kumaresan *et al*. (2012b) studied the modulation of ODF in cryopreserved boar spermatozoa for 6 h in terms of protein tyrosine phosphorylation. The exposure to pre-ovulatory ODF significantly ($P<0.001$) increased the population of spermatozoa showing PTP after 2 h (13.83%), peaked at 4 h (17.50%), and then decreased at 6 h (9.80%). Immediately after thawing, the proportion of spermatozoa (3.64%) displaying pattern A showed an increasing trend (7.64%) in the EODF groups after 6 h incubation; the PTP patterns T and AET were observed only after 4 h incubation with EODF. Hence it was inferred that the percentage of phosphorylated spermatozoa in the pre-ovulatory ODF group was significantly ($P<0.001$) higher than NCM, CM and post-ovulatory ODF.

Kumaresan *et al*. (2014) studied the dynamic quantification of intracellular Ca^{2+} and PTP in frozen boar spermatozoa with minimal incubation in EODF. After a relatively short exposure to EODF, the cryopreserved spermatozoa endured capacitation-like changes and some subpopulations of spermatozoa endured PTP despite having low intra-cellular Ca^{2+} concentrations due to cryopreservation.

Kumaresan *et al*. (2017) incubated spermatozoa of different fertility bulls with EODF for 6 h. From 4 h of incubation and onwards, the sperm from HF bulls showed a lower ($p<0.05$) tyrosine phosphorylation and, high capacitation and acrosome reaction compared to spermatozoa from LF bulls.

EL-Shahat *et al*. (2018) added 50 µL/mL of oviduct fluid on ram spermatozoa and incubated for 4 h which resulted in increased motility and acrosome reaction.

Kumaresan *et al*. (2019) studied the effect of bovine EODF on different sperm functional attributes in cryopreserved bull spermatozoa. At 4 h and 5 h of incubation, the proportion of live tyrosine phosphorylated spermatozoa was higher ($P<0.05$) in EODF; from 4 h to 6 h of incubation the proportion of live acrosome reacted (LAR) spermatozoa was significantly higher ($P<0.05$) in EODF compared to luteal ODF, NCM and CM. It was concluded that cryopreserved bull spermatozoa exposed to EODF induced a higher tyrosine phosphorylation and acrosome reaction status.

Alfradique *et al*. (2019) studied the effects of fresh ram spermatozoa incubated in bovine oviductal fluid (bOF) for 24 h and concluded that ODF from different stages of the estrous cycle improves ram sperm motility for 4 h and the rate of acrosome reaction after 24 h incubation periods, without affecting the sperm viability.

2.9 Capacitation associated genes related with fertility

In-vivo capacitation is an essential phenomenon for any sperm to fertilize an egg. The physiological end points to evaluate successful capacitation include hyperactivated motility and an ability to undergo the acrosome reaction (Naaby-Hansen *et al*., 2002).

Spermatozoal RNAs are involved in various process like sperm motility, capacitation, acrosome reaction, sperm-egg fusion, fertilization induction, and early embryo development (Selvaraju *et al*., 2018). Various mRNAs have directly or indirectly involved in the process of sperm capacitation and acrosome reaction. Some mRNAs like NYD-SP 16 (*SPATA9*) are involved in capacitation and acrosomal reaction and are highly expressed in normal spermatogenesis (Cheng *et al*., 2003). Tyrosine phosphorylation-transactivation of *AKAP3* has shown a progressive response on human sperm motility (Luconi *et al*., 2005); it is vital for sperm capacitation (Vizel *et al*., 2015). *PEBP1* has shown an inhibitory effect on sperm capacitation (Nixon *et al*., 2006). It is positively correlated with SCR and is possibly involved in functions associated with capacitation and fertilization (Arangasamy *et al*., 2011). *CRISP2* regulates sperm capacitation (Gibbs *et al*., 2006). Arangasamy *et al*. (2011) reported that *CRISP2* was positively correlated with sire conception rate and sire with high conception rate had high *CRISP2* expression. Kasimanickam *et al*. (2013) stated that adiponectin and its receptors have numerous functions on sperm structural and functional traits and through cholesterol efflux it is involved in sperm capacitation and positively associated with bull fertility. *CATSPER1* is vital for the hyperactivity of spermatozoa, chemotaxis towards the egg, capacitation and acrosome reaction (Sun *et al*., 2017) and is essential for male fertility. Jin *et al*. (2005). The relevance of mRNA to capacitation and embryo development was studied in selected transcripts like *MYC, CYP19, ADAM2, PRM1, PRM2*. In which, a significant (p<0.05) downregulation of MYC transcript was observed in capacitated spermatozoa of porcine (Hwang *et al*., 2013). *IQCF1* is a recently identified acrosomal protein of spermatozoa and spermatids. It interacts with calmodulin and is involved in tyrosine phosphorylation of sperm proteins during capacitation (Fang *et al*., 2015). *CABYR* is a novel calcium-binding protein involved in capacitation (Naaby-Hansen *et al*., 2002). *CABYR*-knockout male mice have shown severe subfertility (Young *et al*., 2016).

CHAPTER - 3

Materials and Methods

3. MATERIALS AND METHODS

The present study was conducted at the Theriogenology laboratory, SRS of ICAR- National Dairy Research Institute (NDRI), Adugodi, Bengaluru. The study protocol was duly approved by the Institute Animal Ethics Committee (CPCSEA/IAEC/LA/SRS-ICAR-NDRI-2019/No.09) and performed in accordance with relevant guidelines and regulations.

The study had two objectives

1. Differential transcriptome profile between high- and low-fertile crossbred bulls
2. Ability of spermatozoa to undergo capacitation upon exposure to oviductal fluid and its relationship with bull fertility

3.1 Objective 1 - Differential transcriptome profile between high- and low-fertile crossbred bulls

3.1.1 Source of chemicals

DNA loading dye, DNA ladder, Turbo DNA-Free kit (contains DNase enzyme, DNase buffer, DNase inactivation buffer and molecular grade nuclease free water), first strand cDNA synthesis kit, Dream Taq green PCR mastermix (2X) and maxima SYBR green / ROX qPCR master mix (2X) were procured from Thermo Fisher Scientific, USA. RNaseZAp solution from Sigma- Aldrich, USA. Chemicals and reagents viz., molecular grade sodium lauryl sulphate, Triton-X-100, diethylpyrocarbonate (DEPC), chloroform, 2- propanol and ethanol were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Primers were procured from Proteogen Biosciences (India) Pvt. Ltd (Integrated DNA Technologies, USA). All the glassware’s were procured from Borosil glass works Ltd., India and all plastic wares like micro tips and eppendorf tubes were procured from Thermo fisher scientific, Rochester, New York, USA.

3.1.2 Reagent preparation

Molecular biology grade chemicals were used for preparing buffers and reagents using autoclaved distilled water. The buffers and reagents used for RNA work were prepared exclusively in DEPC treated distilled water and autoclaved prior to use. All the plastic and glassware's were thoroughly treated with 0.1% DEPC in MilliQ water overnight at 37°C, autoclaved at 121°C for 15 minutes at 15 psi and completely dried prior to use.

3.1.3 Source of spermatozoa

Cryopreserved semen straws of HF crossbred bulls (n=12) of known fertility status were procured from Artificial Breeding and Research Centre, Karnal, Haryana, India and Kerala Livestock Development Board, Mattupetty, Kerala, India. The conception rates (CR) of the bulls used in the study are given in **Fig 3.1**. Based on CR, high- and low-fertile bulls (two in each category) were identified. For this purpose, bull CRs were plotted and the mean CR and standard deviation was calculated. The mean CR of the bulls was 39.6% and the SD was 11.4%. The bulls with CR higher than mean+1SD (i.e 51%) were considered as high-fertile while the bulls with CR less than mean-1SD (i.e 28%) were considered as low-fertile. Cryopreserved semen from the identified two high- and two low-fertile bulls were individually subjected to high throughput transcriptomic analysis as detailed below.

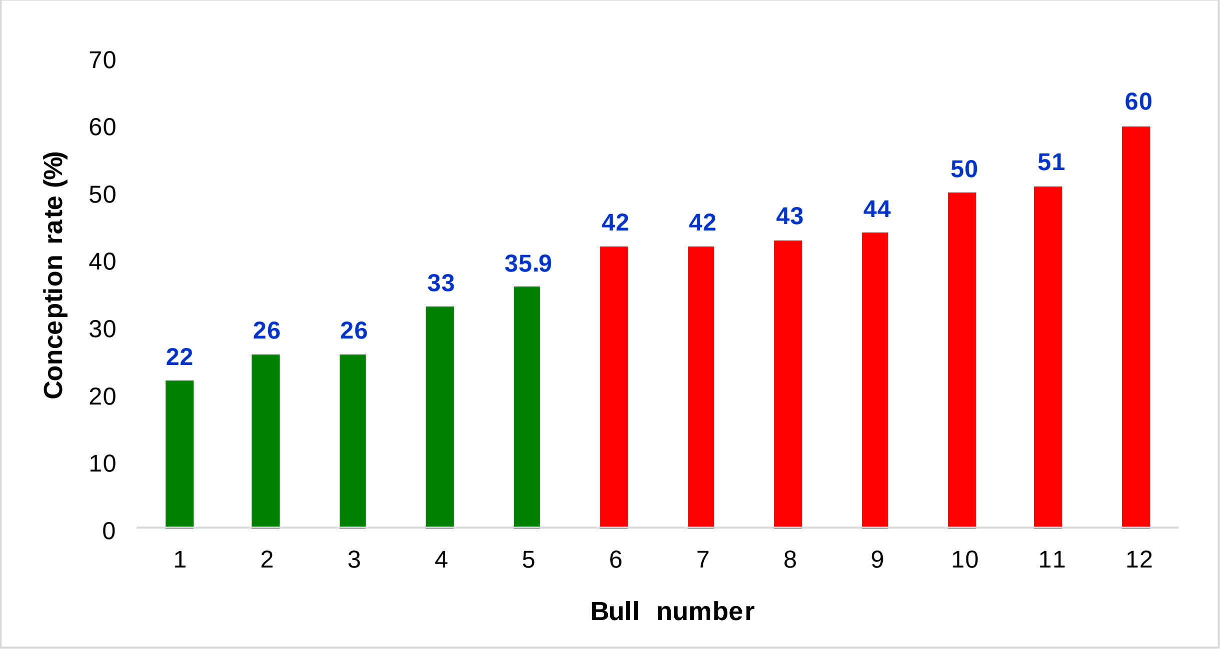


Fig 3.1: Conception rate of study bulls

3.1.4 Total RNA isolation

Total RNA was isolated from sperm using TRIzol (Ambion, Thermo Fisher Scientific, USA) as previously described by Parthiban *et al*. (2015) with minor modifications. In brief, frozen semen straws from experimental bulls ($40x10^6$ spermatozoa/bull) were thawed in a 37°C water bath for 30 sec, centrifuged at 800 x g for 10 min at 4°C, and the supernatant was removed without disturbing the sperm pellet and the pellet was washed twice with 1 mL of DPBS at 800 x g for 15 min at 4°C. The sperm pellet was purified by mixing with 1 ml of somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in DEPC treated water) and It was then kept over ice for 1 hour to remove somatic cell contamination with regular vortexing for every 15 mins and centrifuged at 5939 x g (8000 RPM) for 1 h at 4°C. The pellet was then dissolved in 1 mL DEPC treated water and centrifuged at 5939 x g for 15 min at 4°C.

The washed sperm pellet was suspended in 1 mL pre-warmed TRIzol reagent (65°C) and shredded by sonication process for 1 min and 30 sec (shredding for 6 sec and break for 5 sec). The samples were then vortexed for 5 min and incubated for 1 hour in dry bath (62°C) for complete dissociation of the sperm membrane. To the lysate, 200 µl of chloroform was added, mixed vigorously for 20 sec and allowed to stand without disturbance at room temperature for 15 min. The mixture was centrifuged (13362 x g {12000 RPM}, 4°C, 20 min) without applying a break to separate the phases. Following centrifugation, three layers were formed, the clear aqueous layer containing RNA, the opaque white interphase containing DNA and the red bottom organic layer containing protein. The upper aqueous layer containing RNA was carefully aspirated without disturbing the interphase and transferred to another tube. To this, isopropanol (0.5 mL) was added and gently mixed by inverting the tubes. The mixture was then incubated at room temperature for 15 min and centrifuged at 13362 x g for 15 min at 4°C. The supernatant was discarded, and 1 ml of 70% ethanol was added to the RNA pellet and centrifuged at 13362 x g for 10 min. Ethanol was removed, and the RNA pellet was air-dried to remove traces of ethanol. The pellet was dissolved in 20-40 µl of DEPC treated water and proceeded for RNA quantification.

RNA quantification was done using Nanodrop (ND-1000, Thermo-scientific, USA), in which a 260/280 ratio for was calculated for the RNA samples to assess quality of the RNA isolation. RNA samples with 260/280 ratio of 1.7 to 2.0 were processed immediately for cDNA synthesis, followed by storage at -20°C until further processing.

3.1.5 Transcriptome library preparation

1µg of Total RNA was used to enrich mRNA using NEB Magnetic mRNA Isolation Kit (Illumina, USA). The transcriptome library was prepared using NEB ultraII RNA library prep kit (Illumina, USA) and sequenced using Illumina Next Seq 500 (Illumina, USA) paired end technology.

The enriched mRNA was fragmented (approximately 200 bp) using fragmentation buffer. Random hexamer primers were then added and hybridized to complementary RNA sequences. These short fragments were used as templates to synthesize the first-strand cDNA using reverse transcriptase and dNTP. The DNA-RNA hybrids synthesized during first strand cDNA synthesis were converted into full-length double-stranded of cDNAs using RNase H and *E. coli* DNA Polymerase I and the second strand cDNA was synthesized using second strand enzyme mix and buffer. The double-stranded cDNA fragments were purified using 1.8X Ampure beads. The purified double stranded cDNA was end repaired to ensure that each molecule is free of overhangs and has 5' phosphates and 3′ hydroxyls before the adaptor ligation. The adaptor ligated DNA was purified using Ampure beads and enriched with specific primers, compatible for sequencing on to the Illumina platforms. The final enriched library was purified and quantified by Qubit ® Fluorometer and the size is analyzed by Bio analyzer.

3.1.6 Sperm RNA sequencing and data analysis

The cDNA synthesized samples of high fertile (n=2) and low fertile (n=2) bulls were sequenced using Illumina Nextseq-500 sequencing system (Sandor® Lifesciences Pvt. Ltd. Banjara Hills, Hyderabad, India) to generate paired-end 76bp reads. The sequence analysis was done using the online server tool *Galaxy* (https://usegalaxy.org/) (Shannon *et al*., 2003). Raw data generated from the four samples, read quality was checked using *FastQC* (Galaxy version 0.72) program and the reads were then processed with *Cutadapt* tool (Galaxy Version 1.18) (Martin, 2011). Processing includes removal of adapter (AGATCGGAAGA) sequence, length trimming (>15bp) and quality trimming (30 phred score). Using *HISAT2* (Galaxy Version 2.1.0+galaxy4) (Kim *et al*., 2015) all the four sample processed reads were aligned to the bovine genome (*Bos taurus* UMD 3.1.94/Btau8) and the samples were sorted with aligned sequences using *Samtools* (Galaxy Version 2.0.2) (Li, 2011). The mapped and properly paired sequence to the reference genome was calculated based on tabular descriptive statistics dataset tool *Flagstat* (Galaxy Version 2.0.1) (Li, 2011). Using the tool *Cufflink* (Galaxy Version 2.2.1.2) (Trapnell *et al*., 2010) the presence of individual transcripts and their expression levels were expressed as Fragments per kilobase of exon per million fragments mapped (FPKM). The data's of cufflink assemblies were then merged using *Cuffmerge* (Galaxy Version 2.2.1.2). Between high- and low-fertile groups, significant changes in transcript expression, splicing and promoter were studied using *Cuffdiff* (Galaxy Version 2.2.1.5)

The transcripts expressed between the high- and low-fertile groups were categorized as differentially expressed transcripts based on log2 fold change and FPKM value. They are the transcripts with log2fold change >+1 (upregulated in low fertile), >-1 (downregulated in low fertile) and between >±1 to <±1 (neutral between high- and low fertile). The transcripts with FPKM present only in high fertile group were considered as unique to high-fertile bulls, while transcripts with FPKM present only in low fertile group were classified as unique to low fertile bulls. All the raw data were uploaded and available in the NCBI SRA database under PRJNA516089 (https://www.ncbi.nlm.nih.gov/sra/PRJNA516089).

The total number of sperm transcripts expressed between high- and low-fertile populations were plotted using online tool *Venny* (Version 2.1.0). The differentially expressed sperm transcripts between high and low fertile population was plotted using Volcano plot based on the transcript log2 (fold change) and p value. Selected differentially expressed spermatozoal transcripts between high and low fertile groups were generated as Heat Map using *Clustvis* (r programming) (Metsalu and Vilo, 2015).

Gene ontology (GO) and function annotation of sperm transcripts were analyzed using *Uniport* (https://www.uniprot.org/) and The Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (v6.8) (https://david.ncifcrf.gov/) into three main categories as molecular function (MP), biological process (BP), cellular component (CC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The top 10 biological process, cellular component and molecular functions were plotted as Donut pie chart using *Highcharts* (https://www.highcharts.com/demo/pie-donut). Pathway enrichment was carried out using *Custer Profiler* and *enrichKEGG* functions. Interaction of genes and detailed network analysis of combined GO categories and pathway analysis was performed using *ClueGo* (Version 2.5.4) and *Cluepedia* (Version 1.5.4) plugins in the open source *Cytoscape* (Version 3.7.1) (Cluego.org) platform (Locatelli *et al*., 2019). All the analysis was done with *Bos taurus* as background.

3.1.7 Real time expression analysis of selected genes

Insilico analysis identified thousands of transcripts between high and low fertile crossbred bull spermatozoa from which, few genes (*TPT1, PFN1, ZNF706, MDB4, TNP2, TNP1, CRISP2* and *ADIPOR1*) were selected, based on FPKM and functional relevance to sperm fertilizing potential, were validated using qPCR in all the 12 bulls including the four bulls whose spermatozoa were subjected to NGS. Primers were designed based on published nucleotide sequences obtained from NCBI (www.ncbi.nlm.nih.gov). Primers designing was done using web-based software PRIMER-3 across exon-exon junctions in order to eliminate contaminating genomic DNA amplification. The annealing temperatures of primers for the selected genes were optimized using PCR (Prima-96 plus, Himedia). The primer sequence, product size and annealing temperatures are given in **Table 3.1**. The cDNA prepared from different samples were subjected to RT-qPCR analysis.

Table 3.1: Primers used for Real time quantification

S. No	Genes	Primer sequence	Product size	Annealing temperature	Accession number
1.	*TPT1*	FP-ACCGGGACCTCATTAGCCA	106 bp	60°C	NM_001014388.1
		RP-GTCCTACTGACCATCTTCCCC			
2.	*PFN1*	FP-CCTTCGTCAACATCACGCCT	86 bp	60°C	NM_001015592.1
		RP-CCCAAGTGTTAGCCCGTTCA			
3.	*ZNF706*	FP-TGAGAGAACAAAGGCTGCGAG	181 bp	60°C	NM_001199073.1
		RP-GGGTCTGGCATTTGTGTCCTA			
4.	*MDB4*	FP-TGGAAGCAGGTACACCCTGAA	184 bp	60°C	NM_001034405.2
		RP-CTATACGCCTGGAAAGGTGGT			
5.	*TNP2*	FP-ACAGACACACCATGCACTCC	172 bp	60°C	NM_174200.1
		RP-TCAGTTGTACTTCCGTCCTGAG			
6.	*TNP1*	FP-TGTCGACCAGCCGCAAATTA	149 bp	60°C	NM_174199.2
		RP-ATTGCGATTGGCATCGTCAC			
7.	*CRISP2*	FP-GCCGCTCTGCAACAGAAGA	68 bp	60°C	NM_001038089.1
		RP-AGGAAGAGCATGGTGTTTGGT			
8.	*ADIPOR1*	FP-GATCGGCCTGCTGATCATG	99 bp	60°C	NC_037343.1
		RP-GACGCAGACGATGGAGAGGTA			
9.	*GAPDH*	FP- CTGAGGACCAGGTTGTCTCCTG	141 bp	60°C	NM_001034034.1
		RP- CCCTGTTGCTGTAGCCAAATTC			

First strand cDNA Synthesis

Total RNA from spermatozoa from 12 bulls were carried out as indicated earlier. Only those samples having a 260/280 ratio of 1.8 or more were selected for cDNA synthesis (reverse transcription) which was done using a combination of oligo (dT) and random hexamers with an initial concentration of 50-100 ng of total RNA from each crossbred bull spermatozoa sample and 1000 ng total RNA from bull testis sample using the RevertAid First Strand cDNA Synthesis Kit (Thermo-Scientific, USA, Catalog number K1622) based on the following manufacturer's instructions of 20 µl final volume.

Protocol

The components of the kit were thawed before use, mixed and centrifuged briefly before storing on ice

1. The reagents shown in **Table 3.2** were added into a sterile, nuclease- free tube on ice in the indicated order.
2. The mixture was subjected to brief centrifugation and incubated at 65°C for 5 minutes.
3. The reagents shown in **Table 3.3** were added in the indicated order and further incubated for 60 min at 42°C for oligo (dT) cDNA synthesis.
4. Termination of the reaction was done by heating at 70°C for 5 min. The reverse transcription reaction product was either directly used in PCR applications or stored at -80°C until further use.

Table 3.2: Reagents for first strand cDNA synthesis (First step-Incubation)

Component	Volume/Reaction (µl)
Total RNA	50ng (1 µL)
Oligo (dT) primer	1 µL
Nuclease free water	10 µL
*Total volume	12 µL

Table 3.3: Reagents for first strand cDNA synthesis (Second step)

Component	Volume/Reaction (μl)
*Template (Incubated RNA)	12 μL
5X reaction buffer	4 μL
Ribolock RNase inhibitor (20U/ μL)	1 μL
10mMdNTP Mix	2 μL
RevertAid M-MulV RT (200U/ μL)	1 μL
Total volume	20 μL

Real time Quantitative PCR (RT-qPCR)

The qPCR experiments were performed using Insta Q96 Plus Real Time Machine PCR system (HiMedia, India). Thermal cycling conditions were chosen as per manufacturer's protocol (Maxima SYBR Green/ROX qPCR master mix 2X). The qPCR cycling conditions are given in **Table 3.4** and the components of qPCR reactions are given in **Table 3.5**. All the reactions were performed in duplicates and qPCR amplification of selected genes with their desired product sizes were confirmed by 2% agarose gel electrophoresis.

Table 3.4: qPCR cycling conditions

Steps	Temp (°C)	Time	No. of cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	30 sec	40
Extension	72	30 sec	40

Table 3.5: Components of qPCR

Component	Volume/Reaction (µl)
Template cDNA	2.0 µl
SYBR Premix Ex Taq	10.0 µl
Forward primer (10pmol/ µL)	0.5 µl
Reverse primer (10pmol/ µL)	0.5 µl
Nuclease-free water	7.0 µl
Total per Reaction	20.0 µl

3.2: Objective 2- Ability of spermatozoa to undergo capacitation upon exposure to oviductal fluid and its relationship with bull fertility

3.2.1 Source of chemicals

Chemicals used for the present study viz., Dulbecco's Phosphate Buffered Saline (DPBS) (Ca^{2+} and Mg^{2+} free), Bovine Serum Albumin (BSA), Saponin and Paraformaldehyde (PFA) were procured from Sigma Aldrich, USA. All the chemicals for media preparation were procured from Sigma Aldrich, USA and Sisco Research Laboratories, Pvt. Ltd, Mumbai, India. All the medium and chemicals were freshly prepared on the day of the experiment.

3.2.2 Source of spermatozoa

Cryopreserved semen straws of HF crossbred bulls (n=6) of known field fertility status were procured from Kerala Livestock Development Board, Mattupetty, Kerala, India. The conception rates (CR) of the bulls used in the study are given in **Fig 3.2**. Based on the mean CR, semen straws of crossbred bulls were classified as above average (n=3; CR>50%) and below average (n=3; CR<40%). On the day of experiment, frozen semen straws from each bull were thawed at 37°C and used for experimentation.

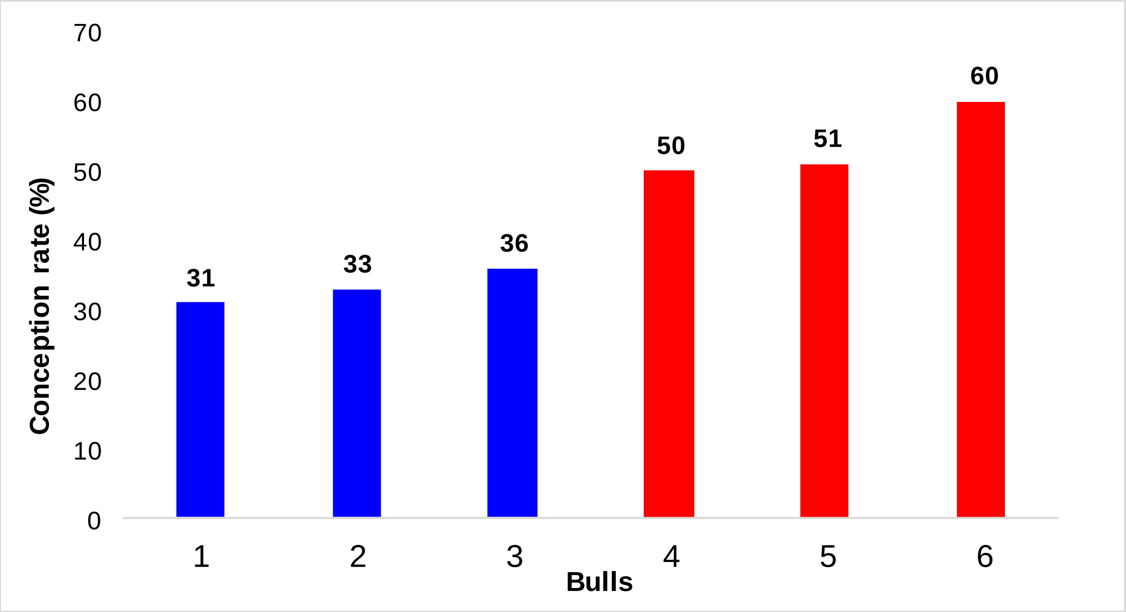


Fig.3.2: Conception rates (CR) of study bulls

3.2.3 Oviductal fluid collection

Slaughter house derived apparently healthy bovine non-luteal oviducts were utilized for extraction of oviductal fluid. The oviducts of slaughtered cows were categorized based on the ovarian morphology according to the criteria given by Orsi *et al*. (2005).

Non-luteal oviducts trimmed off the surrounding tissue and washed twice in normal saline (0.9% NaCl). The dissected oviducts were placed in petri dish over ice until oviductal fluid was collected. Collection of oviductal fluid was done by placing the oviduct on microscopic slide and moving another slide with gentle pressure through the length of the oviduct from utero-tubal junction to ampulla and the fluid was collected. The collected oviductal fluid was pooled and centrifuged under 8000 x g for 10 mins at 4°C to remove any cellular portions. The supernatant fluid was separated and stored immediately at -80°C until further use. The flowchart of oviductal fluid collection from non-luteal stage of ovaries is shown in **Fig 3.3**.

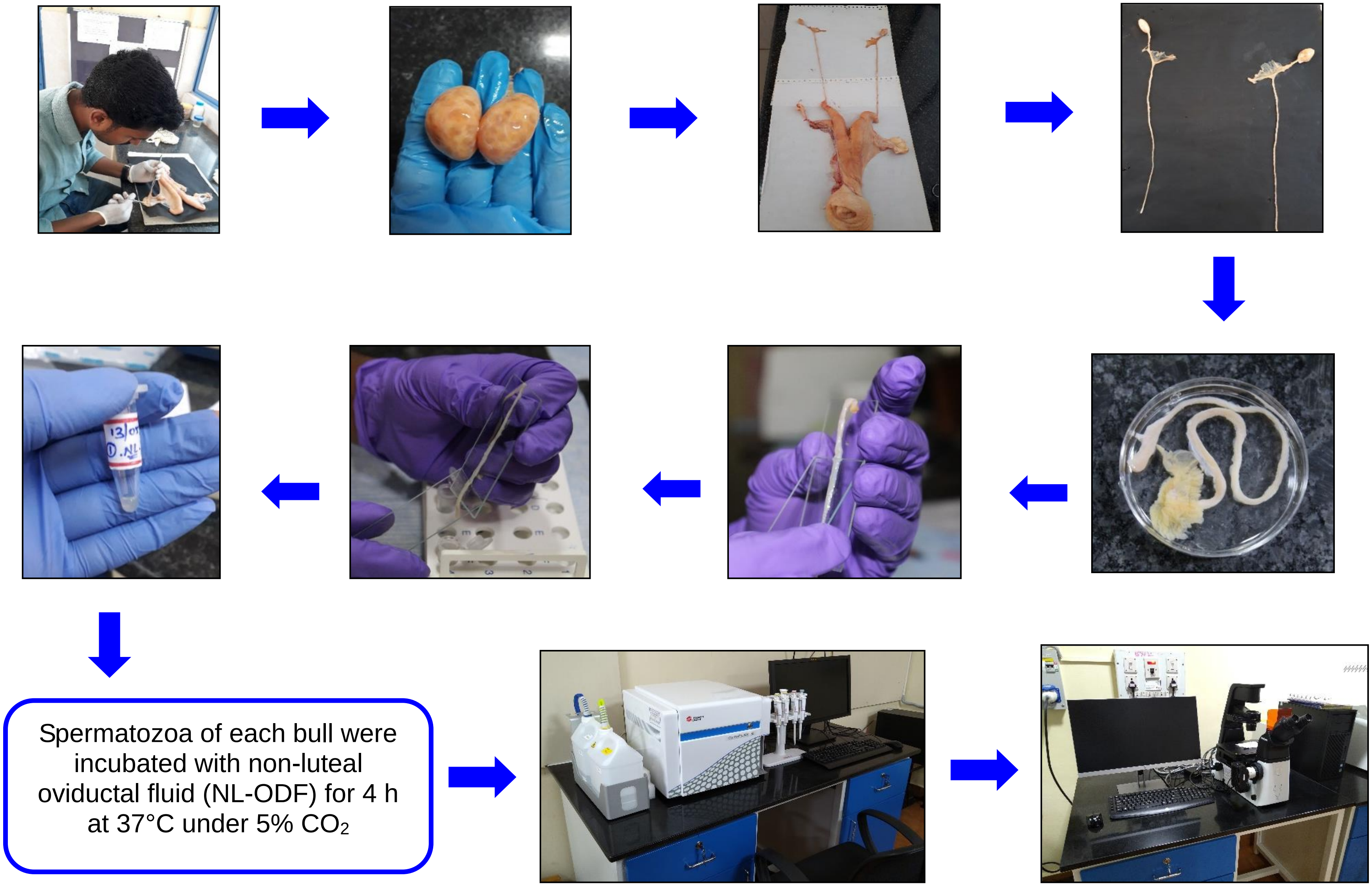


Fig. 3.3: Oviductal fluid collection

3.2.4 Incubation of spermatozoa with non-luteal oviductal fluid (NL-ODF)

Semen samples with more than 50% post-thaw sperm motility were used for experiment. The spermatozoa were washed (300 x g; 10 mins) in pre-warmed (37°C) non- capacitating medium [NCM containing 3.1 mM KCl, 0.3mM NaH_2PO_4, 115 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 21.6 mM sodium lactate, 5 mM Glucose and 1 mM Sodium pyruvate (pH 7.4)] to remove the extender components. The semen samples of treatment group of were re-suspended with NCM containing 20% v/v NL-ODF and incubated in 5% CO_2 for 4 h at 38°C. A control group was maintained under similar conditions except for the absence of NL-ODF. Sperm suspended with NCM only (control) were tightly closed to prevent bicarbonate formation during incubation. The proportion of NL-ODF used was selected according to Kumaresan *et al*. (2019). After 4 h incubation, non-luteal ODF treatment group was further subdivided into 2 million spermatozoa (5 aliquots in each group), re-suspended with 450 µL NCM and subjected to flow cytometric analysis for sperm viability/membrane integrity, intracellular calcium content, acrosome reaction status and global tyrosine phosphorylation. All these assays were carried out with cryopreserved spermatozoa immediately after thawing and after incubation for 4 h.

3.2.5 Estimation of sperm membrane integrity

Sperm membrane integrity was estimated using propidium iodide (PI; LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen, Eugene, Oregon, USA). Spermatozoa (2 million spermatozoa) were suspended in 450 µL of NCM and 3 µL PI (2.4 mM) was added. The sperm suspension was mixed well, incubated for 5 mins in dark at 37°C and analyzed using flow cytometry (CytoFLEX S, Beckman Coulter Life Sciences, Indianapolis, IN). Excitation was induced by a blue laser (488 nm). Green fluorescence (FITC) was detected with a fluorescence channel (FL) 1 band-pass filter (525/40 nm) and red (PI) fluorescence was measured using a FL3 band-pass filter (585/42 nm). A total of 10,000 spermatozoa events were evaluated for each sample. After gating out to exclude debris, spermatozoa were classified, according to the degree of intactness of the plasma membrane, as live and dead. Acquisitions and analyses were made using CytExpert software.

3.2.6 Estimation of intra-cellular calcium concentrations

Intracellular calcium concentration of spermatozoa was assessed using flouroscent probe Fluo-3, AM (Molecular probes, Invitrogen, Eugene, Oregon, USA). (Thermo Fisher Scientific catalog number-23915) according to the procedure described by Kadirvel *et al*. (2009) with slight modifications. Fluo-3, AM stock solution was prepared by mixing 50 µg of Fluo-3 AM with 200µL of DMSO and stored in -20°C. The final concentration of Fluo-3 in the stock solution was 0.25 µg/µL.

To 2 million spermatozoa in 450 µL of NCM, 3 µL of fluorescent probe Fluo-3 stock solution was added. Dye-loaded semen samples were incubated for 10-15 mins in dark at 37°C. After incubation, 3 µL 2.4mM of propidium iodide (PI; Molecular probes, Invitrogen Eugene, Oregon, USA) was added. The sperm suspension was mixed well, incubated for 5 mins in dark at room temperature. After incubation, the samples were analyzed as indicated above. After gating to identify the spermatozoa, four different population were observed as described earlier (Green and Watson, 2001; Harrison *et al*., 1993) as (a) spermatozoa with low Fluo-3 fluorescence signal and PI negative as live low intracellular calcium (b) spermatozoa with high Fluo-3 fluorescent signal and PI negative as live high intracellular calcium (c) sperm cells stained with PI with low Fluo-3 signal as dead cell with low intracellular calcium and (d) sperm cells stained with PI with high Fluo-3 signal as dead cell with high intracellular calcium.

3.2.7 Estimation of sperm acrosome reaction status

Acrosome reaction status was analyzed using fluorogenic stain Fluorescein isothiocyanate-*Pisum sativum agglutinin* (Sigma- Aldrich, St. Louis, MO) and propidium iodide (PI) according to the procedure given by Swain *et al.* (2012) and Kumaresan *et al.* (2017) with little modifications.

To 2 million spermatozoa suspended in 450 µL of NCM, 10 µL FITC-PSA (50µg/ml) was added. The dye-loaded semen samples were incubated at 37°C for 10 mins under dark conditions. After incubation, 3 µL of 2.4mM of propidium iodide (PI; Molecular probes, Invitrogen Eugene, Oregon, USA) was added to the sperm suspension was mixed well and incubated for 5 mins in dark at room temperature. After incubation, analyzed by flow cytometry as mentioned above. After gating to identify the spermatozoa, 4 types of population were observed; live acrosome intact (LAI), live acrosome reacted (LAR), dead acrosome intact (DAI) and dead acrosome reacted (DAR) and expressed in percentages.

3.2.8 Estimation of global protein tyrosine phosphorylation

Global tyrosine phosphorylation of spermatozoa was analyzed by flowcytometry as described earlier by Sidhu *et al*. (2004) with some modifications by Kumaresan *et al*. (2019).

Aliquots of sperm samples (2 million spermatozoa) obtained at 0 h and after 4 h incubation, were suspended in 400 µL of NCM to which 5 µL of 2.4 mM propidium iodide (PI; Molecular probes, Invitrogen Eugene, Oregon, USA) was added. The sperm suspension was incubated at 37°C for 10 mins at room temperature (RT) and centrifuged at 300 x g for 10 mins at 25°C. The sperm pellet was fixed overnight at 4°C with 500 µL of ice cold 1% paraformaldehyde in DPBS (Sigma-Aldrich). Fixed sperm samples were centrifuged at 1000 x g for 3 mins at 25°C and permeabilized in 0.05% saponin (Sigma-Aldrich) for 10 mins in room temperature. After centrifugation (300g, 10 mins, 25°C), the sperm pellet was suspended in 1 mL DPBS containing 0.1% bovine serum albumin (BSA) (Himedia) and 0.1% Tween 20 (Himedia) and incubated for 30 mins at room temperature to block the non-specific binding sites of spermatozoa. Following incubation, the sperm sample was centrifuged at 300 x g for 10 mins at 25°C. Monoclonal anti-phosphotyrosine antibody produced in mouse clone pT-154 (P1869; Sigma) was added (1:300 in 1% BSA) incubated in dark for 45 mins at room temperature and centrifuged (300g, 5 mins, 25°C). Followed by centrifugation, flurorescein isothiocynate (FITC)-conjugated anti-mouse IgG (Fab Specific) antibody produced in goat (F4018, Sigma) was added (1:300 in 1% BSA) and incubated in dark for 45 mins at room temperature and centrifuged (300g, 5 mins, 25°C). After centrifugation, the sperm sample were suspended 1 mL DPS in both (antibody added tubes and control tubes and centrifuged at 300 x g for 10 mins at 25°C). The pellet was again suspended in 500 µL DPBS and analyzed by flow cytometry as mentioned above.

3.2.9 Immuno-localization of protein tyrosine phosphorylation

The localization of protein tyrosine phosphorylation (PTP) in spermatozoa was carried out as described previously by Saraf *et al*. (2017) with slight modifications. From an aliquot of sperm suspension, 3 µl was taken and smeared on the coverslip (18 × 18 mm), fixed with 4% PFA in DPBS for 25 min and then washed twice with DPBS. The spermatozoa were permeabilized with ice-cold methanol at -20°C for 20 min. The smears were washed again with DPBS and air-dried. The smear was blocked with 5% BSA in DPBS for 1 h. After blocking, the smears were incubated with monoclonal anti-phosphotyrosine antibody (P1869; Sigma) produced in mouse (1:100) in 1% BSA for 2 h at 37°C in the humidified box and thoroughly washed with DPBS. After washing, smears were incubated with FITC conjugated anti-mouse IgG (Fab specific) antibody produced in goat (1:200) (F4018; Sigma) in 1% BSA for 1 h at 37°C in the humidified box. After incubation, the coverslips were washed with DPBS and air-dried. Further, an anti-fading agent (1, 4 diazabicyclo [2.2.2] octane (DABCO) in glycerol and PBS) (Himedia) was added in a grease-free slide and covered with the smeared coverslip. The smears were examined under a fluorescence microscope (Nikon ECLIPSE Ts 2R, Japan) in 1000X using FITC filter (Excitation- 465 to 495 nm and Emission- 515 to 555 nm). To assess the proportions of different phosphorylation patterns, at least 200 spermatozoa displaying fluorescence were counted per smear.

3.2.10 Assessment of relative abundance of sperm capacitation related genes

The transcriptional abundance of selected sperm capacitation associated genes (*CRISP2, PEBP1, CATSPER1, ADIPOR1* and *ADIPOR2*), based on earlier reports (Kasimanikam *et al*., 2013; Arangasamy *et al*., 2011), were assessed using RT-qPCR. The primer sequence, product size and annealing temperature are given in **Table 3.6**. The cDNA prepared from different bull spermatozoa samples were subjected to RT-qPCR analysis. The steps and procedures of total RNA isolation, quantification of sperm RNA, synthesis of first strand cDNA and RT-qPCR protocol was discussed earlier under section 3.1.4 and 3.1.7. The relationship of the transcriptional abundance of genes *CRISP2, PEBP1, CATSPER1, ADIPOR1* and *ADIPOR2* with sperm functional attributes and bull fertility was studied.

Table 3.6: Primers for capacitation genes

S. No	Genes	Primer sequence	Product size	Annealing temperature	Accession number
1.	*CATSPER1*	FP-TACTCTGACCCCAAACGCTT	100 bp	60°C	XM_024987570.1
		RP-GGCTGTCCAGGTAGATGAGG			
2.	*PEBP1*	FP-TTTCTCTGCTGTCTGACCCG	178 bp	60°C	NM_001033623.2
		RP-CGGTTTTTAACCTGGGTGGG			
3.	*CRISP2*	FP-GCCGCTCTGCAACAGAAGA	68 bp	60°C	NM_001038089.1
		RP-GGAAGAGCATGGTGTTTGGT			
4.	*ADIPOR1*	FP-GATCGGCCTGCTGATCATG	99 bp	60°C	NC_037343.1
		RP-GACGCAGACGATGGAGAGGTA			
5.	*ADIPOR2*	FP-GTGATCCCTCACGACGTGCTA	99 bp	60°C	NC_037332.1
		RP-TCTTAAAACAGGCCCGGAAA			
6	*IQCF1*	FP-AGGCAGAGGCTCAACAAGAG	116 bp	60°C	NM_001075773.1
		RP-TGCTTCTGGATCACTGACGG			
7.	*GAPDH*	FP- CTGAGGACCAGGTTGTCTCCTG	141 bp	60°C	NM_001034034.1
		RP- CCCTGTTGCTGTAGCCAAATTC			

3.3 Statistical analysis

Relative gene expression was determined using ΔΔCt method as described by Schmittgen & Livak (2008). ΔCt value for each gene was calculated by subtracting the reference genes Ct from target gene Ct. Then ΔΔCt was calculated by subtracting the control ΔCt values from target ΔCt (target ΔCt - control ΔCt). Fold change was calculated using the formula $2^{-\Delta\Delta CT}$. t-Test is used to find out differences between two (high- and low-fertile) groups and correlation of different parameters were performed using SPSS (22.0, IBM, USA) software. The difference was considered as significant when $P<0.05$.

CHAPTER - 4

Results and Discussion

4. RESULTS AND DISCUSSION

In the present study, we assessed the transcriptomic profile of crossbred bull spermatozoa and identified the differential transcriptome profile between high-and low-fertile crossbred bulls. The ability of spermatozoa to undergo capacitation upon exposure to non-luteal oviductal fluid (NL-ODF) and its relationship with bull fertility was assessed. Selected differentially expressed transcripts between high- and low-fertile bulls and genes related to capacitation were validated by qPCR analysis. The objective wise results along with possible explanations are discussed below.

4.1 Objective-1: Differential transcriptome profile between high- and low-fertile crossbred bulls

Bio-analyzer analysis of spermatozoal RNA isolated from the bull spermatozoa showed the lack of 18s and 28s rRNA peaks in sperm **(Fig. 4.1)**, indicating that the total RNA isolated from spermatozoa had no contamination of somatic cells, leucocytes, testicular germ cells and other cells.

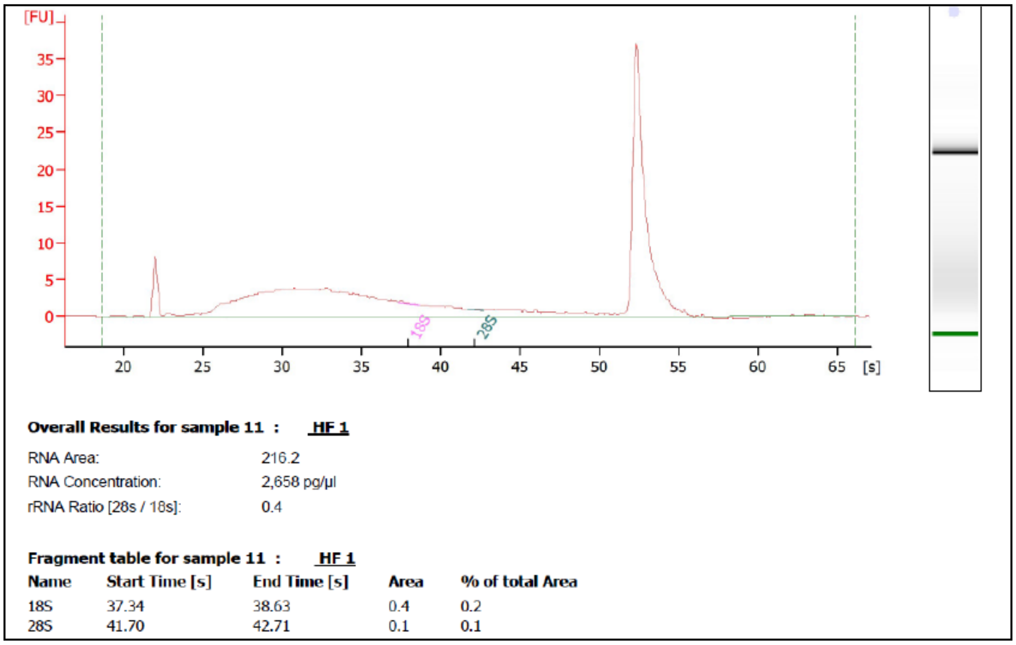
Using Illumina Next Seq-500 RNA sequencing, the raw reads of semen sample of high fertile populations (HF1 and HF2) were found to be 28818082 (28 million) and 30447573 (30 million) respectively, whereas low fertile populations (LF1 and LF2) were 31028839 (31 million) and 26688034 (26 million) reads, respectively. After processing of raw reads, processed reads of high fertile populations (HF1 and HF2) were 26144072 (26 million) and 26454332 (26 million) respectively, with paired end 76 bp reads. The low fertile populations (LF1 and LF2) showed 21063462 (21 million) and 24663094 (24 million) processed reads respectively, with paired end 76 bp. The processed reads were mapped to the *Bos taurus* genome which showed an accuracy of 32.71 % and 67.55% for high fertile bulls, HF1 and HF2, respectively and 45.05% and 74.01% for low fertile bulls LF1 and LF2, respectively. Paired reads of high fertile populations (HF1 and HF2) were 23.74% and 56.35% respectively and that of low fertile populations (LF1 and LF2) were 35.76% and 65.64%.

Here we report the expression of transcriptomic profiling between high- and low-fertile crossbred bull spermatozoa using high-throughput sequencing. The crossbred bull spermatozoa contained transcripts for 13,563 genes; high- and low-fertile bull spermatozoa contained transcripts for 8109 and 11470 genes, respectively. The number of transcripts observed in spermatozoa of crossbred bulls is comparable with earlier findings on bovine spermatozoa (Card *et al*., 2013; Card *et al*., 2017; Selvaraju *et al*., 2017; Vijayalakshmy, 2017; Singh *et al*., 2019; Raval *et al*., 2019). There are wide variations in the total number of transcripts reported in spermatozoa. For example, a global transcriptomic study by Card *et al*. (2013) reported that the total no. of transcripts detected in bovine was 6166, while Raval *et al*. (2019) observed a total no. of 14,711 transcripts in bovine spermatozoa, using the same technique. Similarly, Card *et al*. (2017) reported a total transcript of 3227 and 5336, while Singh *et al*. (2019) observed a total of 3,510 and 6,759 transcripts in high- and low-fertile bulls, respectively. Several factors have been reported to influence the total number of transcripts detected in spermatozoa including season of sample collection (Godia *et al*., 2019), state of spermatozoa (fresh or frozen) (Card *et al*., 2017; Singh *et al*., 2019), method of RNA isolation (Parthiban *et al*., 2015; Frazer *et al*., 2017), integrity of sperm RNA, RNA-Sequencing instrument (Selvaraju *et al*., 2017) and library preparation methods (Mao *et al.*, 2014).

4.1.1 Sperm transcriptome profile of high- and low-fertile bulls

Of the total transcripts detected, 6016 transcripts were common between high- and low-fertile populations. 2093 transcripts were unique to high fertile population and 5454 transcripts were unique to low fertile population **(Fig. 4.2)**. After total hit normalization of data, a total of 776 transcripts were detected; 524 transcripts were common to both high- and low-fertile bulls, while 84 sperm transcripts were unique to high-fertile bulls and 168 transcripts were unique to low-fertile bulls **(Fig. 4.3)**. Among the co-expressed genes, 176 transcripts were upregulated (fold change >1) while 209 were down regulated (fold change <1) in low-fertile bulls.

Based on the transcript log2 (fold change) and *P* value, the differentially expressed transcripts were plotted using Volcano plot **(Fig. 4.4)** in which the transcripts with *P* value 0.8 and above were significantly expressed between high- and low- fertile populations. The transcripts tumor protein translationally-controlled 1 (*TPT1*), homeobox D13 (*HOXD13*) were upregulated while *ORAI* calcium release-activated calcium



Overall Results for sample 11 : **HF 1**

RNA Area: 216.2
RNA Concentration: 2,658 pg/µl
rRNA Ratio [28s / 18s]: 0.4

Fragment table for sample 11 : **HF 1**

Name	**Start Time [s]**	**End Time [s]**	**Area**	**% of total Area**
18S	37.34	38.63	0.4	0.2
28S	41.70	42.71	0.1	0.1

Fig.4.1: RNA integrity assessment of high- and low-fertile bull spermatozoa (showing absence of 28s and 18s)

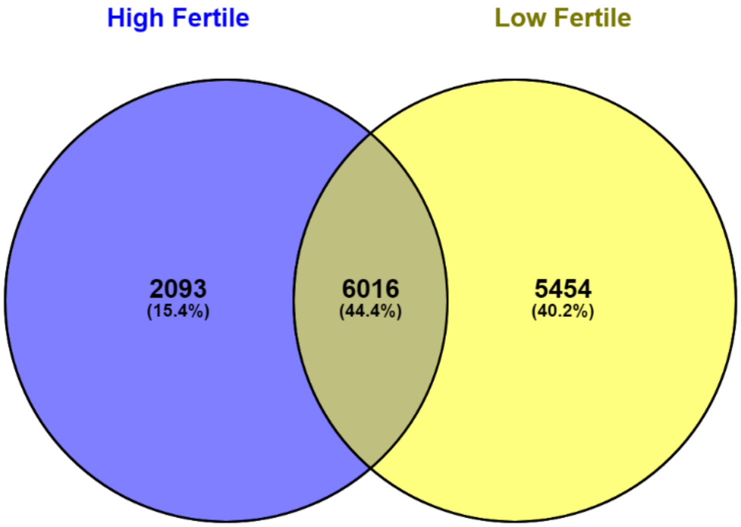


Fig.4.2: Sperm transcriptome profile of high- and low-fertile bull spermatozoa

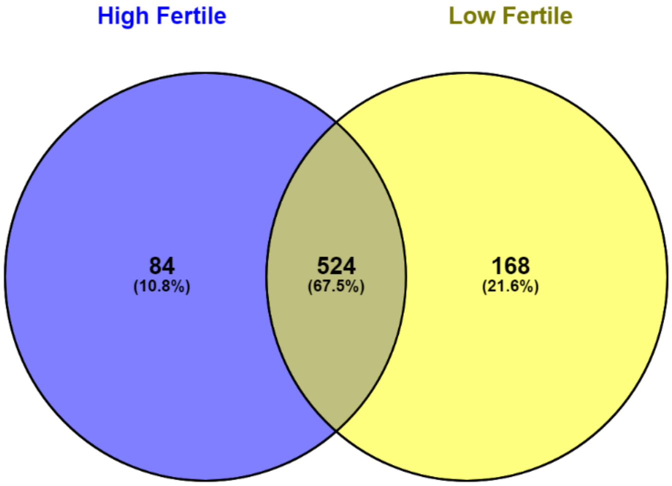


Fig.4.3: Sperm transcriptome profile of high- and low-fertile bull spermatozoa (after total hit normalization)

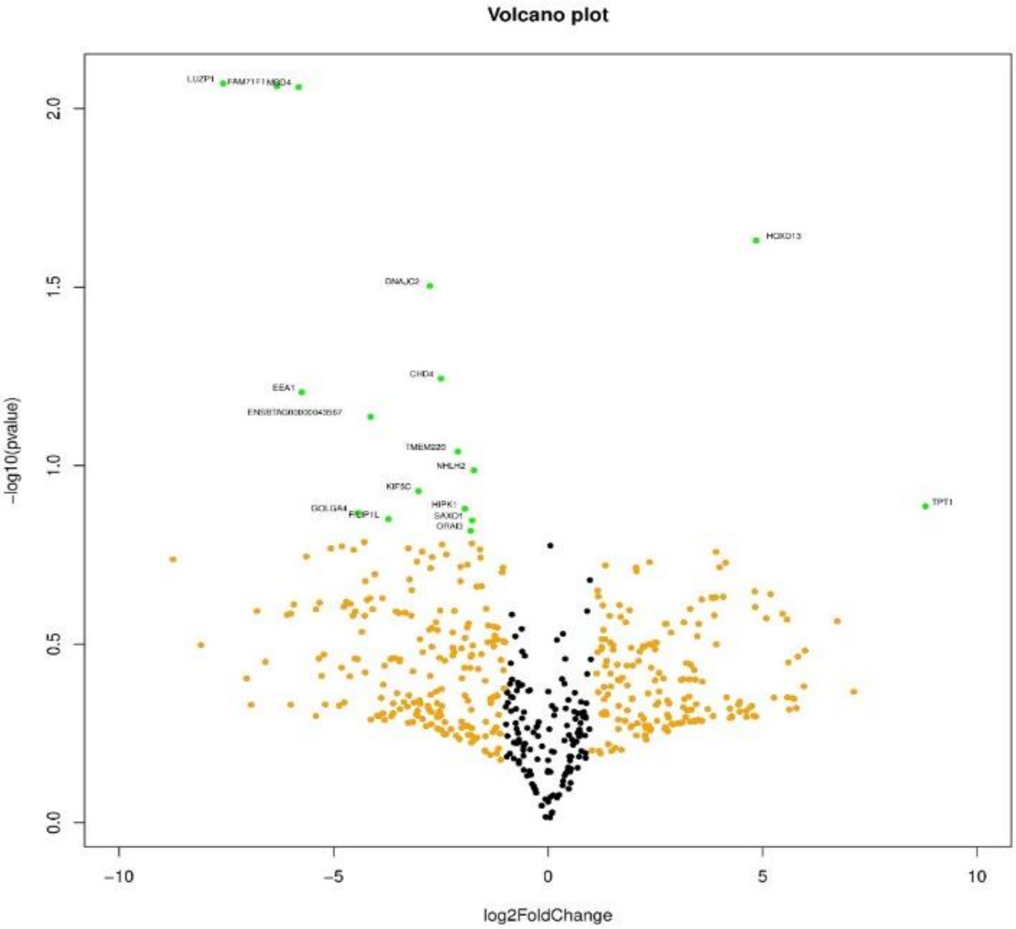


Fig.4.4: Differentially expressed sperm transcripts-Volcano plot

modulator 3 (*ORAI3*), stabilizer of axonemal microtubules 1 (*SAXO1*), filamin A interacting protein 1 like (FILIP1L), golgin A4 (*GOLGA4*), homeodomain interacting protein kinase 1 (*HIPK1*), kinesin family member 5C (*KIF5C*), nescient helix-loop-helix 2 (*NHLH2*), transmembrane protein 220 (*TMEM220*), ENSBTAG00000043567, early endosome antigen 1 (*EEA1*), chromodomain helicase DNA binding protein 4 (*CHD4*), DnaJ heat shock protein family (*Hsp40*) member C2 (*DNAJC2*), methyl-CpG binding domain 4, DNA glycosylase (*MBD4*), family with sequence similarity 71 member F1 (*FAM71F1*), leucine zipper protein 1 (*LUZP1*) were down regulated in low fertile population. Differentially expressed top 20 sperm transcripts plotted using Heat map **(Fig. 4.5)** revealed that ribosomal proteins (*RPS8, RP14*) and *TPT1* was highly upregulated and *ZNF706* was highly down regulated in low fertile bulls.

A complex pool of coding and non-coding RNAs were also observed in the sperm transcriptome. Of the total transcripts (13,563 transcripts), coding RNAs were 13,002 (95.86%), non-coding RNAs were 88 (0.64%), pseudogenes were 375 (2.76%) and processed pseudogene were 98 (0.72%). Among the non-coding RNAs; miscellaneous RNA (misc_RNA) were 48 (0.35%), small nuclear RNA (snRNA) were 21 (0.15%), small nucleolar RNA (snoRNA) were 12 (0.08%) and ribosomal RNA (rRNA) were 7 (0.05%). After total hit normalization, a total 776 transcripts were detected, coding RNA (known/categorized) were 585 (75.38%), coding RNA (unknown/uncategorized) were 148 (19.07) and noncoding RNA were 43 (19.07%). Among the noncoding RNAs, misc_RNA were 19 (2.44%), snRNA were 12 (1.54%), snoRNA were 6 (0.77%) and rRNA were 6 (0.77%). A total of 61 protein coding ribosomal mRNAs were observed among the total transcripts after normalization.

Library preparation specific to coding RNAs has detected a meagre 0.64% of non-coding RNAs in crossbred bull spermatozoa. After total hit normalization, the non-coding RNAs accounted to 19.07%, Further, non-coding RNAs were detected with highest FPKM value among sperm transcripts unique to high- and low-fertile populations. In line with our observation, Card *et al*. (2013) also observed non-coding RNAs among the top transcripts with highest FPKM. Several researchers have reported

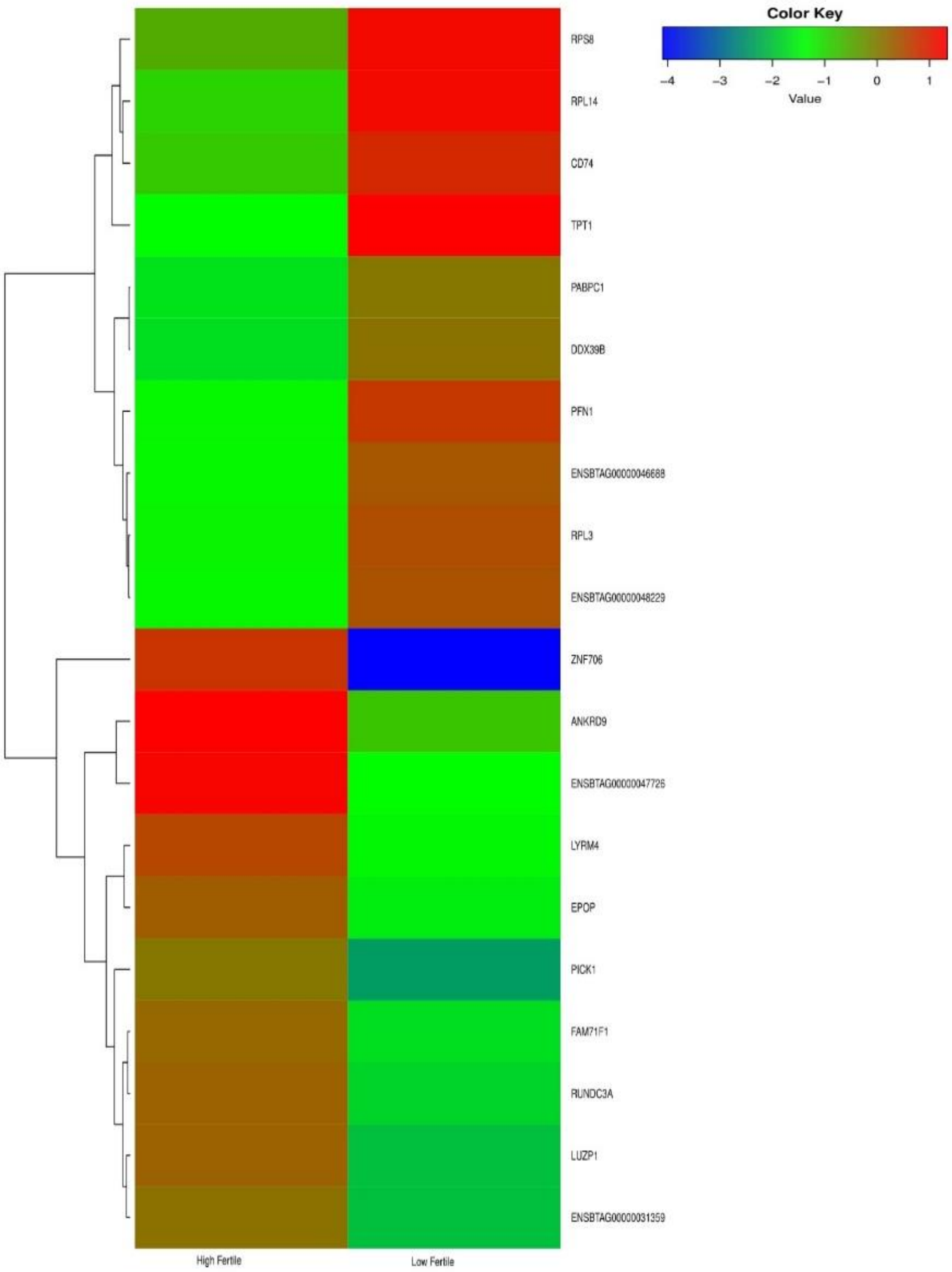


Fig.4.5: Heat map analysis of top 20 differentially expressed sperm transcripts between high- and low-fertile bulls

the presence of non-coding RNAs in livestock and human spermatozoa (Kempisty *et al*., 2008; Das *et al*., 2013; Card *et al*., 2013; Sendler *et al*., 2013; Jodar *et al*., 2013). It was reported that non-coding RNAs have a role in spermatogenesis and embryogenesis (Tscherner *et al*., 2014; McIver *et al*., 2011; Capra *et al*., 2017) as well as epigenetic modification (Jodar *et al*., 2013).

Transcripts for protein coding ribosomal mRNAs were detected in high proportions in the crossbred bull spermatozoa. Specifically, sperm transcripts upregulated and unique to low fertile bulls had more coding ribosomal RNAs compared to high fertile bulls. In bovine spermatozoa ribosomal RNAs are detected as either intact or degraded (Selvaraju *et al*., 2017) and could be packed during the process of spermatogenesis (Garrido *et al*., 2009; Montjean *et al*., 2012). The primary function of ribosomal RNA is protein synthesis; it has a critical role in the production of healthy spermatozoa (Bansal *et al*., 2015). The mitochondria in sperm have their own translational machinery and are the main source of energy. Differential expression (DE) of ribosomal proteins may affect the ribosomal assembly in mitochondria thereby affecting its function in the sperm. Bansal *et al*. (2015) reported that transcripts for ribosomal protein were down-regulated in asthenozoospermic patients compared to normal individuals, indicating disrupted mitochondrial functions in such conditions. In the present study, we profiled the sperm transcriptome in high- and low-fertile bulls that had no significant difference in sperm motility between themselves. The preliminary results from our study indicate the possible role of ribosomal RNAs in infertility/ subfertility.

4.1.2 Top 10 differentially expressed and unique sperm transcripts in high- and low-fertile bull

The top 10 up- and down-regulated transcripts in low fertile bull spermatozoa compared to high fertile bull spermatozoa are given in **Table 4.1 and 4.2** respectively. The top 10 transcripts unique to high- and low-fertile populations after excluding the noncoding RNAs, are listed in **Table 4.3 and 4.4**. Among the sperm transcripts, five transcripts (*HOXD13, LUZP1, MBD4, FAM71F1* and *DNAJC2*) were significantly ($p<0.05$) different between high- and low-fertile populations.

Table 4.1: Top 10 upregulated transcripts in low-fertile bull spermatozoa compared to high-fertile bull spermatozoa

Sl. No	Transcript	Description	Accession number	FPKM HF	FPKM LF	Log2(fold change)	P Value
1.	*TPT1*	Tumor protein translationally-controlled 1	NM_001014388	0.04	19.84	8.79	0.13
2.	*RPL14*	Ribosomal protein L14	NM_001034674	0.11	15.47	7.12	0.43
3.	*PFN1*	Profilin 1	NM_001015592	0.04	4.95	6.74	0.27
4.	*DDX39B*	DEAD-box helicase 39B	NM_001033629	0.01	1.22	5.98	0.32
5.	*RPL3*	Ribosomal protein L3	NM_174715	0.05	3.22	5.96	0.41
6.	ENSBTAG00000048229	Protein_coding	N/A	0.04	2.80	5.82	0.34
7.	*PABPC1*	Poly(A) binding protein cytoplasmic 1	NM_174568	0.02	1.16	5.78	0.47
8.	*RPS8*	Ribosomal protein S8	NM_001025317	0.29	15.9	5.73	0.45
9.	*CD74*	CD74 molecule	NM_001034735	0.14	7.48	5.70	0.44
10.	ENSBTAG00000046688	Protein_coding	N/A	0.04	2.32	5.62	0.48

(HF-High Fertile; LF- Low fertile)

Table 4.2: Top 10 down regulated transcripts in low-fertile bull spermatozoa compared to high-fertile bull spermatozoa

Sl. No	Transcript	Description	Accession number	FPKM HF	FPKM LF	Log2(fold change)	P Value
1.	*ZNF706*	Zinc finger protein 706	NM_001199073	5.47	8.13	-16.03	0.44
2.	ENSBTAG00000047726	Protein_coding	N/A	18.08	0.04	-8.74	0.18
3.	*PICK1*	Protein interacting with PRKCA 1	NM_001035401	1.10	0.004	-8.08	0.31
4.	*LUZP1*	Leucine zipper protein 1	NM_001205828	1.82	0.009	-7.57	0.008
5.	ENSBTAG00000031359	Protein_coding	#N/A	1.25	0.009	-7.02	0.39
6.	*ANKRD9*	Ankyrin repeat domain 9	NM_001102144	21.7	0.17	-6.92	0.46
7.	*RUNDC3A*	RUN domain containing 3A	NM_001076322	1.74	0.01	-6.78	0.25
8.	*LYRM4*	LYR motif containing 4	NM_001076306	3.63	0.03	-6.58	0.35
9.	*FAM71F1*	Family with sequence similarity 71 member F1	NM_001046457	1.60	0.02	-6.31	0.008
10.	*EPOP*	Elongin BC and polycomb repressive complex 2 associated protein	XM_024980838	2.04	0.03	-6.08	0.26

(HF-High fertile; LF- Low fertile)

Table 4.3: Top 10 sperm transcripts unique to high fertile bull spermatozoa

Sl. No	Transcript	Description	Accession number	FPKM	P Value
1.	ENSBTAG00000006046	Protein_coding	N/A	12.76	0.11
2.	ENSBTAG00000038366	Protein_coding	N/A	7.58	0.12
3.	ENSBTAG00000019880	Protein_coding	N/A	7.07	0.24
4.	*TSSK6*	Testis specific serine kinase 6	XM_002688518	6.90	0.11
5.	ENSBTAG00000044154	Protein_coding	N/A	6.07	0.12
6.	*C12H13orf46*	Chromosome 12 C13orf46 homolog	NM_001077039	5.25	0.07
7.	*FABP3*	Fatty acid binding protein 3	NM_174313	3.30	0.12
8.	ENSBTAG00000047199	Protein_coding	N/A	3.07	0.12
9.	*IQCF1*	IQ motif containing F1	NM_001075773	2.89	0.12
10.	ENSBTAG00000030927	Protein_coding	N/A	2.78	0.12

Table 4.4: Top 10 sperm transcripts unique to low fertile bull spermatozoa

Sl. No	Transcript	Description	Accession number	FPKM	P Value
1.	*RPL37*	Ribosomal protein L37	NM_001078132	21.57	0.12
2.	*RPS11*	Ribosomal protein S11	NM_001024568	20.97	0.10
3.	*RPS12*	Ribosomal protein S12	NM_001014387	17.69	0.11
4.	*RPL13A*	Ribosomal protein L13a	NM_001076998	15.35	0.10
5.	*RPS3*	Ribosomal protein S3	NM_001034047	13.66	0.10
6.	*RPS27*	Ribosomal protein S27	NM_001098135	13.33	0.12
7.	*RPL31*	Ribosomal protein L31	NM_001025341	13.30	0.12
8.	*TMSB10*	Thymosin beta 10	NM_174623	12.31	0.12
9.	*RPL30*	Ribosomal protein L30	NM_001034434	10.56	0.11
10.	*RPL32*	Ribosomal protein L32	NM_001034783	8.84	0.11

Only one transcript (*HOXD13*) was upregulated in low fertile and the remaining four transcripts (*LUZP1, MBD4, FAM71F1, DNAJC2*) were down regulated in low fertile bull spermatozoa.

The top most upregulated spermatozoal transcripts in low-fertile crossbred bulls were *TPT1, RPL14, PFN1, DDX39B, RPL3, PABPC1, RPS8* and *CD74*. The tumor protein, translationally-controlled 1 (*TPT1*) also known as Translationally-controlled tumor protein (*TCTP*) is the prime spermatozoal transcript upregulated in low fertile bulls (8.79 fold) compared to high fertile bulls spermatozoa. *TPT1* has a biological process of apoptosis, cellular differentiation, control of sperm functions (Arcuri *et al*., 2004) and molecular functions of calcium ion binding and microtubule binding. It is reported as the one of the most abundant unique transcripts in human ejaculated spermatozoa (Zhao *et al*., 2006) and is also reported in chicken sperm (Singh *et al*., 2015). Montjean *et al*. (2012) stated that *TPT1* was down regulated in oligozoospermic patients compared to normal individuals in human. However, the relationship of *TPT1* to sperm functions and fertility in bovine spermatozoa was not reported earlier. Ribosomal protein L14 (*RPL14*) is the second greatest transcript upregulated in low fertile bulls with over 7.12 fold compared to high fertile bull spermatozoa. It was reported that *RPL13A* was highly enriched in testis and extracellular part (Selvaraju *et al*., 2018). Raval *et al*. (2019) reported that *RPL14* was the highly abundant sperm transcript in both *Bos taurus* and *Bos indicus* bulls. We observed that, several ribosomal proteins were upregulated in low fertile bulls compared to high fertile bull spermatozoa signifying that detailed studies on ribosomal proteins are required for a thorough understanding of their role on fertility. Profilin 1 (*PFN1*) are small actin binding proteins (Witke, 2004) with biological process of actin cytoskeleton organization, regulation of actin filament polymerization and positive regulation of actin filament bundle assembly. Profilin are ubiquitous proteins, extensively distributed in every species including human (Rawe *et al*., 2006). By modulating actin it has a role on oocyte maturation, fertilization, embryo development (Rawe *et al*., 2006) and spermatogenesis (Selvaraju *et al.*, 2018). In mice, it is reported to be essential for cell functions (Witke *et al.*, 2001).

DEAD (Asp-Glu-Ala-Asp) -box helicase 39B (*DDX39B*) are ATP-dependent RNA binding proteins and RNA-dependent ATPases involved in all events of RNA metabolism (Raj *et al*., 2019). Poly (A) binding protein cytoplasmic 1 (*PABPC1* also known as *PABP1*) is largely localized in cytoplasm of elongating spermatids and is involved in mRNA metabolism during spermatogenesis (Xu *et al*., 2014; Ozturk *et al*., 2012). Singh *et al*. (2015) reported that *PABPC1* was differentially expressed between sperm and testes of chicken and is down regulation in sperm compared to testes. Ribosomal protein S8 (*RPS8*), gene encodes a ribosomal protein that is a component of the 40s subunit. Zhao *et al*. (2006) reported that, *RPS8* is one of the most abundant unique transcripts in human ejaculated spermatozoa. *CD74* molecule (*CD74*) is a gene associated with class II major histocompatibility complex (MHC). It controls the antigen presentation for immune responses. Hu *et al*. (2017) reported that *CD74* was differentially expressed in testes of diploid and tetraploid cyprinid fish. In chicken it was differentially expressed in sperm and testes and is down regulated in sperm compared to testes (Singh *et al*., 2015).

The top most spermatozoal transcripts down-regulated in low-fertile crossbred bull were *ZNF706, PICK1, LUZP1, ANKRD9, RUNDC3A, LYRM4, FAM71F1* and *EPOP*. The zinc finger protein *ZNF706*, is the predominant spermatozoal transcript downregulated in low fertile bulls compared to high fertile bulls. Zinc is important for male and female fertility. Zinc-containing metalloenzymes are associated with sperm functions (Kerns *et al*., 2018). Protein interacting with *PRKCA 1* (*PICK1*), is a peripheral membrane protein highly expressed in round spermatids and localized to golgi-derived pro-acrosomal granules (Xiao *et al*., 2009) necessary for acrosome formation (Chen *et al.,* 2016b) and has a role in spermatogenesis and cytoskeletal organization (Selvaraju *et al.*, 2017). Knockout of gene *PICK1* in mice leads to infertility and deletion or mutation of *PICK1* gene leads to globozoospermia condition in human (Liu *et al*., 2010). Similar to our results, Singh *et al*. (2019) reported that high fertile crossbred bull spermatozoa showed upregulation of *PICK1* transcripts compared to low fertile bulls. Ankyrin repeat domain 9 (*ANKRD9*) is a protein coding gene whose exact function on spermatozoa is not known. It could be involved in intracellular lipid accumulation and lipid metabolism (Wang *et al*., 2009). Similar to our results, Jodar *et al*. (2011) also reported that

ANKRD9 is down regulated in asthenozoospermic than in fertile humans. RUN domain containing 3A (*RUNDC34*) is a protein coding gene. LYR motif containing 4 (*LYRM4*), is a protein coding gene. It is involved in nuclear and mitochondrial iron-sulfur protein biosynthesis. Family with sequence similarity 71 member F1 (*FAM71F1*), was detected in leydig cells and in germ cells (Malcher *et al*., 2013). Similar to our results, Malcher *et al*. (2013) also reported that the expression levels of *FAM71F1* gene in human was down regulated in azoospermic males than normal males. Elongin BC and polycomb repressive complex 2 associated protein (*EPOP*) is a novel that has not been reported in bull spermatozoa.

The top spermatozoal transcripts unique to high fertile bulls were *TSSK6*, *C12H13orf46, FABP3, IQCF1*. Among the top sperm transcripts unique to high fertile bulls, testis specific serine kinase 6 (*TSSK6*) is the predominant spermatozoal transcript present only in high fertile bulls. It is involved in fertilization through the regulation of actin polymerization and is mostly expressed in male germ cells (Sosnik *et al*., 2009). It has a biological process of protein phosphorylation; sperm chromatin condensation; intracellular signal transduction and on sperm function (Selvaraju *et al*., 2018), sperm motility (Bissonnette *et al*., 2009) and gamete fusion (Sosnik *et al*., 2009). Mondal *et al*. (2013) reported that *TSSK6* transcript was associated with high-motility status ($P<0.01$) and their relative gene expressions were higher in fresh than cryopreserved sperm. Chromosome 12 C13orf46 homolog (*C12H13orf46*), is a novel transcript whose functions are not yet reported. Fatty acid binding protein 3 (*FABP3*), is a member of intracellular FABPs family. It plays have a role in the intracellular transport of long-chain fatty acids (Yathish *et al*., 2017; Roy *et al*., 2003), modulation of cell growth and proliferation (Bauersachs *et al*., 2012), in remodeling of member polar lipids in spermatogenesis (Furuhashi and Hotamisligil, 2008) and is generally needed for male fertility. Contradictory to our results, *FABP3* was the top upregulated (approx. 16 fold) transcript in crossbred bull fresh semen with poor motility compared to good motility semen (Yathish *et al*., 2017). IQ motif containing F1 (*IQCF1*) is an acrosomal protein mostly localized in the acrosome of spermatozoa and spermatids. It is associated with sperm capacitation and acrosome reaction is also involved in tyrosine phosphorylation of sperm proteins through interaction with calmodulin. *IQCF1* null mice had reduced sperm motility and acrosome reaction (Fang *et al*., 2015).

The top sperm transcripts unique to low fertile bulls had ribosomal proteins (*RPL37, RPS11, RPS12, RPL13A, RPS3, RPS27, RPL31, RPL30, RPL32*) and Thymosin beta 10 (*TMSB10*). The prevalence of ribosomal proteins and its significance was discussed earlier. *TMSB10* is possibly involved in cellular remodeling during the implantation period and trophoblast adhesion (Cammas *et al*., 2005). It is also involved in sperm capacitation and spermatozoa egg fusion (Selvaraju *et al*., 2017). Raval *et al*. (2019) reported that *TMSB10* was one of the most abundant transcripts in common, between frozen semen of *Bos taurus* and *Bos indicus*. Kropp *et al*. (2017) reported that *TMSB10* expression was high in embryos of high fertile sires (4.88 fold) compared to embryos of low fertile sires.

Among the significant gene differentially expressed between high- and low-fertile bulls, homeobox D13 (*HOXD13*) belonging to the homeobox family of genes, is the one significantly (p=0.023) upregulated genes in low fertile bulls compared to high fertile bull spermatozoa (fold change 4.85). Hox genes encode a highly conserved family of transcription factors with key roles in body patterning during embryogenesis (Debeer *et al.,* 2002). Deletion or mutation of *HOXD13* gene cluster or the 5' end of this cluster is linked to with limb (synpolydactyly) and genital defects (Debeer *et al*., 2002; Johnson *et al*., 2003). Transcripts significantly downregulated in low fertile bulls than high fertile bulls were *LUZP1* (p=0.008), *MDB4* (p=0.008), *FAM71F1* (p=0.008) and *DNAJC2* (p=0.031). Among the down regulated transcripts in low fertile bulls, leucine zipper protein 1 (*LUZP1*) gene encodes a leucine zipper motif containing protein whose exact function is unknown. Methyl-CpG binding domain 4, DNA glycosylase (*MBD4*) gene is a member of nuclear protein family linked to the presence of a methyl-CpG binding domain (*MBD*), also known as *MED1*. These proteins are capable of specific binding to methylated DNA and suppress transcription from methylated gene promoters (Kondo *et al*., 2005). *MBD4* is a candidate tumor suppressor which is associated with DNA mismatch repair (Bellacosa, 2001); it participates in meiotic recombination during spermatogenesis (Terribas *et al*., 2010). Supporting to our result, Bellacosa (2001) and

Terribas *et al*. (2010) reported that expression of *MBD4* decreased in spermatogenic failure, and that it is reduced in maturation arrest at the spermatocyte level. Family with sequence similarity 71 member F1 (*FAM71F1*) has a role in spermatogenesis (Razavi *et al*., 2017). Malcher *et al*. (2013) reported that this gene was 4 fold down-regulated ($P<0.05$) in infertile males and significantly lower ($P<0.001$) azoospermicmales compared to normal subjects. DnaJ heat shock protein family (Hsp40) member C2 (*DNAJC2*) has a role in spermatogenesis (Selvaraju *et al*., 2018). Singh *et al*. (2015) reported that *DNAJC2* was differentially expressed between sperm and testes of chicken and showed down regulation in the sperm compared to testes.

4.1.3 Gene Ontology (GO) analysis

Gene ontological analysis of 176 (139 functionally annotated) sperm transcripts upregulated in low fertile population revealed their involvement 16 molecular functions (MF), 39 biological process (BP) **(Table 4.5)**, 24 cellular components (CC) and 12 KEGG pathways. GO analysis of 209 (178 functionally annotated) sperm transcripts downregulated in low fertile population revealed their involvement in 08 MF, 17 BP **(Table 4.6)**, 12 CC and 08 KEGG pathways. The 524 (436 functionally annotated) sperm transcripts common to high- and low-fertile populations revealed their involvement in 29 MF, 71 BP, 39 CC categories and 15 KEGG pathways. The 84 sperm transcripts (50 functionally annotated) unique to high fertile population revealed their involvement in 02 MF, 01 BP as innate immune response with three genes (*PYCARD, APCS*, ENSBTAG00000039963) and 03 KEGG pathways while 168 sperm transcripts (118 functionally annotated) unique to low fertile population had 10 MF, 12 BP **(Table 4.7)**, 20 CC and 04 KEGG pathways. The top 10 GO categories of biological process, cellular components and molecular functions of differentially expressed transcripts between high- and low-fertile populations are given in **Fig. 4.6ab** and transcripts unique to high- and low-fertile populations is given in **Fig. 4.7**.

Table 4.5: Biological process of sperm transcripts upregulated in low fertile bulls

Term	Count	Genes
GO:0006412~translation	12	*RPL14, RPL18, RPL23, RPL3, RPL36, RPL37A, RPL4, RPS2, RPS28, RPS8, SLC25A6, UBA52*
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	11	*DDX3X, DDX5, JUNB, PPARGC1A, JUN, HMGB1, PIN1, PCBP1, PROX1, TGFB1, TNFSF8*
GO:0098609~cell-cell adhesion	9	*RAB11B, EIF3E, HSPA8, HNRNPK, PCBP1, RACK1, RPS2, TAGLN2, YWHAE*
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	9	*DDX5, JUN, JUNB, NRARP, CALR, EZR, PROX1, TGFB1, ZBTB18*
GO:0008380~RNA splicing	6	*ALYREF, HSPA8, HNRNPF, HNRNPK, PABPC1, SRSF7*
GO:0006397~mRNA processing	6	*ALYREF, HSPA8, HNRNPF, HNRNPK, PABPC1, SRSF7*
GO:0002181~cytoplasmic translation	5	*RPL36, RPL8, RPLP0, RPLP1, RPLP2*
GO:0010501~RNA secondary structure unwinding	4	*DDX17, DDX3X, DDX5, DDX39B,*
GO:0051726~regulation of cell cycle	4	*JUN, JUNB, HSPA8, RACK1*
GO:0006457~protein folding	4	*ACTB, CALR, HSP90AB1, PPIA*
GO:0070374~positive regulation of ERK1 and ERK2 cascade	4	*CD74, JUN, HMGB1, TGFB1*
GO:0006414~translational elongation	3	*EEF1G, RPLP1, RPLP2*
GO:0006611~protein export from nucleus	3	*CALR, NUTF2, TGFB1*
GO:0045070~positive regulation of viral genome replication	3	*DDX3X, PPIA, PABPC1*
GO:0000060~protein import into nucleus, translocation	3	*SEC61B, NUTF2, TGFB1*

GO:0050714~positive regulation of protein secretion	3	*EZR, PPIA, TGFB1*
GO:0051028~mRNA transport	3	*ALYREF, NUTF2, SRSF7*
GO:0006413~translational initiation	3	*DDX3X, EIF3E, EIF4G2*
GO:0021762~substantia nigra development	3	*ACTB, CALM2, YWHAE*
GO:0042752~regulation of circadian rhythm	3	*PPARGC1A, HNRNPD, PROX1*
GO:0048146~positive regulation of fibroblast proliferation	3	*CD74, JUN, TGFB1*
GO:0009615~response to virus	3	*DDX3X, EEF1G, STMN1*
GO:0032922~circadian regulation of gene expression	3	*PPARGC1A, BHLHE41, HNRNPU*
GO:0001938~positive regulation of endothelial cell proliferation	3	*JUN, NRARP, PROX1*
GO:0001934~positive regulation of protein phosphorylation	3	*PIN1, RACK1, TGFB1*
GO:0010468~regulation of gene expression	3	*DDX3X, DDX39B, TENT5A*
GO:0039694~viral RNA genome replication	2	*PCBP2, PCBP1*
GO:0045581~negative regulation of T cell differentiation	2	*CD74, NRARP*
GO:0051343~positive regulation of cyclic-nucleotide phosphodiesterase activity	2	*CALM2, RACK1*
GO:1990441~negative regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress	2	*JUN, TMBIM6*
GO:0010941~regulation of cell death	2	*JUN, JUNB*
GO:0051085~chaperone mediated protein folding requiring cofactor	2	*CD74, HSPA8*
GO:0007184~SMAD protein import into nucleus	2	*JUN, TGFB1*
GO:0031953~negative regulation of protein auto phosphorylation	2	*ERRFI1, JUN*
GO:0017015~regulation of transforming growth factor beta receptor signaling pathway	2	*NREP, TGFB1*
GO:1901224~positive regulation of NIK/NF-kappaB signaling	2	*CALR, HMGB1*
GO:0051591~response to cAMP	2	*JUN, JUNB*
GO:0009612~response to mechanical stimulus	2	*JUN, JUNB*
GO:2000249~regulation of actin cytoskeleton reorganization	2	*ARHGDIB, TGFB1*

Table 4.6: Biological process of sperm transcripts down-regulated in low fertile bulls

Term	Count	Genes
GO:0007275~multicellular organism development	9	*QKI, ODF1, TNP1, PRM2, CFDP1, TNP2, ODF2, SPEM1, MEA1*
GO:0007283~spermatogenesis	7	*ODF1, BCL2L11, PRM2, TNP2, ODF2, SPEM1, MEA1*
GO:0030154~cell differentiation	6	*QKI, ODF1, TNP2, ODF2, SPEM1, MEA1*
GO:0001701~in utero embryonic development	5	*YBX1, UBE2B, BCL2L11, MYH10, RBBP6*
GO:1902600~hydrogen ion transmembrane transport	4	*MT-CYB, MT-CO2, COX3, COX1*
GO:0008380~RNA splicing	4	*YBX1, QKI, EIF4A3, LSM1*
GO:0006397~mRNA processing	4	*YBX1, QKI, EIF4A3, LSM1*
GO:0008360~regulation of cell shape	4	*CSNK1G2, CCL11, CFDP1, MYH10*
GO:0006281~DNA repair	4	*UBE2B, UBE2W, BRCA1, MBD4*
GO:0042773~ATP synthesis coupled electron transport	3	*MT-CO2, ND5, MT-ND4*
GO:0006513~protein mono-ubiquitination	3	*UBE2B, UBE2W, UBE2R2*
GO:0051028~mRNA transport	3	*QKI, KIF5C, EIF4A3*
GO:0090036~regulation of protein kinase C signaling	2	*CAPZB, AKAP12*
GO:0006369~termination of RNA polymerase II transcription	2	*SSU72, PCF11*
GO:0090084~negative regulation of inclusion body assembly	2	*DNAJB8, DNAJA4*
GO:0045717~negative regulation of fatty acid biosynthetic process	2	*BRCA1, INSIG2*
GO:0006301~postreplication repair	2	*UBE2B, BRCA1*

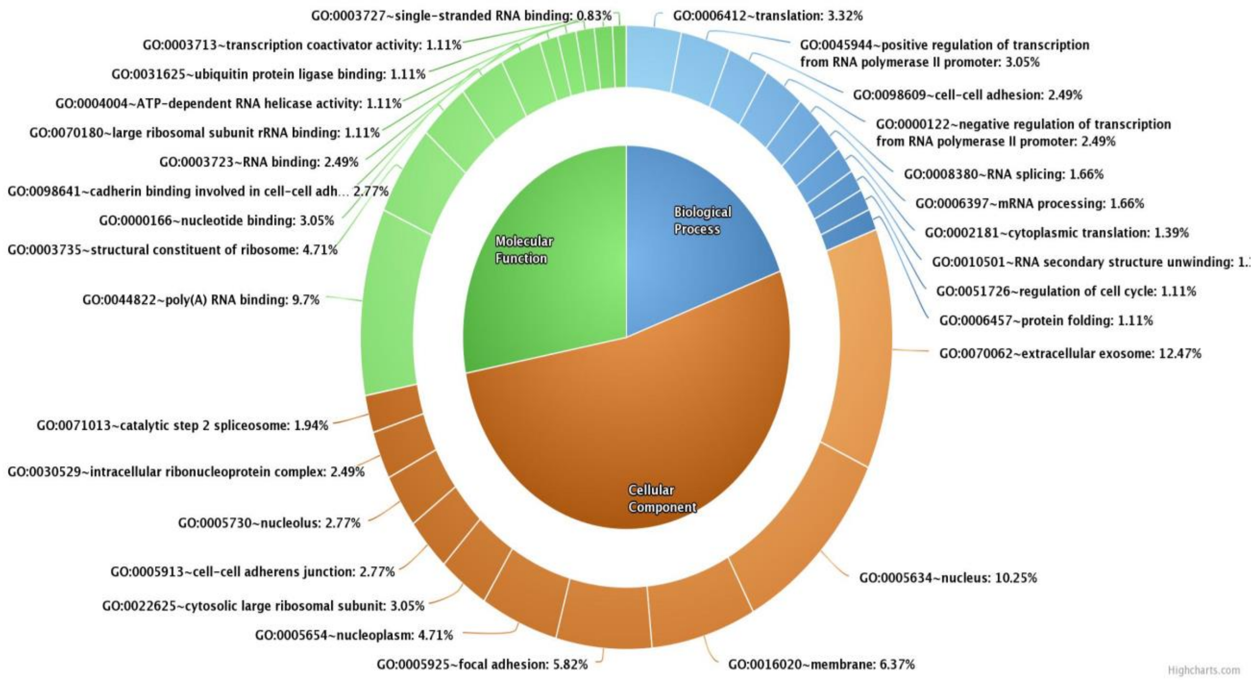


Fig.4.6a: Top 10 Gene Ontology categories of sperm transcripts upregulated in low fertile bulls

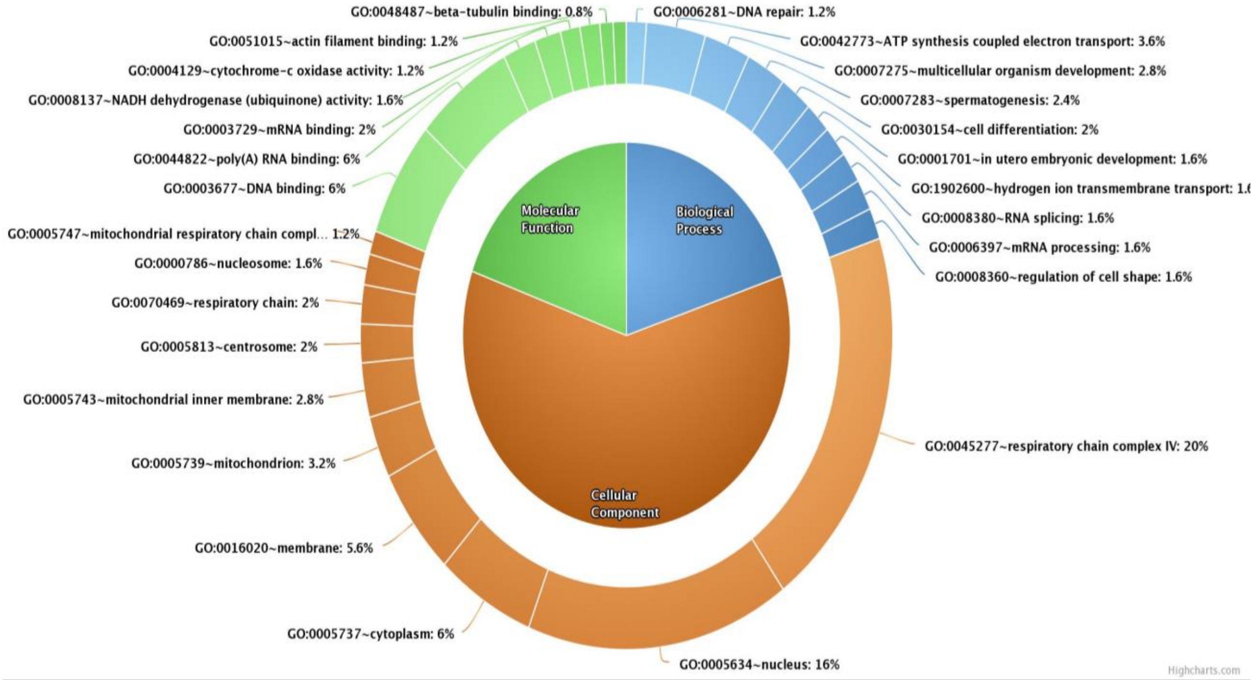


Fig.4.6b: Top 10 Gene Ontology categories of sperm transcripts down-regulated in low fertile bulls

Table 4.7: Biological process of sperm transcripts unique to low fertile bulls

Term	Count	Genes
GO:0006412~translation	39	*RPL21, RPL38, RPS20, RPL11, RPSA, RPS3, RPL10A, RPL34, RPL13A, RPS23, RPL27A, RPL32, EEF2, RPS27, RPS18, RPS17, RPL37, RPL10, RPL28, RPS14, RPS5, RPL24, RPS15, MRPL13, RPS12, RPS27A, RPL30, RPS21, RPS7, ENSBTAG00000033887, RPL7A, RPL18A, RPL27, RPS11, RPS9, RPL5, RPS24, ENSBTAG00000019007, ENSBTAG00000040435*
GO:0000028~ribosomal small subunit assembly	6	*RPS15,ENSBTAG00000040435, RPS27, RPS17, RPSA, RPS5*
GO:0002181~cytoplasmic translation	5	*RPL7, RPL29, RPL35A, RPL26, RPL31*
GO:0000027~ribosomal large subunit assembly	4	*RPL24, RPL11, RPL10, RPL5*
GO:0098609~cell-cell adhesion	4	*RPL24, EEF2, RPL29, RPL7A*
GO:0000398~mRNA splicing, via spliceosome	4	*U2AF1, ZMAT2, SYF2, HNRNPA2B1*
GO:0006364~rRNA processing	3	*RPL11, RPL26, RPS7*
GO:0008380~RNA splicing	3	*RBM8A, C1QBP, SRSF3*
GO:0000461~endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	2	*RPSA, RPS21*
GO:0000447~endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	2	*RPSA, RPS21*
GO:0032695~negative regulation of interleukin-12 production	2	*C1QBP, ACP5*
GO:0006414~translational elongation	2	*EEF1B2, EEF2*

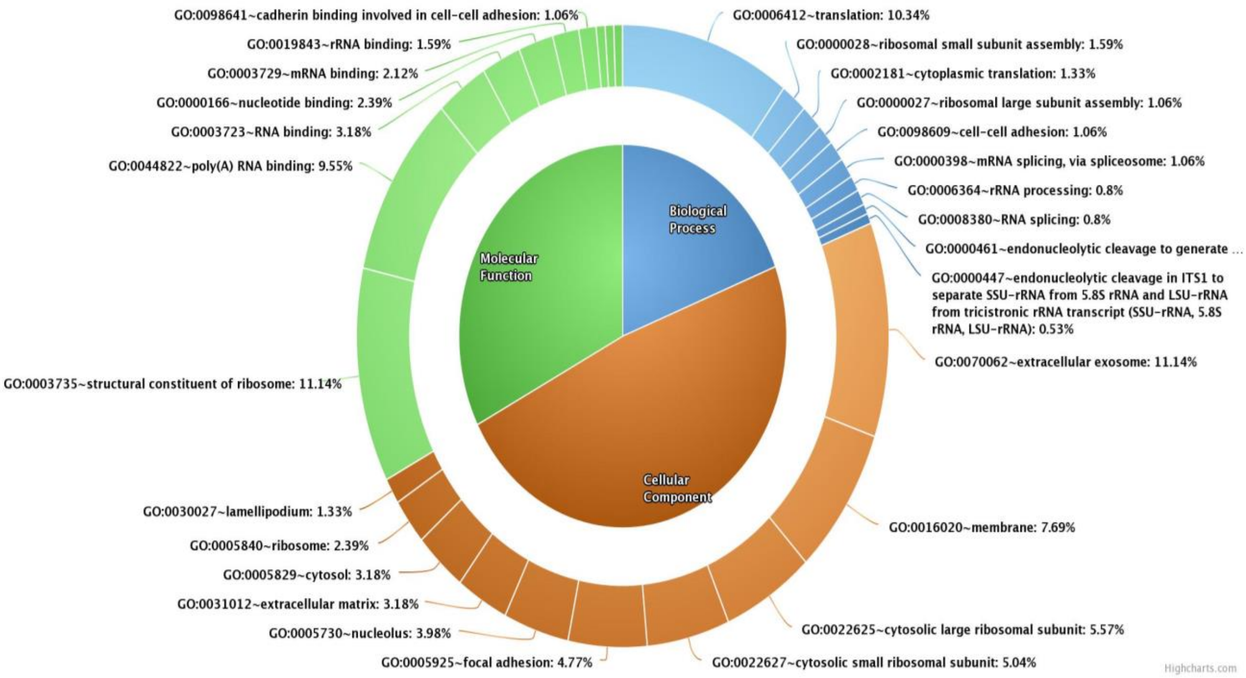


Fig.4.7: Top 10 Gene Ontology categories of sperm transcripts unique to low fertile bulls

Among the GO categories, normalized transcripts unique to high fertile bulls were related to molecular functions such as proton-transporting ATPase activity-rotational mechanism (4%, 2 count, 0.0447), ubiquitin conjugating enzyme activity (4%, 2 count, 0.0522) and biological process such as innate immune response (6%, 3 count, 0.0767). More than 50% of the transcripts unique to low fertile bulls were related to molecular functions such as structural constituent of ribosome (35.5%, 42 count, 9.83E-48), poly (A) RNA binding (30.05%, 36 count, 2.26E-17); biological process such as translation (33.05%, 39 count, 2.50E-47), ribosomal small subunit assembly (5.08%, 6 count, 5.34E-08), cytoplasmic translation (4.23%, 5 count, 3.02E-05), ribosomal large subunit assembly (3.38%, 4 count, 3.94E-04), cell-cell adhesion (3.38%, 4 count, 0.012), mRNA splicing via spliceosome (3.38%, 4 count, 0.0257) and were mostly localized to cellular components of extracellular exosome (35.59%, 42 count, 2.41E-10) and membrane (24.57%, 29 count, 7.78E-12).

The functional annotation of sperm transcripts upregulated in low fertile and unique in low fertile bulls have translation as a predominant biological process. In previous studies too, sperm transcripts associated with translation had a predominant biological process in *Bos indicus* (Raval *et al*., 2019), *Bos taurus* (Selvaraju *et al*., 2017) and in high-and low-fertile HF bulls (Card *et al*., 2017). Ribosomal proteins are mostly involved in the translation process and are necessary for protein synthesis. If any disorganizations occur in transcription and/or translation, it leads to decreased sperm quality (Platts *et al*., 2007). Sperm transcripts down-regulated in low fertile bulls compared to high fertile bulls had biological process of multicellular organism development (*QKI, ODF1, TNP1, PRM2, CFDP1, TNP2, ODF2, SPEM1, MEA1*) and spermatogenesis (*ODF1, BCL2L11, PRM2, TNP2, ODF2, SPEM1, MEA1*) both of which are highly essential for fertility.

4.1.4 Gene pathway and network analysis

Pathway enrichment of genes specific to sperm transcripts upregulated in low fertile populations (12 KEGG pathway) **(Table 4.8) (Fig. 4.8)** indicated that they are highly involved in the ribosome pathway **(Fig. 4.9)** (12.23%, 17 count, 1.09E-12). The transcripts down regulated in low fertile population (08 KEGG pathways) **(Table 4.9)** were highly involved in oxidative phosphorylation **(Fig. 4.10)** (5.05%, 9 count, 7.67E-06). The common transcripts between high- and low-fertile (15 KEGG pathways) were highly involved in ribosome pathway (4.59%, 20 count, 2.73E-09). Transcripts unique to high fertile (03 KEGG pathways) **(Table 4.10; Fig. 4.11)** were involved in huntington disease (8%, 4 count, 0.009), oxidative phosphorylation **(Fig 4.12)** (6%, 3 count, 0.038), Parkinson's disease (6%, 3 count, 0.044) and transcripts unique to low fertile (04 KEGG pathways) **(Table 4.11; Fig. 4.13)** were involved in ribosome **(Fig. 4.14)** (35.59%, 42 count, 1.86E-51), spliceosome (5.08%, 06 count, 1.86E-51), antigen processing and presentation (4.23%, 5 count, 0.009) and phagosome (4.23%, 5 count, 0.098) pathways.

Network analysis of gene interactions of sperm transcripts upregulated in low fertile expressed 120 BP **(Fig. 4.15)**, 27 CC, 26 MF and 14 KEGG pathways **(Fig. 4.16)**. Transcripts downregulated in low fertile bull spermatozoa revealed 28 BP **(Fig. 4.17)**, 06 CC, 05 MF and 10 KEGG (minimum gene as 3) pathways **(Fig. 4.18)**. Transcripts unique to high- and low-fertile bull spermatozoa showed 90 BP **(Fig. 4.19)**, 30 CC, 10 MF, 35 KEGG pathways and 46 BP **(Fig. 4.20)**, 12 CC, 04 MF and 03 KEGG pathways**,** respectively.

Table 4.8: KEGG pathway analysis of sperm transcripts upregulated in low fertile bulls

KEGG pathway terms	Count	Genes
bta03010:Ribosome	17	*ENSBTAG00000018987, RPL14, RPL18, RPL23, RPL3, RPL36, RPL37A, RPL4, RPL8, RPS10, RPS2, RPS28, RPS8, RPLP0, RPLP1, RPLP2, UBA52*
bta03040:Spliceosome	11	*ALYREF, DDX5, DDX39B, HSPA8, HNRNPK, HNRNPM, HNRNPU, PCBP1, PRPF38B, SRSF5, SRSF7*
bta05168:Herpes simplex infection	7	*ALYREF, CD74, JUN, HNRNPK, BOLA-DRA, SRSF5, SRSF7*
bta05164:Influenza A	6	*DDX39B, JUN, ACTB, ACTG1, HSPA8, BOLA-DRA*
bta05205:Proteoglycans in cancer	6	*DDX5, ACTB, ACTG1, EZR, SDC1, TGFB1*
bta04612:Antigen processing and presentation	5	*CD74, CALR, HSP90AB1, HSPA8, BOLA-DRA*
bta05132:Salmonella infection	5	*JUN, ACTB, ACTG1, ARPC2, PFN1*
bta04915:Estrogen signaling pathway	5	*GNAI2, JUN, CALM2, HSP90AB1, HSPA8*
bta05145:Toxoplasmosis	5	*GNAI2, BIRC3, HSPA8, BOLA-DRA, TGFB1*
bta04921:Oxytocin signaling pathway	5	*GNAI2, JUN, ACTB, ACTG1, CALM2*
bta05416:Viral myocarditis	4	*ACTB, ACTG1, EIF4G2, BOLA-DRA*
bta05323:Rheumatoid arthritis	4	*JUN, LTB, BOLA-DRA, TGFB1*

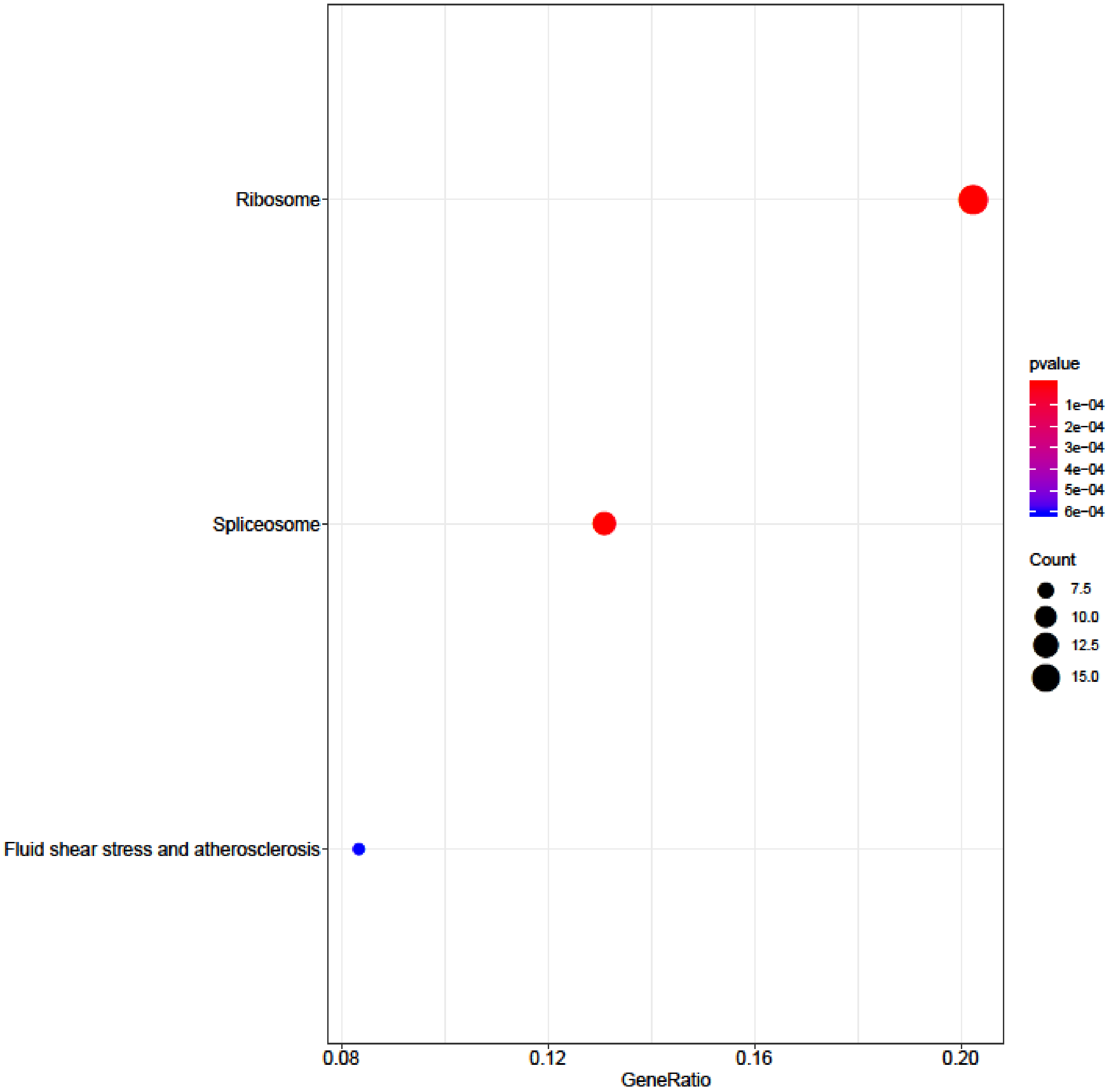


Fig.4.8: Pathway enrichment of sperm transcripts upregulated in low fertile bulls

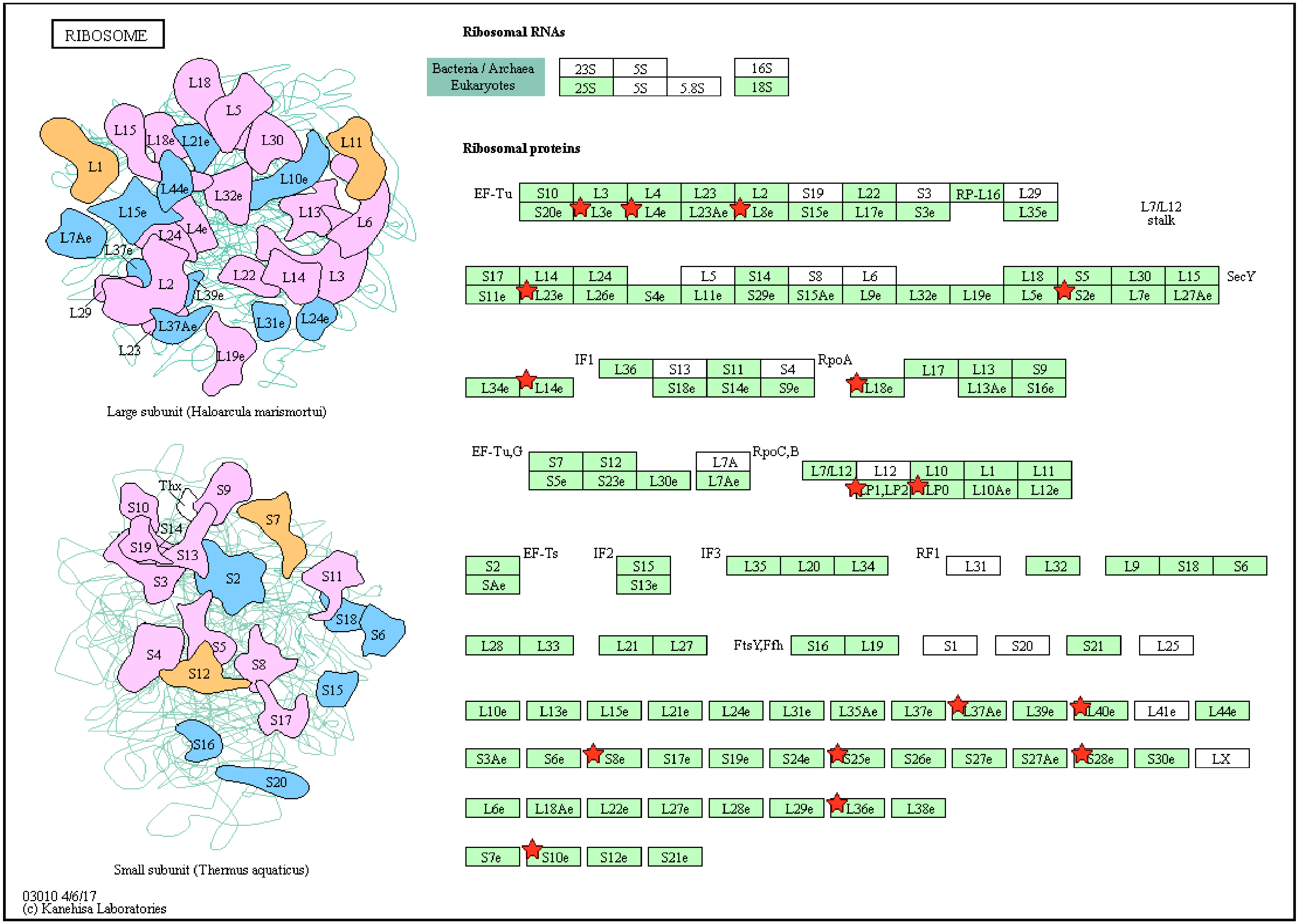


Fig.4.9: Ribosome pathway with sperm transcripts upregulated in low fertile bull spermatozoa

Table 4.9: KEGG pathway involved in sperm transcripts down-regulated in low fertile bulls

KEGG pathway terms	Count	Genes
bta00190:Oxidative phosphorylation	9	*MT-ATP6, ND1, MT-ND2, MT-ND4, ND5, MT-CYB, COX1, MT-CO2, COX3*
bta05012:Parkinson's disease	9	*MT-ATP6, ND1, MT-ND2, MT-ND4, ND5, MT-CYB, COX1, MT-CO2, COX3*
bta04932:Non-alcoholic fatty liver disease (NAFLD)	6	*BCL2L11, ADIPOR1, MT-CYB, COX1, MT-CO2, COX3*
bta05010:Alzheimer's disease	5	*MT-ATP6, MT-CYB, COX1, MT-CO2, COX3*
bta05016:Huntington's disease	5	*MT-ATP6, MT-CYB, COX1, MT-CO2, COX3*
bta04260:Cardiac muscle contraction	4	*MT-CYB, COX1, MT-CO2, COX3*
bta03040:Spliceosome	4	*LSM6, EIF4A3, SF3B1, TRA2B*
bta04120:Ubiquitin mediated proteolysis	4	*BRCA1, UBE2B, UBE2R2, UBE2W*

Table 4.10: KEGG pathway involved in sperm transcripts unique to high fertile bulls

KEGG pathway terms	Count	Genes
bta05016:Huntington's disease	4	*ATP5F1D, CLTA, MGC148714, TFAM*
bta00190:Oxidative phosphorylation	3	*ATP5F1D, ATP6V1C1, MGC148714*
bta05012:Parkinson's disease	3	*ATP5F1D, MGC148714, UBE2J1*

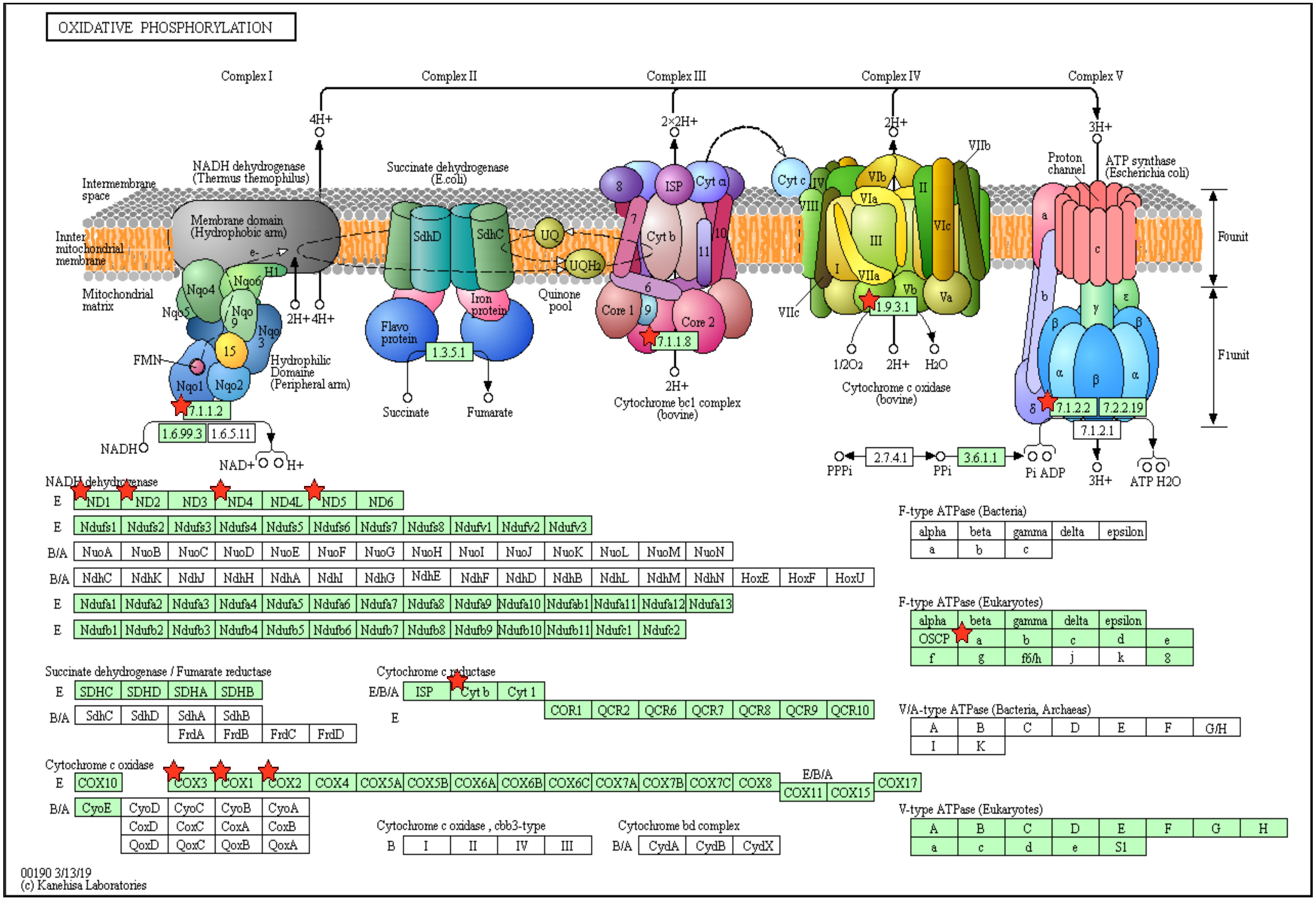


Fig.4.10: Oxidative phosphorylation pathway with sperm transcripts down-regulated in low fertile bull spermatozoa

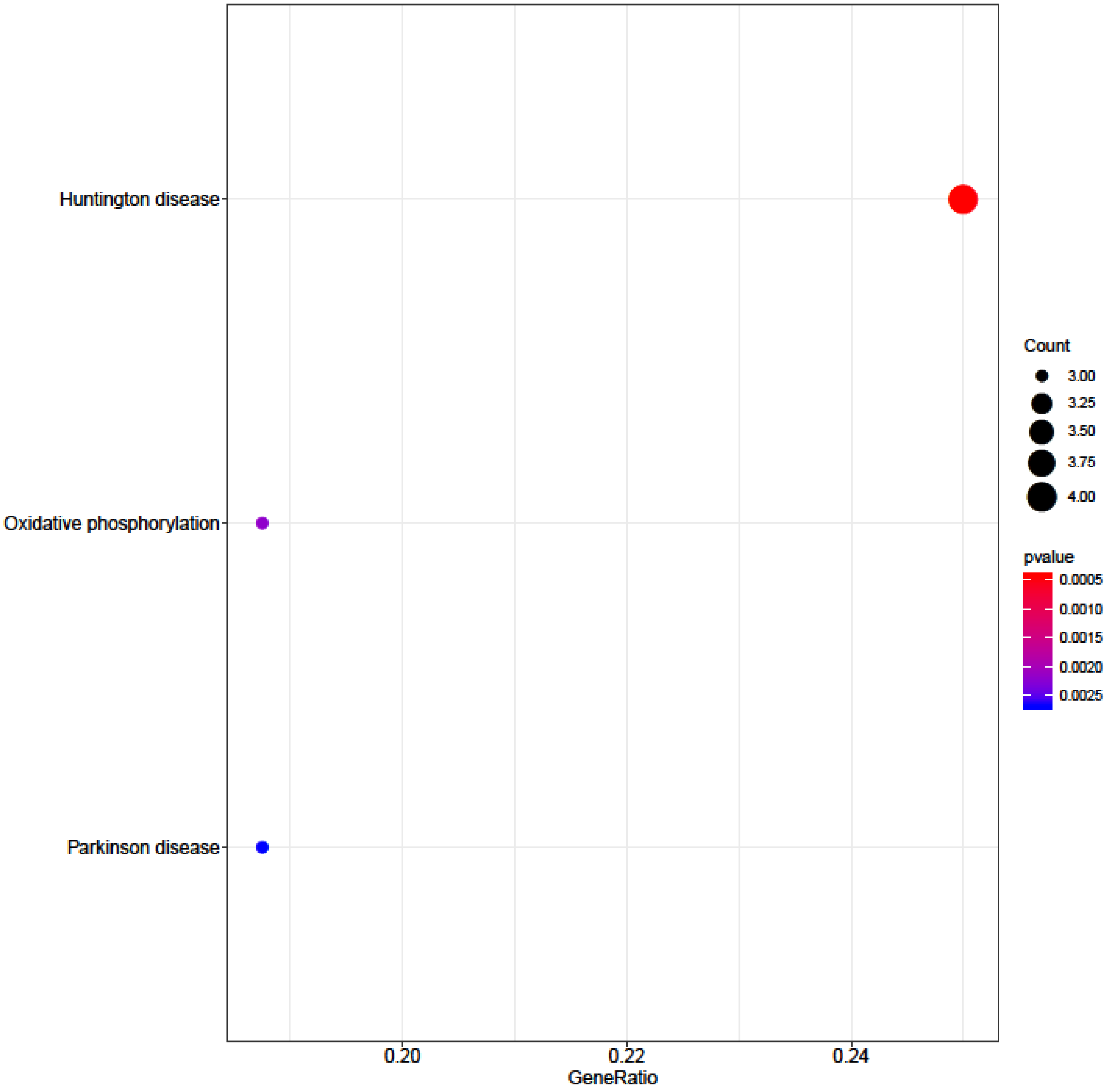


Fig.4.11: Pathway enrichment of sperm transcripts unique to high fertile bulls

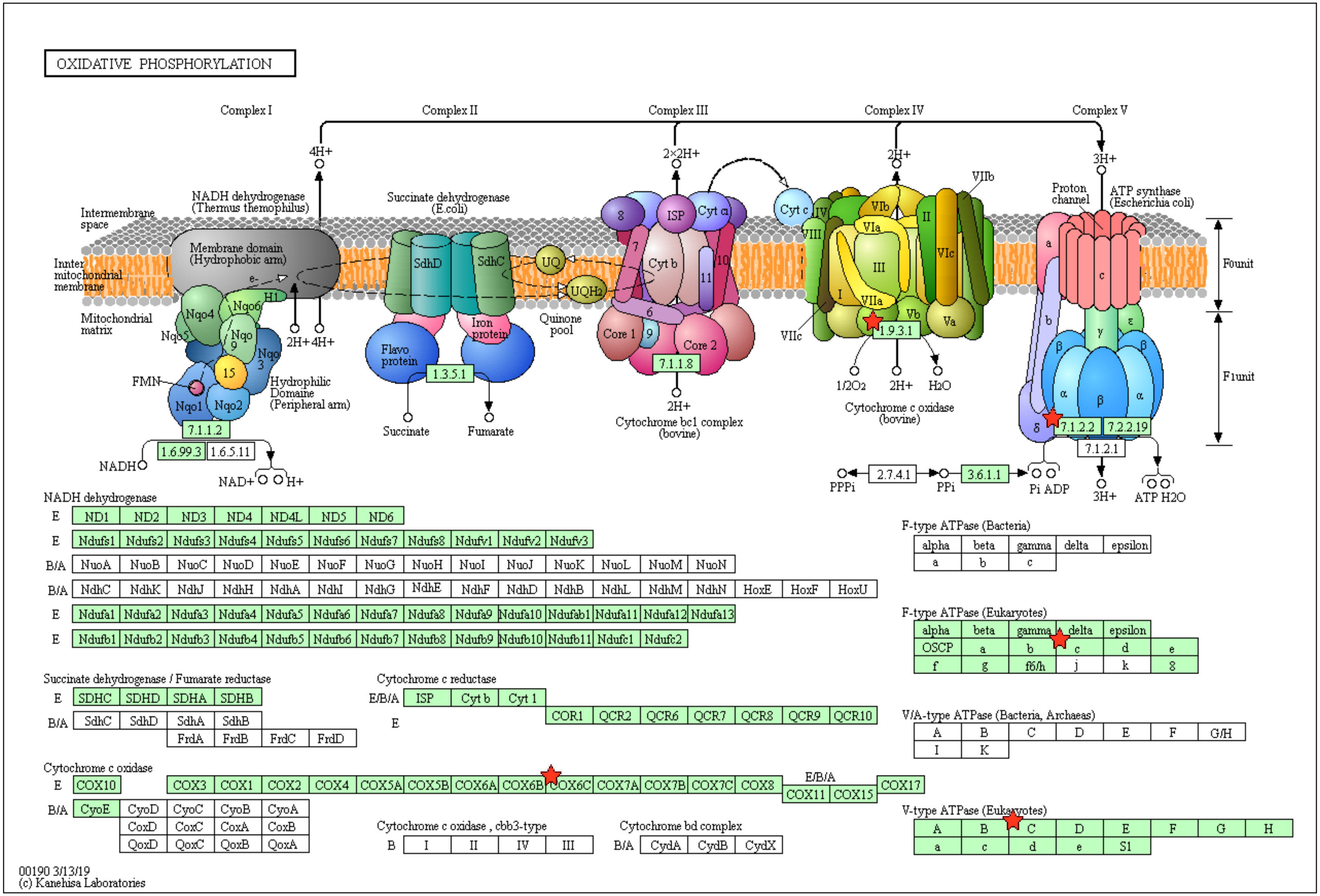


Fig.4.12: Oxidative phosphorylation pathway with sperm transcripts unique to high fertile bull spermatozoa

Table 4.11: KEGG pathway analysis of sperm transcripts unique to low fertile bulls

KEGG pathway terms	Count	Genes
bta03010:Ribosome	42	*ENSBTAG00000040435, MRPL13, RPL10, RPL10A, RPL11, RPL13A, RPL18A, RPL21, RPL24, RPL26, RPL27, RPL27A, RPL28, RPL29, RPL30, RPL31, RPL32, RPL34, RPL35A, RPL37, RPL38, RPL5, RPL7, RPL7A, RPS11, RPS12, RPS14, RPS15, RPS17, RPS18, RPS20, RPS21, RPS23, RPS24, RPS25, RPS27, RPS27A, RPS3, RPS5, RPS7, RPS9, RPSA*
bta03040:Spliceosome	6	*RBM8A, SYF2, U2AF1, HNRNPC, SRSF3, ZMAT2*
bta04612:Antigen processing and presentation	5	*JSP.1, B2M, BOLA-DQA2, PSME1, PDIA3*
bta04145:Phagosome	5	*JSP.1, CORO1A, BOLA-DQA2, RAC1, TUBB*

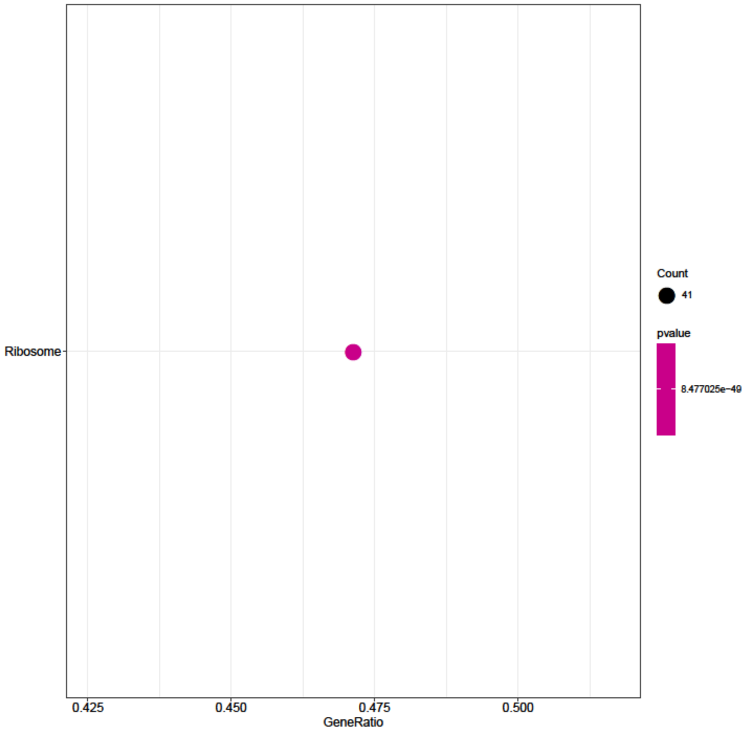


Fig.4.13: Pathway enrichment of sperm transcripts unique to low fertile bulls

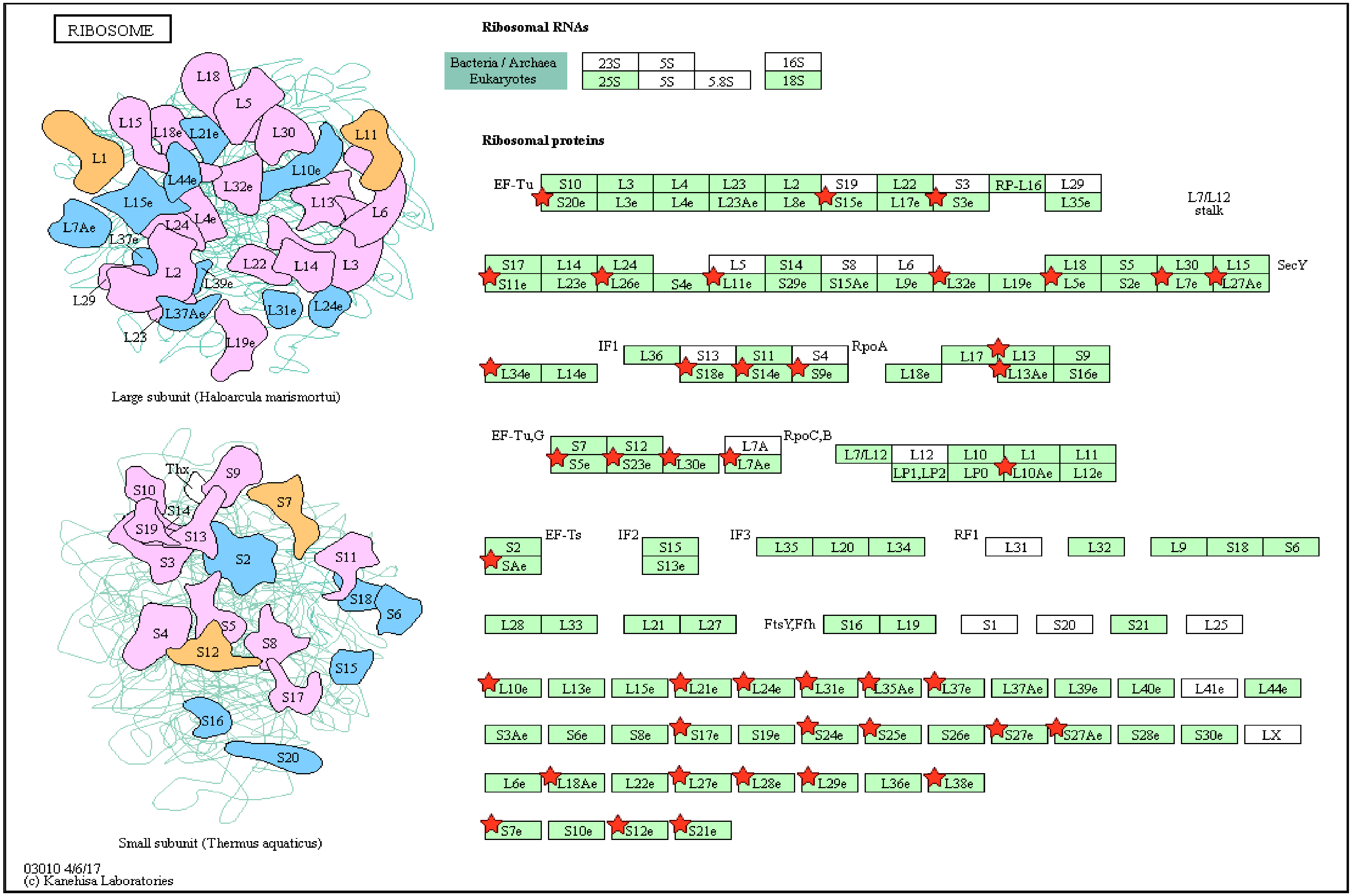


Fig.4.14: Ribosome pathway with sperm transcripts unique to low fertile bull spermatozoa

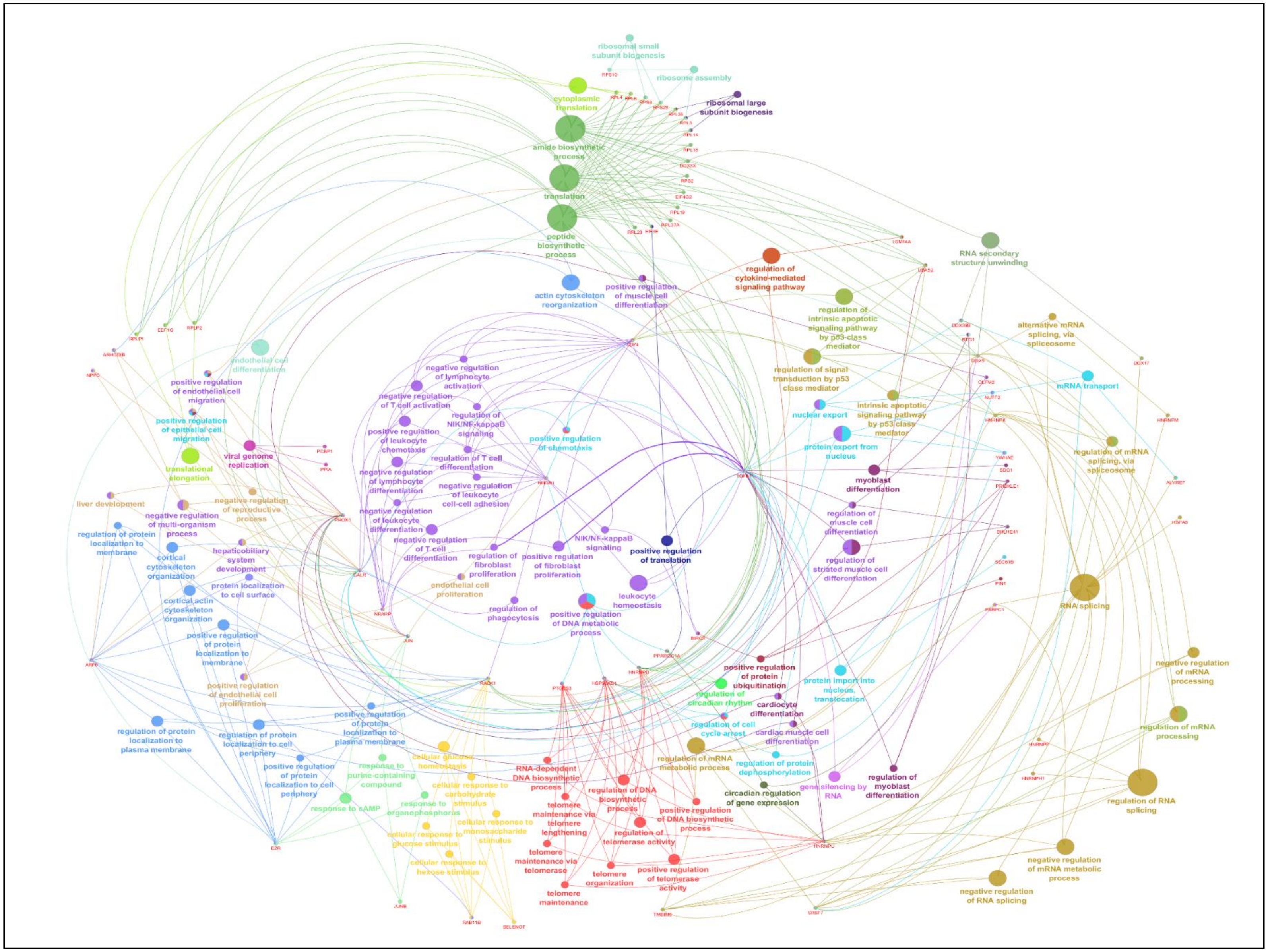


Fig.4.15: Interaction of genes involved in biological process of sperm transcripts upregulated in low fertile bull

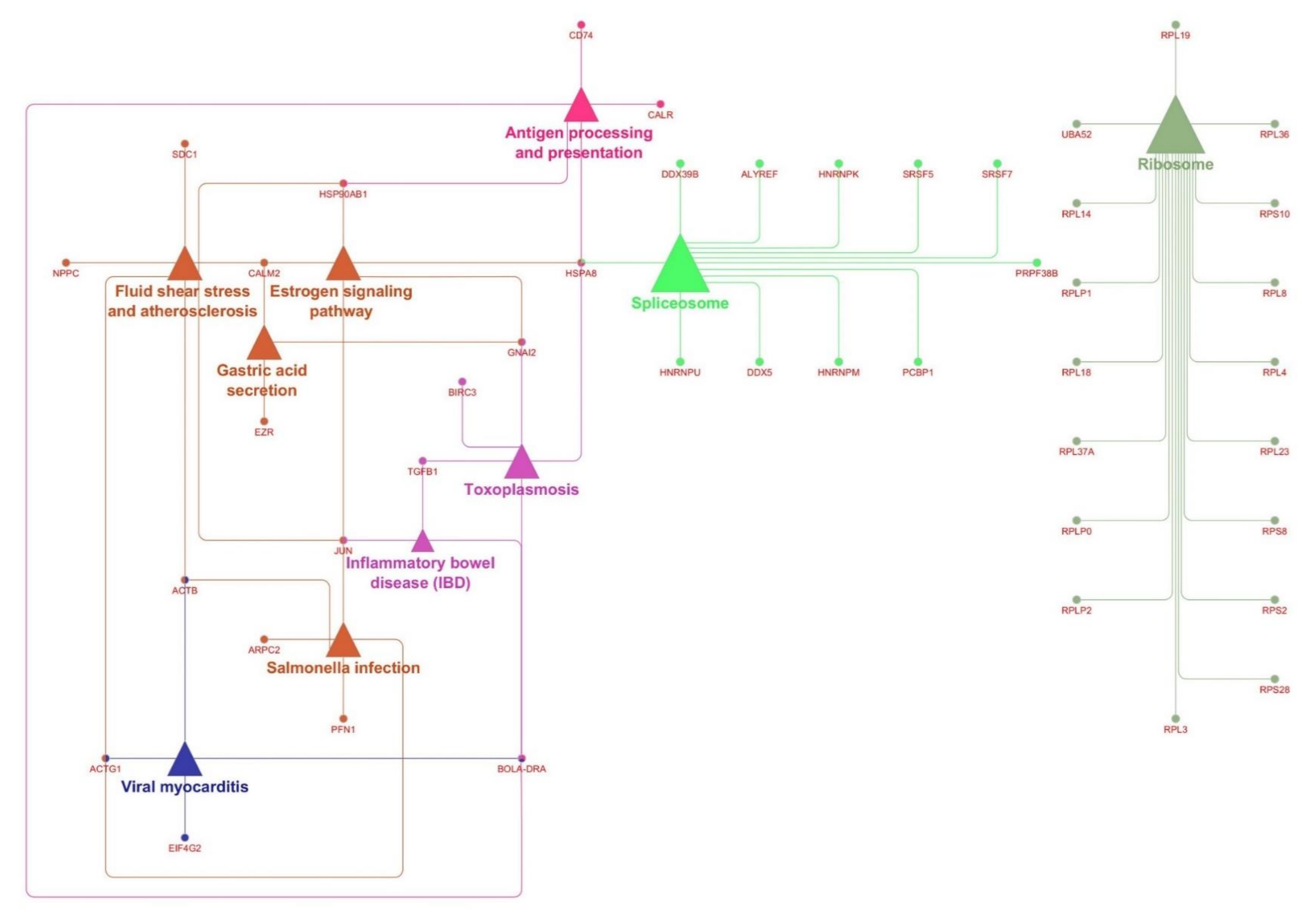


Fig.4.16: Interaction of genes involved in KEGG pathway of sperm transcripts upregulated in low fertile bull

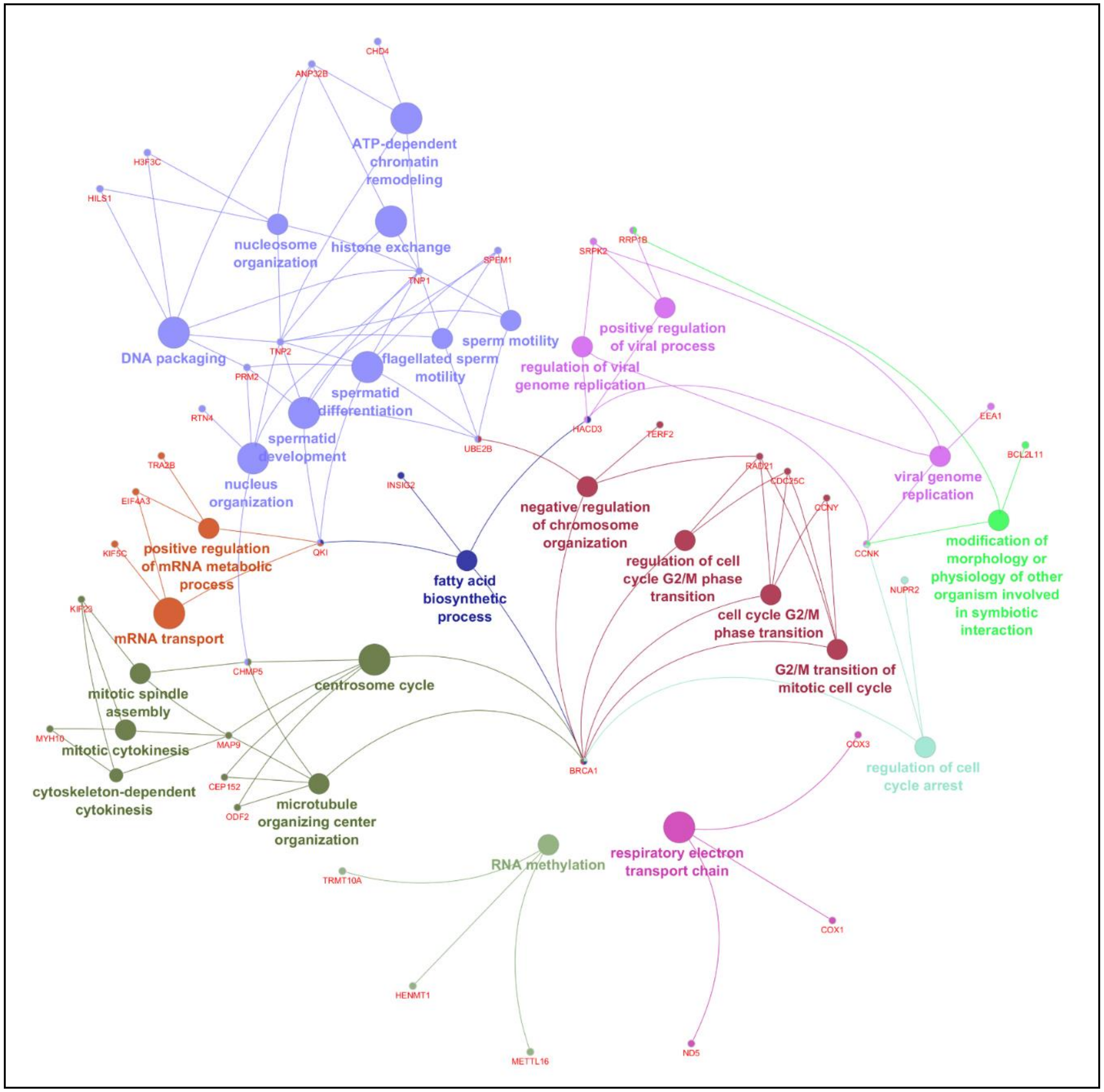


Fig.4.17: Interaction of genes involved in biological process of sperm transcripts down-regulated in low fertile bull

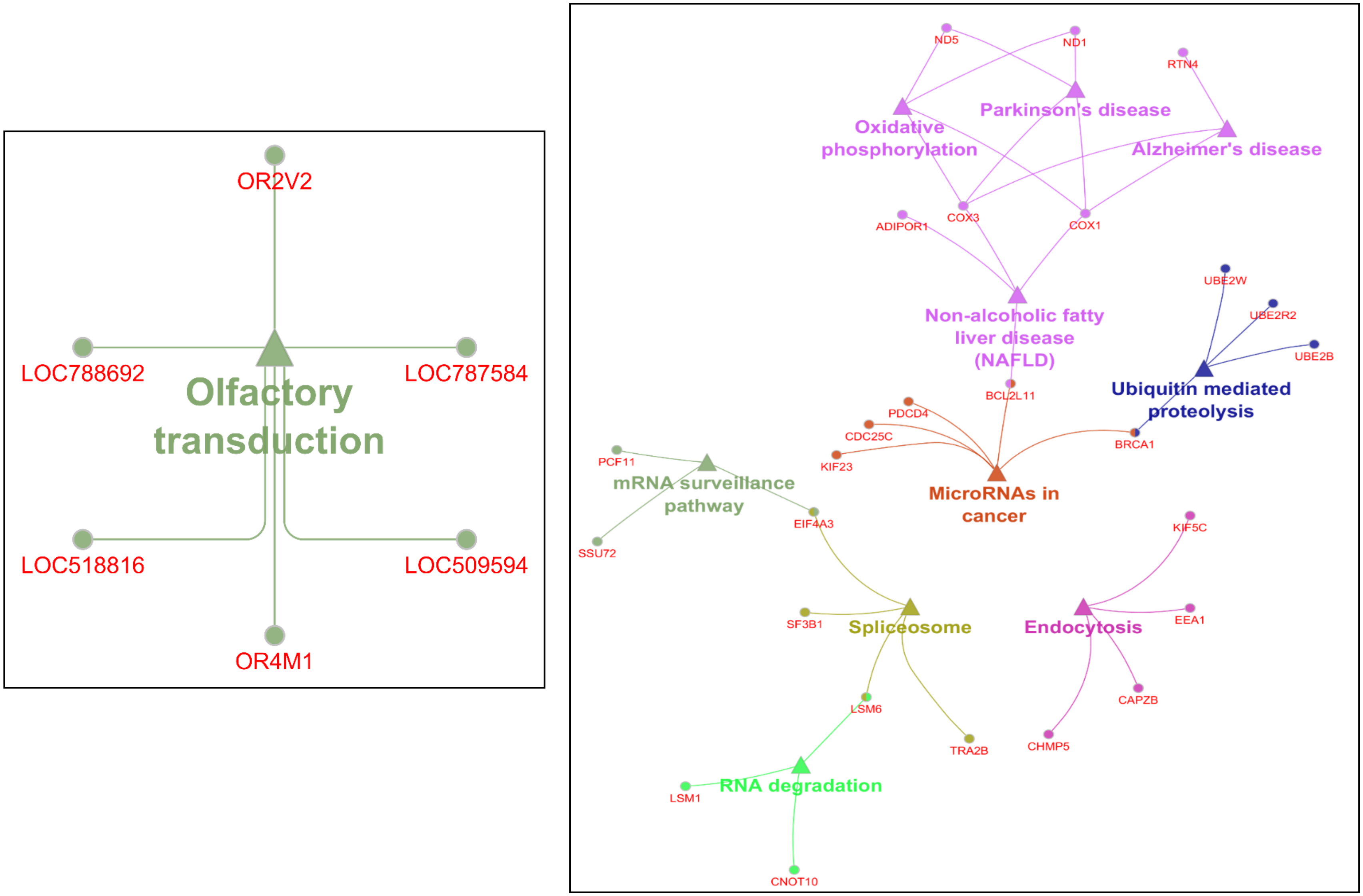


Fig.4.18: Interaction of genes involved in KEGG pathway of sperm transcripts down-regulated in low fertile bull

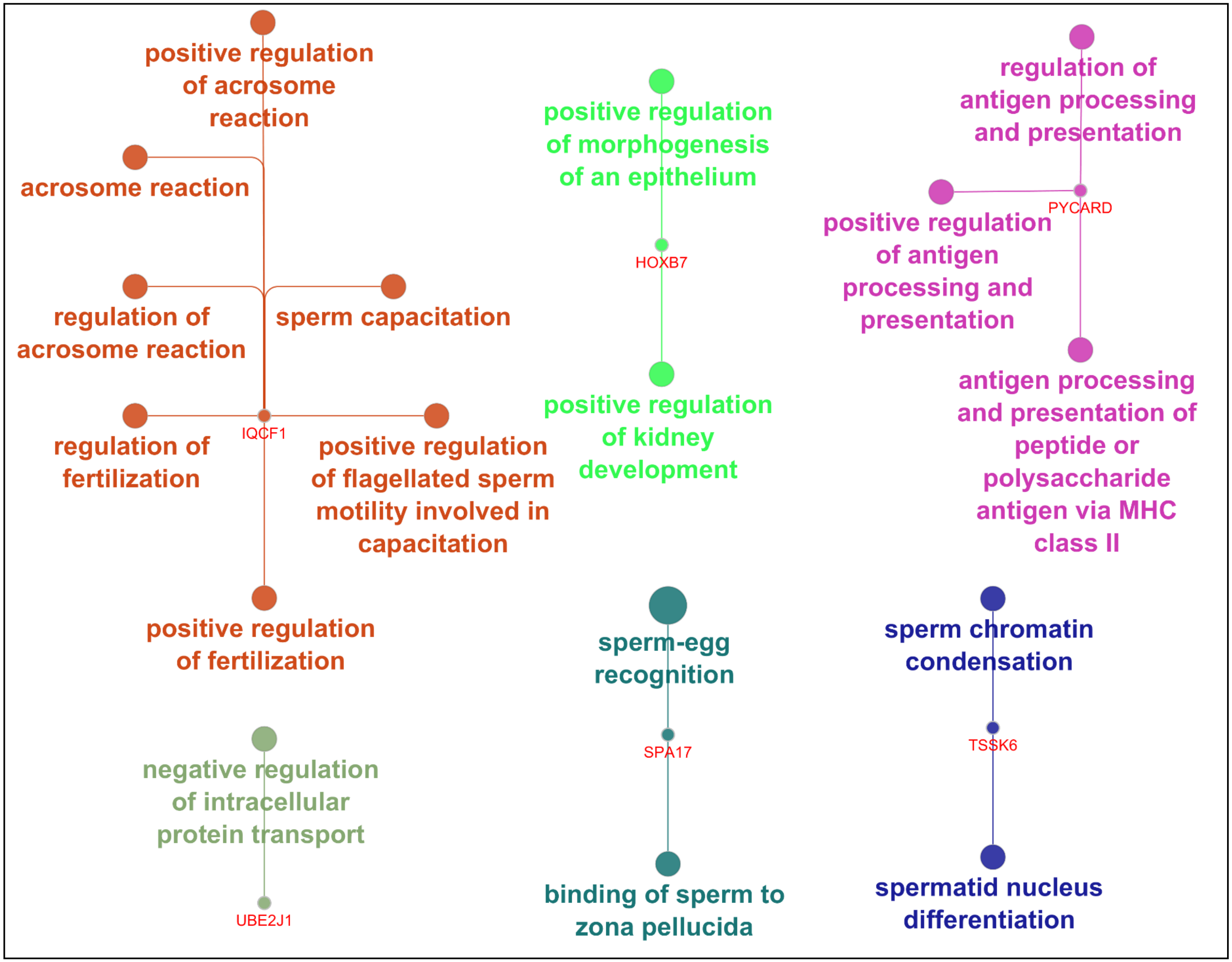


Fig.4.19: Interaction of genes involved in biological process of sperm transcripts unique to high fertile bull

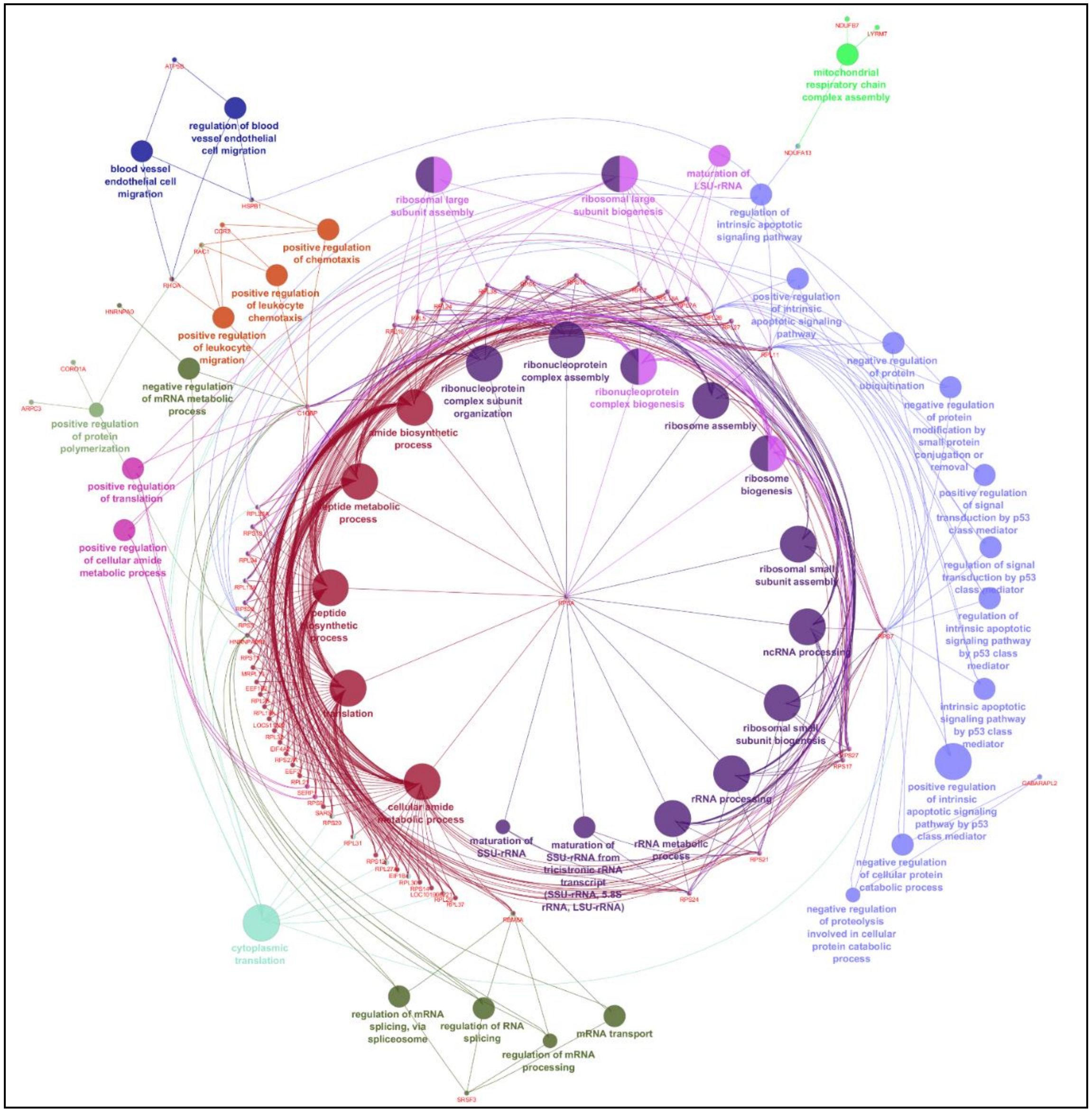


Fig.4.20: Interaction of genes involved in biological process of sperm transcripts unique to low fertile bull

Sperm transcripts upregulated and unique to low fertile bulls are involved in the ribosomal pathway. The genes involved in this pathway are ribosomal proteins and this pathway interacts with any other pathway. Hence, the ribosomal pathway in low fertile bull is so unique. As discussed earlier, the primary function of ribosomal RNA is protein synthesis; it has a major role in producing healthy spermatozoa (Bansal *et al*., 2015). Sperm mitochondria are the chief source of energy and they have their own translational mechanism. Differential expression of the ribosomal proteins could affect ribosomal assembly in mitochondria thereby affecting its functions. Though the ribosomal proteins are having good implications in sperm function, their expression is higher in the present study. In terms of ribosomal pathway it can be said that even with having an efficient energy source to synthesize protein effectively in low fertile bull's spermatozoa, there is still a deficiency in some other sperm functional characteristics compared to high fertile bulls in relevance to ribosomal pathway.

Oxidative phosphorylation is the predominant pathway involved with transcripts down-regulated in low fertile bull spermatozoa (*MT-ATP6, ND1, MT-ND2, MT-ND4, ND5, MT-CYB, COX1, MT-CO2, COX3*) compare to high fertile bull spermatozoa and transcripts unique to high fertile bulls (*ATP5F1D, ATP6V1C1, MGC148714*). Oxidative phosphorylation is responsible for active sperm energy production and ATP synthesis in all mammalian species including bovine (Garrett *et al*., 2008). It improves the sperm functional characters (reviewed in Storey, 2008). Any dysfunction in the oxidative phosphorylation process leads to inefficiency in sperm functional activities like impaired teratospermic ejaculates (Terrell *et al*., 2011). In humans, infertility (asthenospermia) conditions are reported with impaired oxidative phosphorylation in spermatozoa with abnormal mitochondrial organization (Rawe *et al*., 2007; Pelliccione *et al*., 2011) and reduced oxygen consumption (Ferramosca *et al*., 2008). According to previous reports, low fertile bull spermatozoa has reduced oxidative phosphorylation.

This study clearly shows that sperm transcripts in low fertile bulls are involved in the ribosomal pathway to generate protein synthesis in the mitochondria where oxidative phosphorylation process is lacking in the same group of bulls. Though low fertile bulls had an effective and efficient protein synthesis ability, the energy resource for the process is deficient.

4.1.5 Real time expression analysis of selected genes

Six differentially expressed genes (*TPT1, PFN1, ZNF706, MDB4, TNP2* and *TNP1*) were selected based on fold change for qPCR expression analysis on the same four (2HF & 2LF) bulls whose spermatozoa were suspected to NGS. The results of qPCR expression analysis of selected genes are shown in **Fig. 4.21**. Among the six genes validated using qPCR, the expression of *TPT1, PFN1, ZNF706, MDB4, TNP2* and *TNP1* followed the same trend as observed in RNA-Seq analysis.

Relative gene expression analysis using qPCR on different set of bulls was studied on eight genes (*TPT1, PFN1, ZNF706, MDB4, CRISP2, TNP2, ADIPOR1* and *TNP1*) differentially expressed between high- and low-fertile bulls. The details of genes selected for qPCR analysis based on fold change and its functional relevance on spermatogenesis, sperm function and fertility are given in **Table 4.12**. Results of qPCR expression analysis and melting peaks of select genes are shown in **Fig. 4.22ab**. The qPCR amplification of select genes was confirmed by polyacrylamide agarose gel electrophoresis **(Fig. 4.23)**. Among the selected genes validated using qPCR, expression of *TPT1, PFN1, ZNF706, MDB4, CRISP2, TNP2, ADIPOR1* and *TNP1* followed the same trend as observed in RNA-Seq analysis. Five genes (*ZNF706, MDB4, CRISP2, TNP2, TNP1*) were significantly ($p<0.05$) down regulated in low fertile bull spermatozoa. The relationship of selected transcripts with conception rate is given in **Table 4.13**. The relationship of *ZNF706, CRISP2, MDB4, TNP2* and *TNP1* genes with CR was highly significant ($P<0.01$) and positive.

The zinc finger protein *ZNF706*, is the predominant spermatozoal transcript downregulated in low fertile bulls compared to high fertile bulls. The zinc is important for male and female fertility. Zinc-containing metalloenzymes are essential for sperm functions (Kerns *et al*., 2018).

Table 4.12: List of genes selected for real time expression analysis

Sl. No	Gene	Log2(fold change) in NGS	Reported functional roles	References
1.	*TPT1*	8.79	Process of apoptosis, cellular differentiation and control of sperm functions	Arcuri *et al*., 2004
2.	*PFN1*	6.74	Regulation of actin filament polymerization, oocyte maturation	Rawe *et al*., 2006
3.	*ZNF706*	-16.03	Sperm functions and male fertility	Kerns *et al*., 2018
4.	*MDB4*	-5.81	DNA repair and spermatogenesis	Ruddock-D'Cruz *et al*., 2008; Terribas *et al*., 2010
5.	*CRISP2*	-5.19	Spermatogenesis, sperm motility, acrosome reaction, capacitation and fertilization	Card *et al*., 2013; Zhou *et al*., 2015; Brukman *et al*., 2016; Legare *et al*., 2017
6.	*TNP2*	-2.74	Acrosome reaction, penetration of zona pellucida, spermatogenesis	Selvaraju *et al*., 2018; Yathish *et al*., 2018
7.	*TNP1*	-2.52	Chromatin remodeling, spermatid development, spermatogenesis	Meistrich *et al*., 2003; Yathish *et al*., 2018
8.	*ADIPOR1*	-2.06	Capacitation and Sire fertility	Kasimanickam *et al*., 2013; Kadivar *et al*., 2016

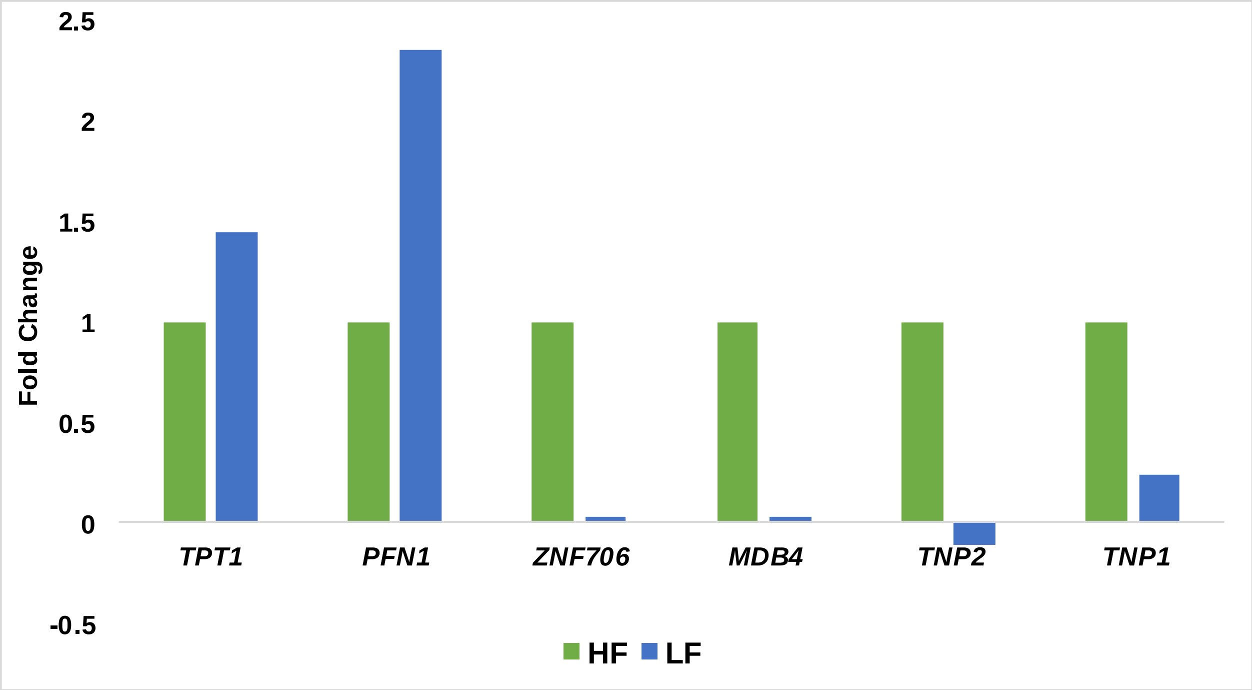


Fig.4.21: Relative expression of selected genes in high- and low-fertile bull

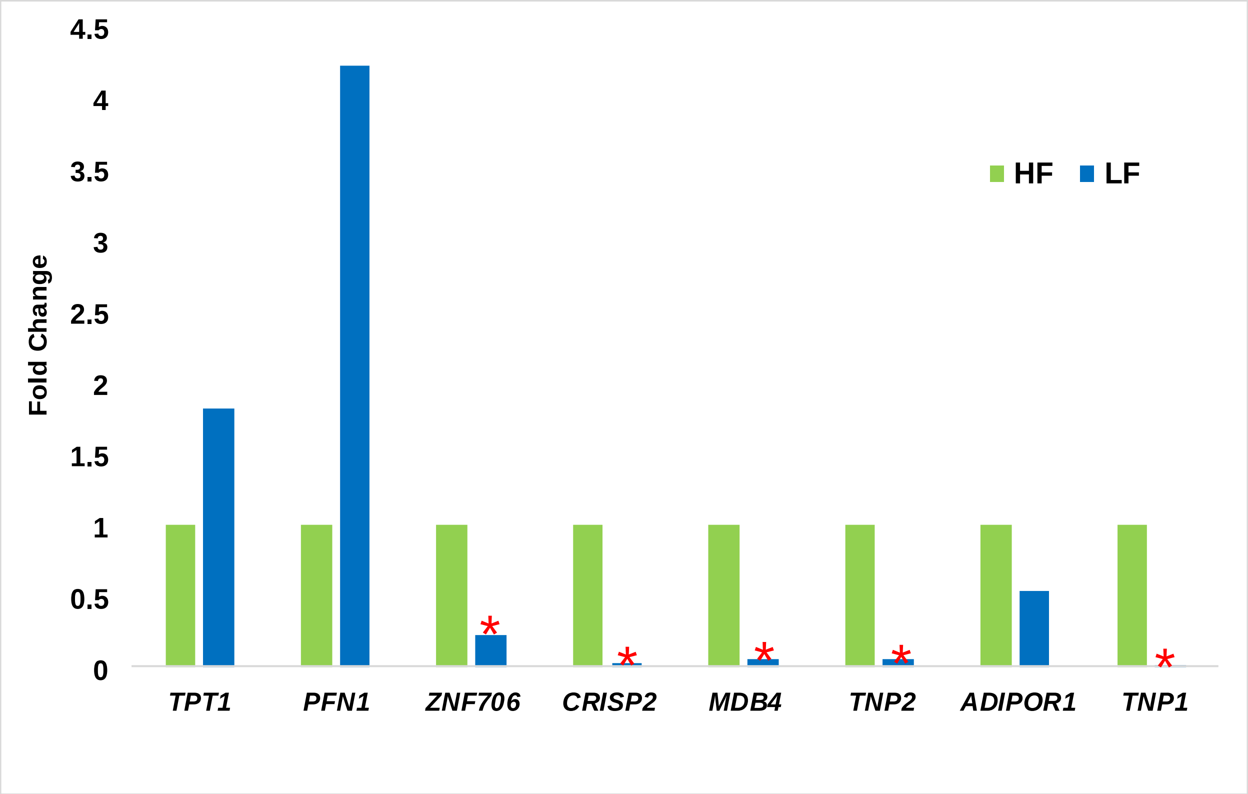


Fig.4.22a: Relative expression of selected genes in high- and low-fertile bulls (Star denotes significant difference ($p<0.05$))

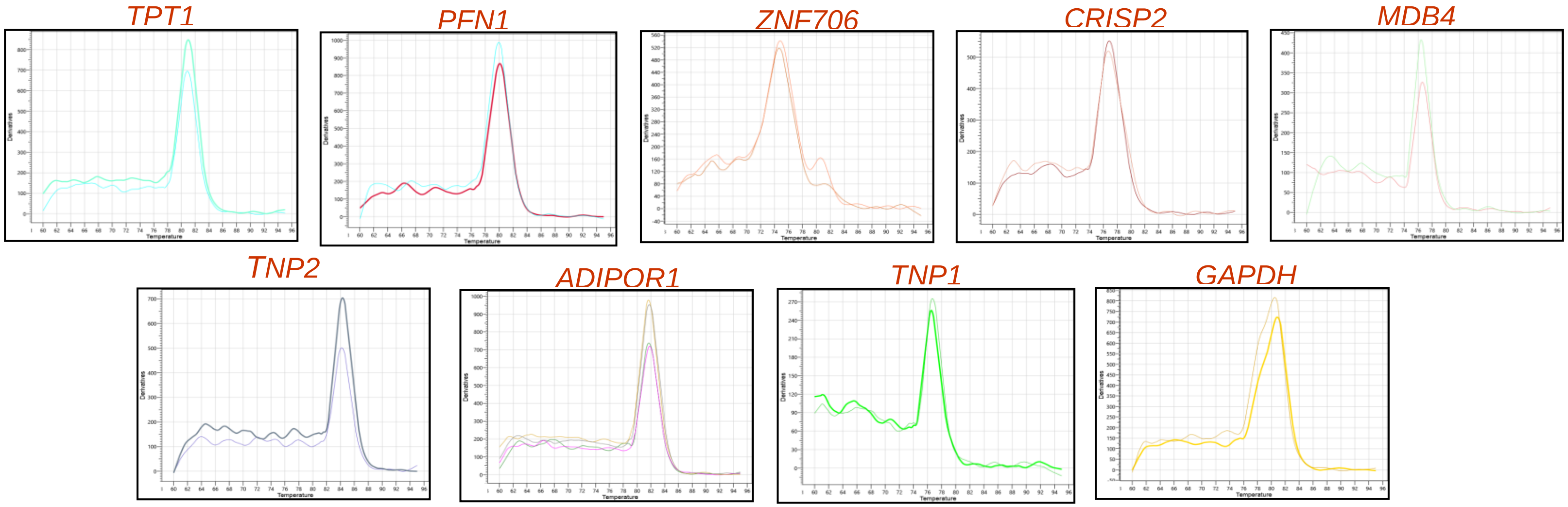
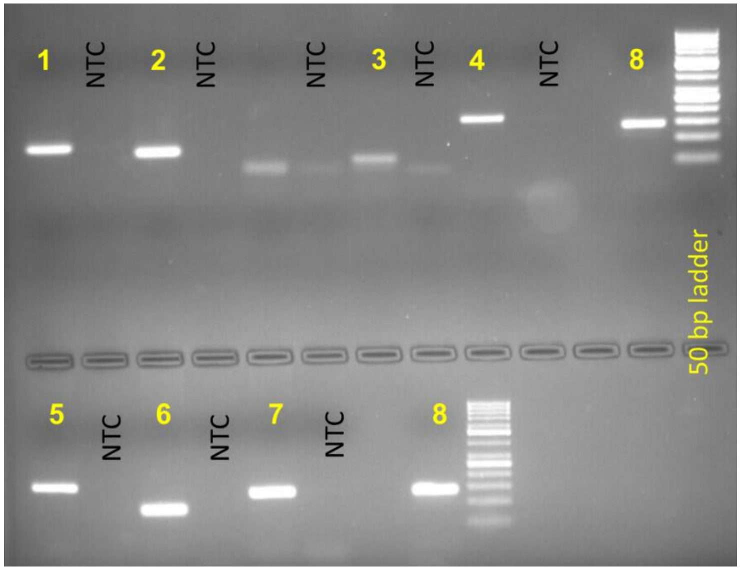


Fig.4.22b: Melting peaks of selected genes using qPCR



No.	Genes	Base pair (bp)
1	*TPT1*	106
2	*PFN1*	86
3	*CRISP2*	68
4	*MBD4*	184
5	*TNP2*	172
6	*ADIPOR1*	99
7	*TNP1*	149
8	*GAPDH*	141
NTC	Non template control	

Fig.4.23: Confirmation of qPCR amplified selected genes by agarose gel electrophoresis

Table 4.13: Relationship among transcriptional abundance of selected genes and conception rate

	TPT1	***PFN1***	***ZNF706***	***CRISP2***	***MDB4***	***TNP2***	***ADIPOR1***	***TNP1***	**CR**
TPT1	1	0.943** **(p=0.00)**	-0.233 (p=0.51)	-0.103 (p=0.77)	-0.264 (p=0.46)	-0.155 (p=0.66)	0.659* **(p=0.03)**	-0.245 (p=0.49)	-0.293 (p=0.41)
PFN1		1	-0.429 (p=0.21)	-0.172 (p=0.63)	-0.333 (p=0.34)	-0.238 (p=0.50)	0.613 (p=0.06)	-0.310 (p=0.38)	-0.344 (p=0.33)
ZNF706			1	0.853** **(p=0.00)**	0.877** **(p=0.00)**	0.891** **(p=0.00)**	0.417 (p=0.23)	0.873** **(p=0.00)**	0.807** **(p=0.00)**
CRISP2				1	0.986** **(p=0.00)**	0.996** **(p=0.00)**	0.655* **(p=0.04)**	0.989** **(p=0.00)**	0.822** **(p=0.00)**
MDB4					1	0.992** **(p=0.00)**	0.519 (p=0.12)	0.999** **(p=0.00)**	0.839** **(p=0.00)**
TNP2						1	0.612 (p=0.06)	0.994** **(p=0.00)**	0.843** **(p=0.00)**
ADIPOR1							1	0.540 (p=0.10)	0.421 (p=0.22)
TNP1								1	0.843** **(p=0.00)**
CR									1

**Significant ($p<0.01$); *Significant ($p<0.05$)

Cysteine rich secretory protein 2 (*CRISP2*), also called as *TPX1* is located in the acrosome and sperm tail (Busso *et al*., 2005; Jamsai *et al*., 2010). It is an important protein associated with fertility and sperm functions like spermatogenesis, modulation of flagellar motility, acrosome reaction, capacitation, gamete fusion and fertilization (Card *et al*., 2013; Zhou *et al*., 2015; Brukman *et al*., 2016; Legare *et al*., 2017). Similar to our results, studies in human reported that reduced *CRISP2* protein levels were found in azoospermia or oligoasthenoteratospermia (Du *et al*., 2006), asthenospermia (Jing *et al*., 2011) or asthenozoospermia (Heidary *et al*., 2019) conditions. Decreased *CRISP2* expression is significantly correlated with decreased motility, abnormal sperm morphology and infertility (Zhou *et al*., 2015). In bovines, *CRISP2* had positively correlated with sire fertility (Arangasamy *et al*., 2011; Zhao *et al*., 2006) and showed upregulation in high fertile bulls compared to low fertile bulls (Kasimanickam, 2011). Card *et al*. (2013) also found that *CRISP2* was among the top 10 transcripts with highest FPKM in frozen HF bulls using RNA-Seq.

Methyl-CpG binding domain 4, DNA glycosylase (*MBD4*), also known as *MED1* gene is a member of the nuclear protein family. These proteins are capable of specific binding methylated DNA and suppress transcription from methylated gene promoters (Kondo *et al*., 2005). *MBD4* is involved in DNA mismatch repair (Bellacosa, 2001) and spermatogenesis (Terribas *et al*., 2010). Supporting to our result, Bellacosa (2001) and Terribas *et al*. (2010) reported that expression of *MBD4* is decreased in spermatogenic failure.

Nuclear transition protein 2 (*TNP2*) has a role in acrosome reaction, multicellular organism development, penetration of *zona pellucida*, positive regulation of protein processing, spermatogenesis, exchange of chromosomal proteins and molecular functions of DNA binding and zinc ion binding (Selvaraju *et al*., 2018; Yathish *et al*., 2018; Meistrich *et al*., 2003). During spermatogenesis, the TNPs should replace protamine; any defective processes will lead to spermatid chromatin condensation resulting in sperm dysfunction (Jedrzejczak *et al*., 2007). This could be a reason for low fertility rate in bulls. Contrary to our finding, *TNP2* was upregulated in bulls with poor semen quality (Yathish *et al*., 2017) and upregulated in humans with teratozoospermia

(Savadi-shiraz *et al*., 2015). Jedrzejczak *et al*. (2007) also reported that *TNP2* transcripts may be linked to asthenozoospermia. Substantial to our reports, the *TNP2* gene knockout in mice leads to defects in sperm head and subsequent reduction in sperm motility (Adham *et al*., 2001).

The spermatid nuclear transition protein 1 (*TNP1*) has a functional role in chromatin remodeling, chromatin silencing, flagellated sperm motility, multicellular organism development, negative regulation of transcription, spermatid development, spermatid nucleus elongation, spermatogenesis and exchange of chromosomal proteins (Yathish *et al*., 2018; Shirley *et al*., 2004; Meistrich *et al*., 2003; Fox *et al*., 2003). *TNP1* was observed in fresh and frozen semen of bovine (Selvaraju *et al*., 2017; Card *et al*., 2013; Ganguly *et al*., 2016), swine (Yang *et al*., 2009) and mouse (Iguchi *et al*., 2006). Shirley *et al*. (2004) reported that the deficiency of *TNP1* leads to impaired spermatogenesis, sperm function and fertility.

4.2 Objective-2: Ability of spermatozoa to undergo capacitation upon exposure to oviductal fluid and its relationship with bull fertility

Cryopreserved semen from all the experimental bulls (n=6) used in the study showed more than 50% post-thaw sperm progressive motility. No significant difference in post-thaw sperm motility was observed between above- and below-average bulls.

4.2.1 Sperm functional attributes incubated before and after NL-ODF

Dot plot of live and dead spermatozoa in flow cytometric analysis is given in **Fig. 4.24**. The mean (±SEM) live and dead spermatozoa in above- and below-average bulls at 0 h and 4 h incubation with non-luteal oviduct fluid (NL-ODF) are shown in **Fig. 4.25**. Immediately after thawing there was no significant difference in progressive sperm motility between above- (63.11±7.1) and below-average (55.24±2.34) bull spermatozoa. The extent of reduction in sperm viability was higher in below average bulls (50.84% reduction) compared to above average fertility bulls (17.5% reduction) from the initial value observed immediately after thawing **(Table 4.14)**. After 4 h incubation with NL-ODF, the sperm viability was significantly ($p<0.05$) higher in above average fertile bulls (52.07±2.4) compared to below average fertile bulls (26.66±4.36).

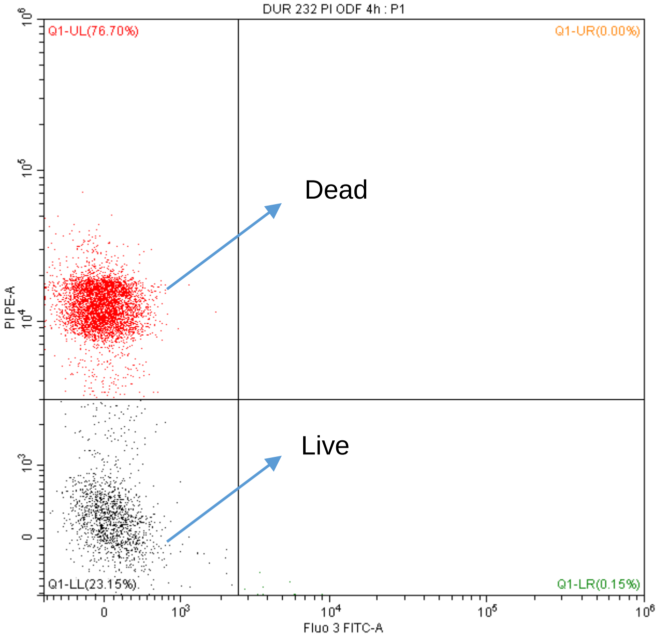


Fig.4.24: Dot plot analysis of sperm viability status using Propidium Iodide

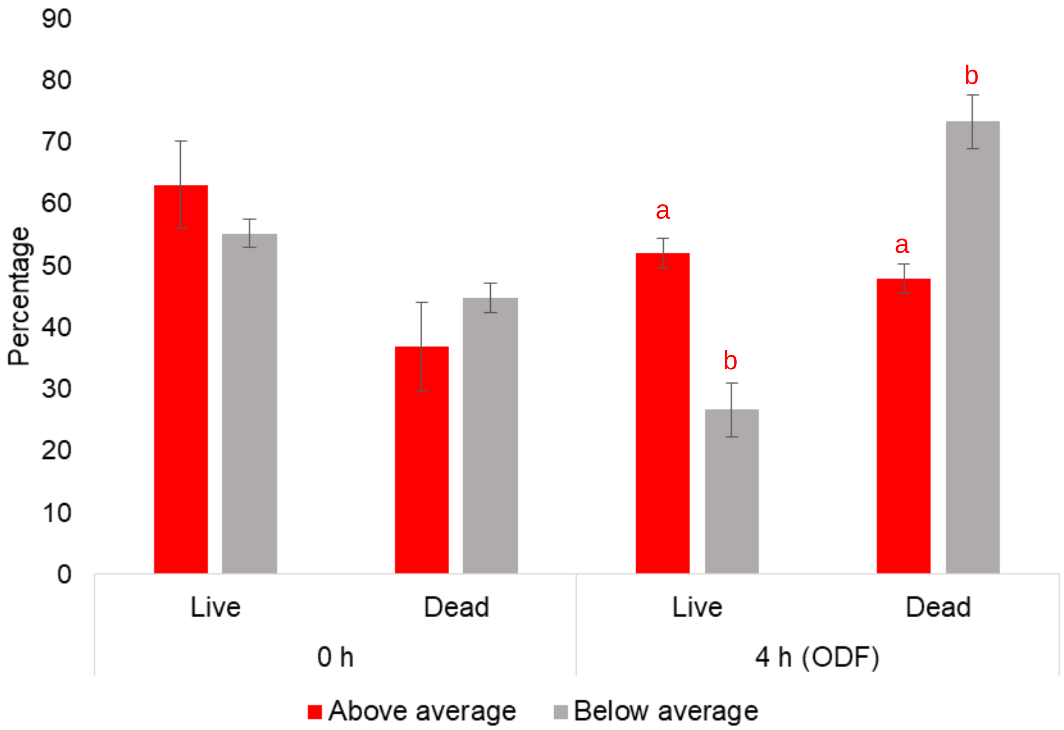


Fig.4.25: Proportion viable spermatozoa in above- and below-average bulls before and after incubation with NL-ODF (Bars with different superscripts (a & b) differ significantly ($p<0.05$) between above- and below-average bull)

Table 4.14: Proportionate change in sperm phenotypic characteristics after 4 h incubation with NL-ODF

Fertility status	Viability	Live low intracellular calcium	Live high intracellular calcium	Live acrosome intact	Live acrosome reacted	Live PTP
Above average	↓17.50%	↑49.31%	↓31.98%	↓28.65%	↑56.39%	↑40.39%
Below average	↓50.84%	↓8.83%	↓40.32%	↓40.92%	↑35.89%	↓48.28%

Flow cytometric analysis of proportion of live and dead spermatozoa with high or low intra-cellular calcium concentrations is depicted in **Fig. 4.26**. Live and dead spermatozoa were differentiated using PI. The proportions of mean (±SEM) spermatozoa with high- or low-intra-cellular calcium in above- and below-average bulls at 0 h and 4 h incubation in NL-ODF are shown in **Fig. 4.27**. Immediately after thawing, the proportion of viable spermatozoa with high intracellular calcium was significantly ($p<0.05$) higher in above average fertility bulls compared to below average fertility bulls. Again, at 4 h incubation in NL-ODF, also the proportion of viable spermatozoa with high intracellular calcium was significantly ($p<0.05$) higher in above average fertility bulls (42.9±2.03) compared to below average fertility (20.08±4.26) bulls.

The proportionate changes in different sub-populations of spermatozoa in respect to intracellular calcium concentrations before and after incubation with NL-ODF are given in **Table 4.14**. After 4 h incubation in NL-ODF, the proportion of viable spermatozoa with low intracellular calcium concentration increased by 49.31% in above average fertility bulls while 8.83% decrease was observed in below average bulls. After 4 h incubation in NL-ODF, the proportion of viable spermatozoa with high intracellular calcium concentration decreased by 31.98 and 40.32% in above average and below average fertility bulls, respectively as compared to the initial value immediately after thawing.

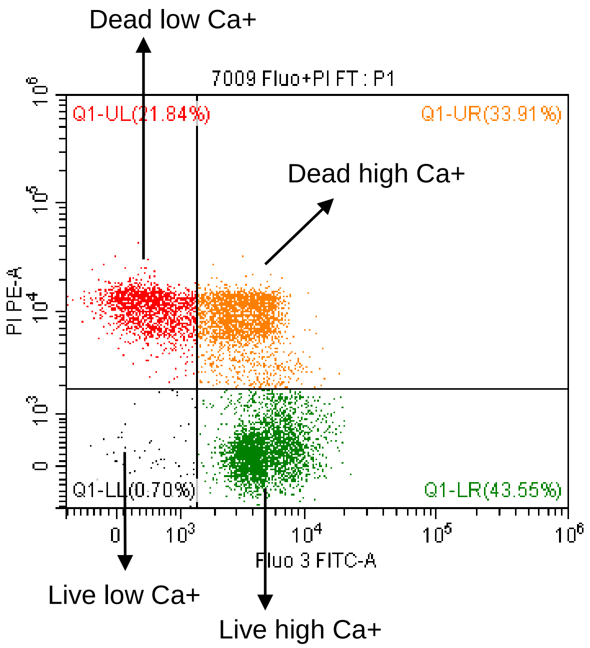


Fig.4.26: Flow cytometric assessment of sperm intra-cellular calcium concentration using Fluo-3

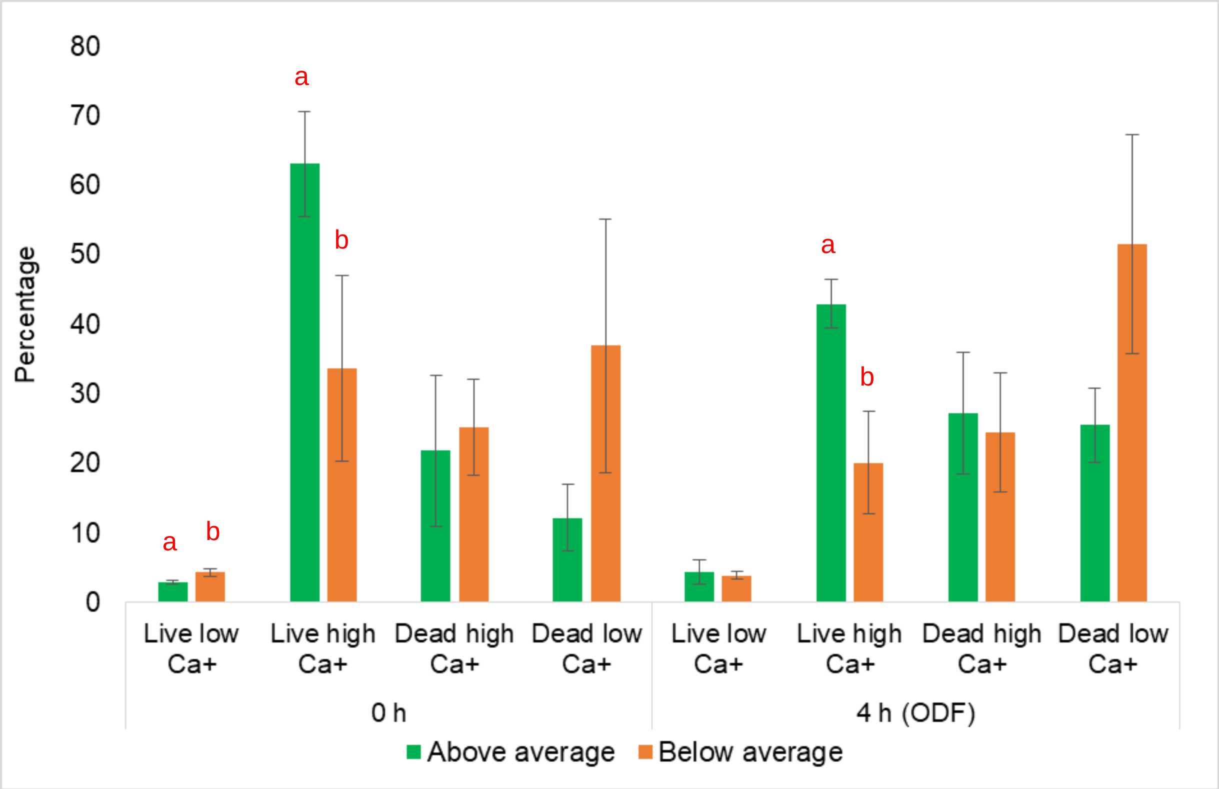


Fig.4.27: Proportion of viable spermatozoa in above- and below-average bulls before and after incubation with NL-ODF (Bars with different superscripts (a & b) differ significantly ($p<0.05$) above- and below-average bull)

Flow cytometric analysis of sub-populations of acrosome intact and acrosome reacted spermatozoa is depicted in **Fig. 4.28**. Live and dead spermatozoa were differentiated using PI. The proportions of mean (±SEM) spermatozoa with intact acrosome and reacted acrosome in above- and below-average bull at 0 h and 4 h incubation in NL-ODF are shown in **Fig. 4.29**. The changes in the acrosome reaction status of viable spermatozoa immediately after thawing and at 4 h incubation with NL-ODF are shown in **Table 4.14**. There was no significant difference between viable acrosome intact and reacted sperm populations, immediately after thawing. At 4 h incubation, the proportion of viable and acrosome intact spermatozoa was significantly ($p<0.05$) higher in above average (44.44±1.94) bulls than in below average (20.92±2.97) bulls. After 4 h incubation in NL-ODF, the proportion of viable spermatozoa with intact acrosome decreased by 28.65% in above average bulls and 40.92% in below average bulls while the proportion of viable spermatozoa with reacted acrosome increased by 56.39% in above average and 35.89% in below average bulls.

Flow cytometric analysis of proportion of live spermatozoa with protein tyrosine phosphorylation is depicted in **Fig. 4.30**. Only live phosphorylated spermatozoa were analyzed while dead cells were gated out using PI staining. The proportions of mean (±SEM) of live tyrosine phosphorylated spermatozoa in above- and below-average bulls at 0 h and 4 h incubation in NL-ODF are shown in **Fig. 4.31**. The proportion of live tyrosine phosphorylated spermatozoa showed no significant difference between above (32.68±21.14) and below average (28.35±15.33) bulls, immediately after thawing. However, at 4 h incubation in NL-ODF, the proportion of live tyrosine phosphorylated spermatozoa was significantly ($p<0.05$) higher in above average (45.84±8.01) bulls compared to below average (14.66±2.37) bulls. The proportionate changes in the live tyrosine phosphorylated spermatozoa immediately after thawing and at 4 h incubation with NL-ODF are shown in **Table 4.14**. After 4 h incubation in NL-ODF, the proportion of live tyrosine phosphorylated spermatozoa increased by 40.39% in above average bulls, which on other hand decreased by 48.28% in below average bulls.

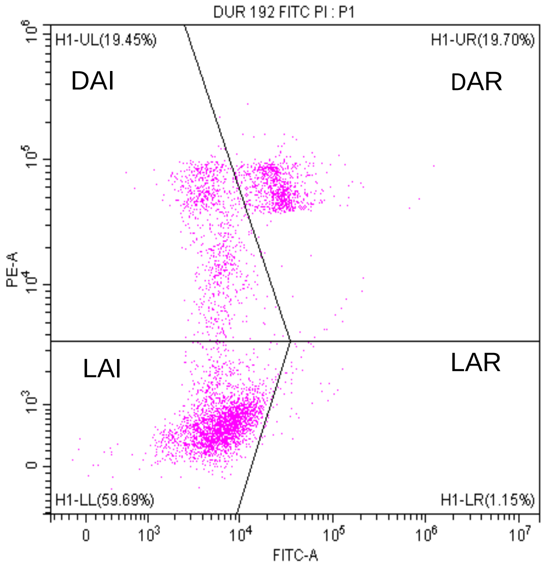


Fig.4.28: Flow cytometric assessment of sperm with acrosome reaction status using FITC-PSA

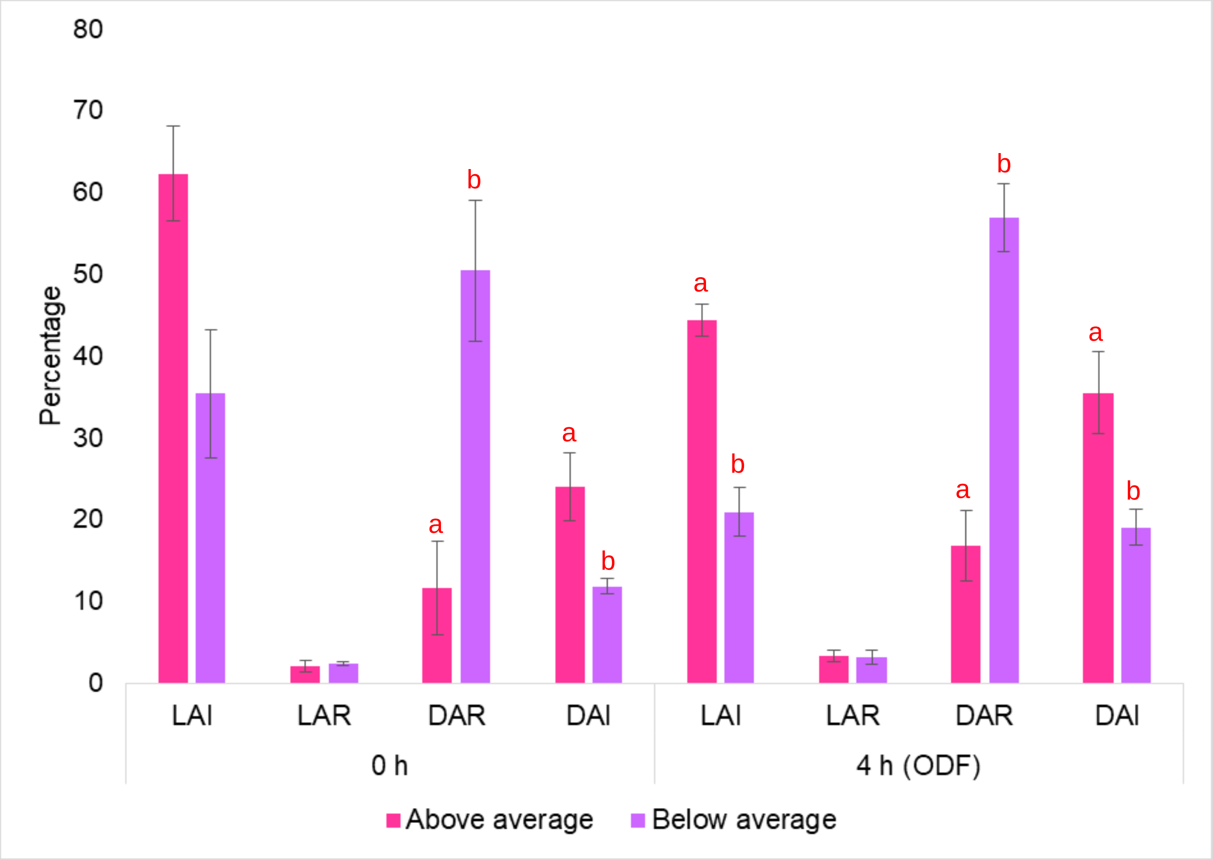


Fig.4.29: Proportion of acrosome reaction status in above- and below-average bulls before and after incubation with NL-ODF (Bars with different superscripts (a & b) differ significantly (p<0.05) above- and below-average bull)

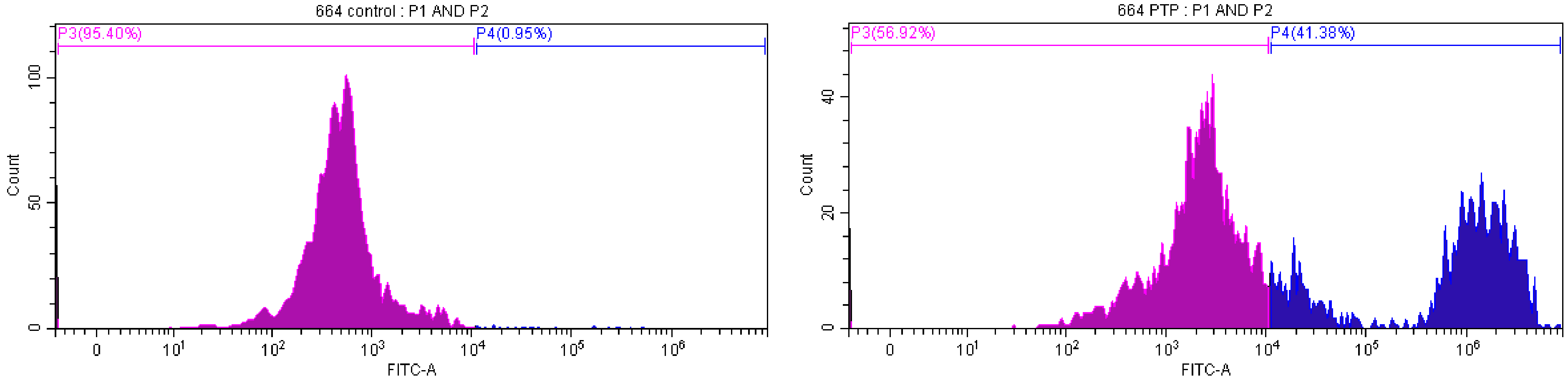


Fig.4.30: Flow cytometric assessment of global tyrosine phosphorylation status

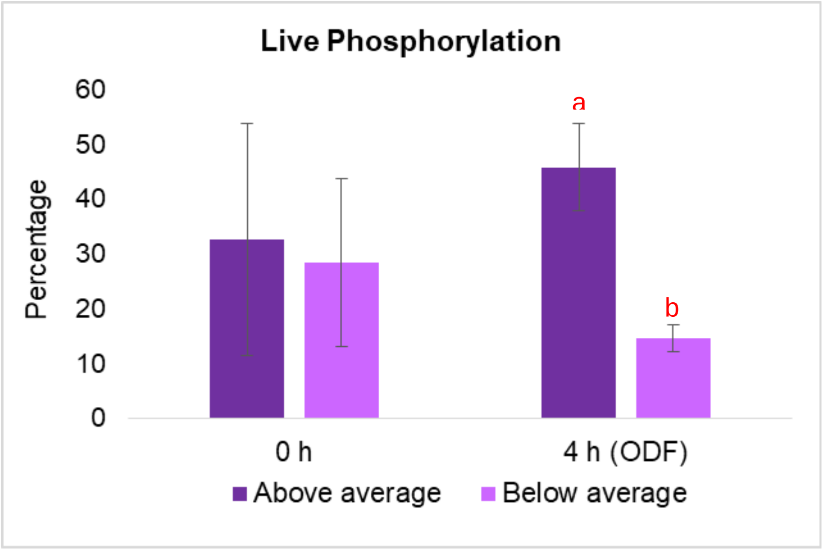


Fig.4.31: Proportion of live phosphorylated spermatozoa in above- and below-average bulls before and after incubation with NL-ODF (Bars with different superscripts (a & b) differ significantly ($p<0.05$) between above- and below-average bull)

Indirect immunofluorescence technique was used to read the localization of different patterns of protein tyrosine phosphorylation between above- and below-average bulls. The same antibody used for detecting tyrosine phosphorylation in flow cytometry, was used to confine tyrosine phosphorylation in spermatozoa. Seven types of immuno-fluorescent labeling patterns were observed in the present study and are displayed in **Fig. 4.32**. The different PTP patterns observed were 1) fluorescence over the acrosomal region (Pattern A); 2) fluorescence in the post-acrosomal and equatorial region (Pattern PA); 3) fluorescence at both acrosomal and post-acrosomal region (pattern APA); 4) fluorescence in tail (pattern T); 5) fluorescence over acrosomal region and tail (pattern AT); 6) fluorescence in the post-acrosomal or equatorial region and tail (pattern PAT); 7) fluorescence over the acrosome, post-acrosome and tail (pattern APAT). Although numerical differences were observed between above average and below average fertility bulls in terms of different patterns of tyrosine phosphorylation, none of the pattern differed significantly between the two categories of the bulls **(Fig. 4.33)**.

Transcriptional abundance of selected sperm-capacitation associated genes (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2* and *IQCF1*) was assessed using qPCR in frozen thawed spermatozoa of bulls with different conception rates. Results of qPCR expression analysis and melting peaks of capacitation associated genes are shown in **Fig. 4.34 a** and **b**. The qPCR amplification of select genes were confirmed by polyacrylamide agarose gel electrophoresis **(Fig. 4.35)**. Based on fold change, the expression of all the capacitation associated selected genes (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2* and *IQCF1*) were upregulated in below average as compared to above average bull spermatozoa, among which *CATSPER1* gene was significantly ($p<0.05$) upregulated in below average bulls than above average bulls.

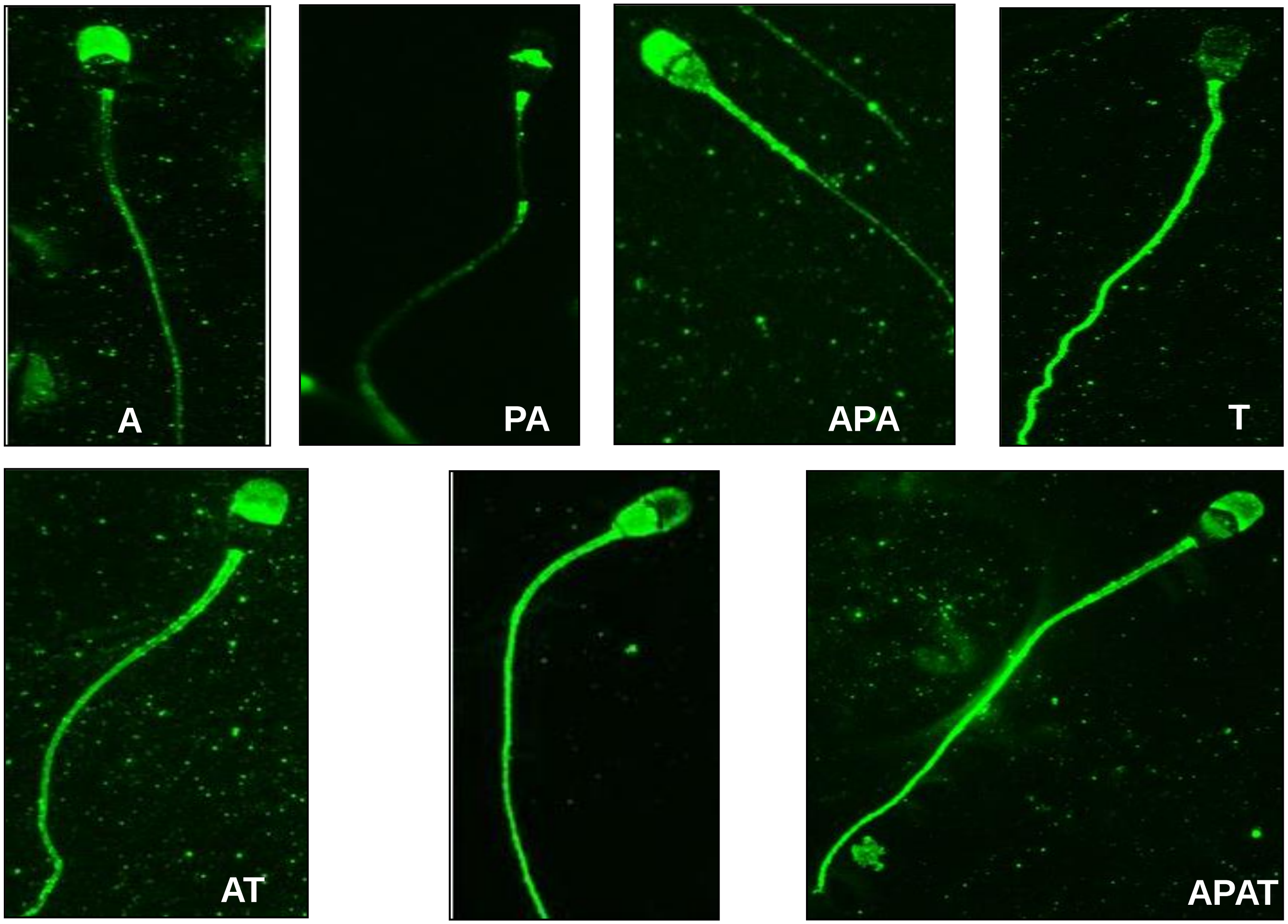


Fig.4.32: Different patterns of protein tyrosine phosphorylation in crossbred bull spermatozoa

(Fluorescence over the acrosome region-pattern A; fluorescence in the post-acrosome and equatorial region-pattern PA; fluorescence at acrosome and post-acrosome region- pattern APA; fluorescence in tail- pattern T; fluorescence in acrosome and tail-pattern AT; fluorescence in post-acrosome and tail-pattern PAT; fluorescence over the acrosome, post-acrosome and tail-Pattern APAT)

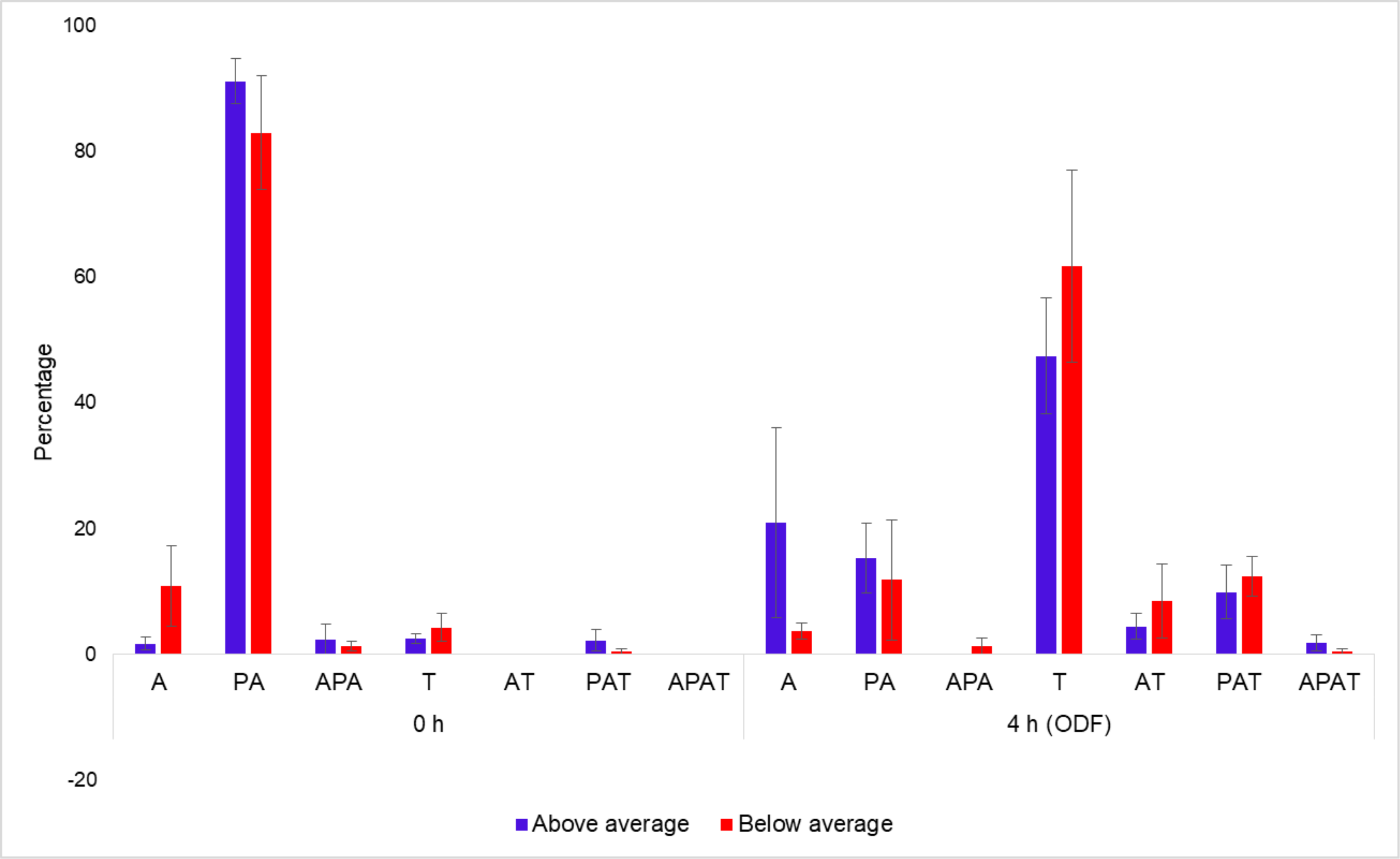


Fig.4.33: Proportion of spermatozoa with different PTP patterns in above- and below-average bulls before and after incubation with NL-ODF

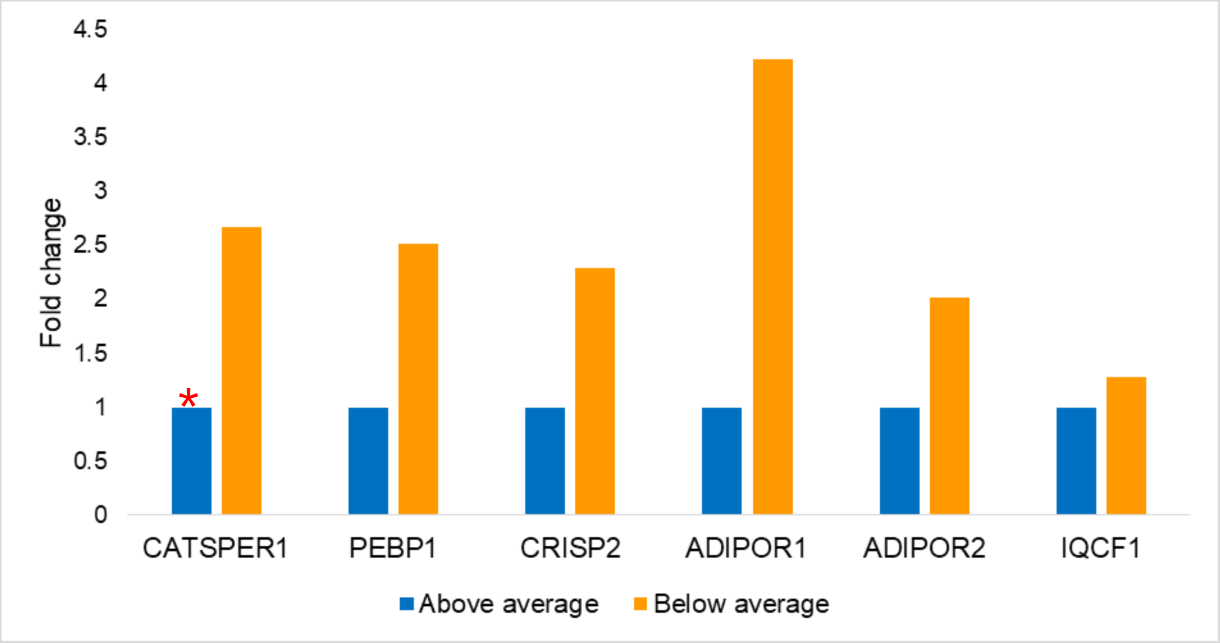
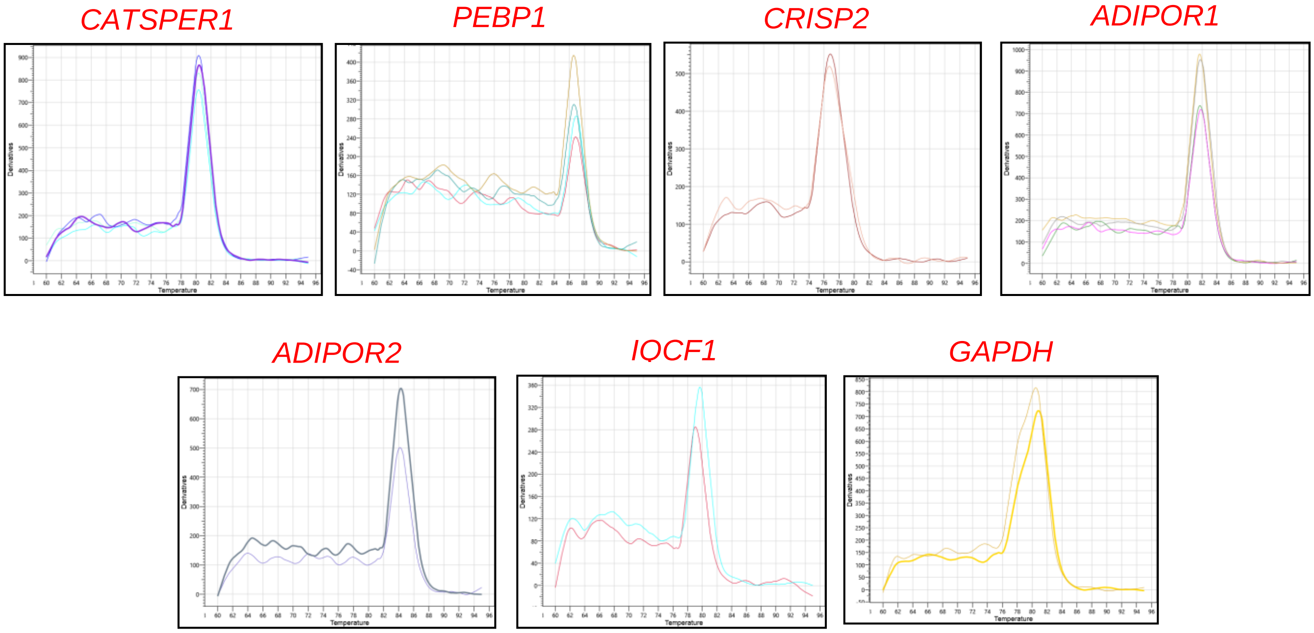


Fig.4.34a: Relative expression of capacitation associated genes in above- and below-average bull spermatozoa. (Star denotes significant difference ($p<0.05$) between above- and below-average bulls


CATSPER1

PEBP1

CRISP2

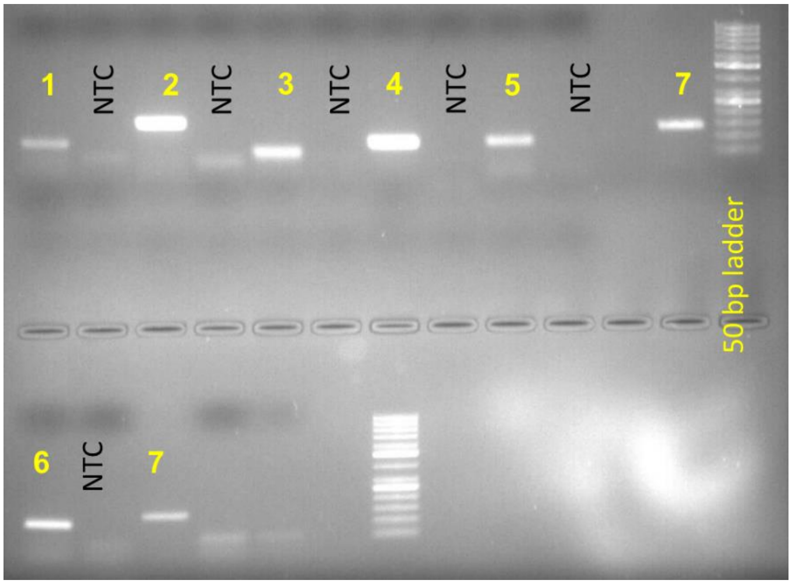
ADIPOR1

ADIPOR2

IQCF1

GAPDH

Fig.4.34b: Melting peaks of capacitation associated genes (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2, IQCF1, GAPDH*) using qPCR



No.	Genes	Base pair
1	*CATSPER1*	100
2	*PEBP1*	178
3	*CRISP2*	68
4	*ADIPOR1*	99
5	*ADIPOR2*	99
6	*IQCF1*	116
7	*GAPDH*	141
NTC	Non template control	

Fig.4.35: Confirmation of qPCR amplified selected genes by agarose gel electrophoresis

The resident time of spermatozoa in the female reproductive tract is the most crucial period during its life span (Naresh and Atreja, 2015). During this period, the spermatozoa undergo a several changes known as capacitation, to acquire the fertilizing ability. Oviduct and its secretions facilitate sperm capacitation before fertilizing oocyte. Sperm capacitation depends upon several molecular biochemical events. Recently, Kwon *et al*. (2015ab) indicated that sperm capacitation associated molecules could serve as a tool for male fertility prediction. Further, it has been reported that the ability of the spermatozoa to undergo capacitation differed among males with different fertility status in boar (Kumaresan *et al*., 2012b) and in bulls (Kumaresan *et al*., 2017). Considering that the ability of a sperm to fertilize an egg is mostly dependent on molecular biochemical characteristics and that the ability of the spermatozoa to undergo capacitation is related to male fertility, we, in this study, assessed the transcriptional abundance of capacitation associated genes in spermatozoa of bulls and related to the ability to undergo capacitation upon exposure to NL-ODF.

Significant differences were observed in sperm phenotypic characteristics, between above average and below average bulls, after incubation with NL-ODF for 4 h.

While there was no significant difference in the proportion of membrane intact spermatozoa immediately after thawing, after 4 h incubation with NL-ODF, the proportion of membrane intact spermatozoa was significantly ($p<0.05$) higher in in above average bulls compared to below average bulls. Similarly, after 4 h incubation with NL-ODF, the proportion of live acrosome intact spermatozoa was significantly higher in above average fertility bulls as compared to below average fertility bulls. Moreover, the extent of decrease from immediately after thawing till 4 h incubation with NL-ODF is the proportion of membrane intact and acrosome intact spermatozoa was higher in below average bulls compared to above average bulls. These findings indicate that the NL-ODF protected spermatozoa from alterations in membrane and acrosome integrity, however such effect was more pronounced in above average bulls compared to below average bulls. Immediately after thawing and after 4 h incubation with NL-ODF, the proportion of viable spermatozoa with high intracellular calcium concentration was significantly higher in above average bulls than below average bulls. However, the percentage decline in the proportion of viable spermatozoa with high intracellular calcium was higher in below average bulls than in above average bulls. On the other hand, there was no significant difference between above average and below average bulls, immediately after thawing. However after 4 h incubation with NL-ODF, spermatozoa from above-average fertility bulls had significantly higher proportion of live tyrosine phosphorylated spermatozoa. These findings indicate that NL-ODF induced capacitation in spermatozoa, but again, the effect was more pronounced in above average bulls compared to below average bulls. Combined together, all these findings indicate the dual role of oviductal fluid i.e maintaining sperm viability, membrane integrity and acrosomal integrity on one hand, and inducing capacitation on the other.

It is not surprising to observe that a significant proportion of spermatozoa had high intracellular Ca^{2+} and tyrosine phosphorylation of proteins. It is well documented that during cryopreservation spermatozoa is prone for cryo-damage leading to reduced selective permeability by influencing cryo-capacitation with huge influx of Ca^{2+} (Bailey *et al*., 2000). Kadirvel *et al*. (2009) reported that cryopreserved semen had significantly ($P<0.01$) higher percentage of spermatozoa with high intracellular Ca^{2+} than that of fresh semen. Increased intracellular Ca^{2+} activates adenylate cyclase to produce cyclic

AMP, which in turn activates protein kinase A to phosphorylate certain proteins (Urner and Sakkas, 2003; Kumaresan *et al*., 2014). Cryopreservation process induced protein tyrosine phosphorylation in spermatozoa has been reported in bull (Bailey *et al*., 2000) and boar (Tardif *et al*., 2001; Kumaresan *et al*., 2012b) spermatozoa. Our results indicate that spermatozoa from different bulls might behave differently during the process of cryopreservation or different bulls might have different cryo-tolerance capacity. Also, we observed that a proportion of viable spermatozoa had low intra-cellular calcium concentration. Cryopreserved spermatozoa having low intra-cellular calcium concentration indicates that among the viable group of spermatozoa, this proportion of spermatozoa was not affected largely due to cryo-damage. Bull to bull variations in different populations of spermatozoa with high- or low-intra cellular calcium might be due to the subpopulations of the spermatozoa with different levels of cryo-susceptibility.

After 4 h incubation with NL-ODF, spermatozoa from above average fertility bulls maintained significantly higher viability and membrane integrity along with high intracellular calcium and tyrosine phosphorylation, which is considered as a hall mark of sperm capacitation. The protective capacity of NL-ODF on sperm motility and viability during incubation with NL-ODF was already reported in cattle (Grippo *et al*., 1995), buffalo (Kumaresan *et al*., 2005), and pigs (Coy *et al*., 2010). Besides membrane integrity, the ability of a sperm population to undergo acrosome reaction can also affect the fertilizing capacity of the bull (Januskauskas *et al*., 2000; Birck *et al*., 2010; Kumaresan *et al*., 2017). We observed that the proportion of live acrosome intact spermatozoa declined greatly, after 4 h incubation with NL-ODF, in below average fertility bulls compared to above average fertility bulls. Our results are to some extent in line with Kumaresan *et al*. (2017; 2019) in which the proportionate increase in the number of live acrosome-reacted spermatozoa incubated at 4 h and after compared with at 0 h of incubation was very large in high fertile bulls compared with lower fertility. The population of dead acrosome-reacted spermatozoa was significantly higher in below average fertility bulls compared to above average fertility bulls after 4 h incubation with NL-ODF. Taft *et al*. (1992) also reported that DAR population of spermatozoa incubated with non-luteal ODF was high in low fertile bulls than high fertile bulls. The findings of the present study also specify that the population of live acrosome reacted (LAR) was high in above-average fertile bulls and dead acrosome reacted (DAR) was high in below-average fertile bulls at 4 h incubation in NL-ODF.

It is reported that intracellular calcium concentration and tyrosine phosphorylation is related to the capacitation status of spermatozoa (Visconti *et al.*, 2002) and it directly related with sperm motility and fertility (Kumaresan *et al*., 2014; Espino *et al*., 2009). Sperm capacitation is related to protein tyrosine phosphorylation characterized by an increase in tyrosine residues of several sperm proteins (Visconti *et al*., 1995, Urner and Sakkas, 2003) and fertilizing potential (Kumaresan *et al*., 2012b). After 4 h incubation with NL-ODF, the proportion of viable spermatozoa displaying tyrosine phosphorylation was significantly ($p<0.05$) higher in above average bulls than below average bulls. Our findings are in line with Kumaresan *et al*. (2019) who observed that EODF induced tyrosine phosphorylation in bull spermatozoa. Kumaresan *et al*. (2012b) reported that boar spermatozoa exposed to porcine EODF significantly ($P<0.001$) increased the population of spermatozoa showing protein tyrosine phosphorylation at 4 h. The findings of the present study indicate that although cryopreservation induced tyrosine phosphorylation was observed in considerable proportion of spermatozoa immediately after thawing (indicating cryo-capacitation), the proportion of live spermatozoa with tyrosine phosphorylation increased after incubation with NL-ODF in above average fertility bulls but not in below average fertility bulls. This study clearly indicates that spermatozoa from bulls with different fertility responded differently on exposure to non-luteal ODF.

Having inferred that spermatozoa from bulls with different fertility ratings differed in their ability to undergo capacitation upon exposure to NL-ODF, we assessed the transcriptional abundance of select capacitation associated genes (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2* and *IQCF1*) to find out differences, if any between the two categories of the bulls. Except for the gene *CATSPER1*, whose transcriptional abundance was significantly ($P<0.05$) higher in below average fertility bulls compared to above average fertility bulls, the expression of all other genes did not vary between the two categories of bulls. Cation Channel Sperm Associated 1

(*CATSPER1*) control the intracellular Ca^{2+} concentration and thereby, sperm motility (Ren *et al*., 2001). It is required for the hyperactivity of sperm, movement towards the egg, capacitation and acrosome reaction (Sun *et al*., 2017) and is essential for male fertility (Jin *et al*., 2005). Even it has been reported that mutations in the *CATSPER1* Channel Protein led to human male infertility (Avenarius *et al*., 2009). Although non-significant, the expression of all other genes was numerically higher in spermatozoa of below average fertility bulls. We could not observe a significant relationship among the transcriptional abundance of studied genes, sperm capacitation status and bull fertility. Earlier reports also indicate diverse findings about the relationship of sperm capacitation associated genes with male fertility. For instance, while few authors report that Phosphatidylethanolamine-binding protein 1 (*PEBP1*) has an inhibitory effect on sperm capacitation (Nixon *et al*., 2006), other researchers reported that *PEBP1* was positively correlated with sperm capacitation and sire conception rate (Arangasamy *et al*., 2011; Kasimanickam *et al*., 2013). D'Amours *et al*. (2010) reported that the protein expression of *PEBP1* was higher in high fertile bulls than in low fertile bulls.

Although, it has been reported that spermatozoa expression of Cystine rich secretary protein 2 (*CRISP2*) was positively correlated with sire conception rate (Arangasamy *et al*., 2011) and decreased *CRISP2* content in sperm was associated with male infertility in human (Zhou *et al*., 2015) and horses (Gottschalk *et al*., 2016) due to compromised ability of sperm to manifest normal motility and acrosomal reaction (Lim *et al*., 2019). We could not observe any difference between above- and below-average fertility bulls. Similarly, IQ Motif Containing F1 (*IQCF1*) is a recently identified acrosomal protein of spermatozoa and spermatids and it is reported to interact with calmodulin and involved in the process of tyrosine phosphorylation of sperm proteins during capacitation (Fang *et al*., 2015). However, we did not observe any significant difference in the transcriptional abundance of this gene between bulls responding significantly to NL-ODF and those responding poorly to NL-ODF in terms of sperm capacitation.

Taken together, it is evident that spermatozoa from above average fertility bulls maintained viability, membrane integrity, acrosomal integrity along with high intracellular Ca^{2+} and protein tyrosine phosphorylation indicating a typical dual role of oviduct on spermatozoa. However, such capacitation response was comparatively lesser in below average fertility bulls. On the other hand, we did not observe significant differences, between the two categories of the bulls, in the transcriptional abundance of selected capacitation associated genes except for *CATSPER1* gene indicating that sperm transcripts associated with capacitation may not directly influence bull fertility. Since spermatozoa are considered as transcriptionally and translationally silent, it may be advantageous to evaluate the protein expression of these genes in spermatozoa in relation to sperm capacitation and bull fertility.

4.2.2 Relationship of bull conception rate with sperm functional attributes and transcriptional abundance of capacitation associated genes

The relationship of conception rate with different sperm functional attributes (viability, intra-cellular calcium concentration, acrosome reaction status and protein tyrosine phosphorylation) after incubation for 4 h with NL-ODF, and sperm transcriptional abundance of capacitation associated selected genes (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2* and *IQCF1*) are shown in **Table 4.15**.

Table 4.15: Relationship among sperm phenotypic characteristics (after incubation for 4 h with NL-ODF), transcriptional abundance of select genes and bull conception rate

Correlation Parameters (4 hour)		R value	p value (Significance)
Conception rate	Live	0.949**	0.004
	Live High Intracellular Calcium	0.963**	0.002
	Live Acrosome Intact	0.976**	0.001
	Total Phosphorylation	0.846*	0.034
	ADIPOR2	-0.830*	0.041
Live High Intracellular Calcium	Live	0.989**	0.000
Live Acrosome Intact	Live	0.994**	0.000

	Live High Intracellular Calcium	0.992^{**}	0.000
Total Phosphorylation	Live	0.836^{*}	0.038
	Live High Intracellular Calcium	0.836^{*}	0.038
	Live Acrosome Intact	0.869^{*}	0.025
Live phosphorylation	Post Thaw Motility	0.918^{**}	0.010
	Total Phosphorylation	0.919^{**}	0.001
APA pattern	PA pattern	0.742^{*}	0.035
CATSPER1	Total Phosphorylation	-0.813^{*}	0.049
PEBP1	Live	-0.886^{*}	0.019
	Live High Intracellular Calcium	-0.899^{*}	0.015
	Live Acrosome Intact	-0.851^{*}	0.031
CRISP2	*PEBP1*	0.878^{*}	0.021
ADIPOR1	Live	-0.824^{*}	0.044
	Live High Intracellular Calcium	-0.835^{*}	0.039
	ADIPOR2	0.922^{**}	0.009
	IQCF1	0.943^{**}	0.005
ADIPOR2	Live	-0.926^{**}	0.008
	Live High Intracellular Calcium	-0.935^{**}	0.006
	Live Acrosome Intact	-0.905^{*}	0.013
	ADIPOR1	0.982^{**}	0.001
	CRISP2	0.870^{*}	0.024
	IQCF1	0.961^{**}	0.002
IQCF1	APA pattern	0.860^{*}	0.028

Conception rate was positively and significantly correlated to sperm viability (p=0.004), live high intracellular calcium (p=0.002), live acrosome intactness (p=0.001) and total phosphorylation (p=0.034) and negatively ($p<0.05$) correlated with transcriptional abundance of *ADIPOR2* gene. Sperm viability was positively and significantly correlated to live high intracellular calcium (p=0.000), live acrosome intactness (p=0.000), total phosphorylation (p=0.038) and, negatively and significantly correlated to *PEBP1* (p=0.019), *ADIPOR1* (p=0.044), *ADIPOR2* (p=0.008).

Similarly, live high intracellular calcium was positively and significantly correlated with viability (p=0.000), live acrosome intactness (p=0.000), total phosphorylation (p=0.038) and negatively correlated with *PEBP1* (p=0.015), *ADIPOR1* (p=0.039), *ADIPOR2* (p=0.006). Live phosphorylation was positively and significantly correlated with post thaw motility (p=0.010). Among the patterns, APA had a positive correlation with PA pattern (p=0.035) and *IQCF1* gene. Transcriptional abundance of *CATSPER1* gene was negatively correlated with total phosphorylation (p=0.049). *CRISP2* gene was positively correlated with *PEBP1* (p=0.021) and *ADIPOR2* (p=0.024). *ADIPOR1* was positively correlated with *ADIPOR2* (p=0.009) and *IQCF1* (p=0.005). Similarly *ADIPOR2* was positively correlated with *ADIPOR1* (p=0.001) and *IQCF1* (p=0.002).

CHAPTER - 5

Summary and Conclusion

5. SUMMARY AND CONCLUSION

Male infertility/ subfertility, especially in crossbred cattle, is a threat for dairy industry leading to reduced conception rates with artificial insemination and associated losses to the farmers. Although male and female contributes equally to the end result of AI, the role of males is assumed big because semen from a single male is used to breed several thousands of females. Thus, ensuring the fertility of bull assumes immense role in improving the reproductive success. In spite of advancements in semen analytical tools, bull fertility prediction remains a dream. A majority of the studies that aimed to predict bull fertility, quantified the expression of few molecules at gene or protein level and tried to associate with fertility. However, such attempts have not always yielded the desired results since the fertility is governed my several molecules and their interaction. In this direction, understanding the global molecular differences between spermatozoa from high- and low-fertile bulls would give an idea about the alterations in low fertile spermatozoa. Further, the spermatozoa acquire the fertilizing ability during their stay at female reproductive tract and it is reported that the ability of spermatozoa to respond to female tract secretions is related to bull fertility. With this backdrop, the present study was undertaken to identify the transcriptomic differences between spermatozoa from high- and low-fertile bulls using high throughput RNA sequencing technique and to identify the subtle differences between spermatozoa from above- and below-average bulls in terms of their ability to undergo capacitation upon exposure to oviductal fluid.

To understand the global transcriptomic profile, cryopreserved spermatozoa from two high- and two low-fertile bulls were subjected to NGS using Illumina NextSeq (500). After detailed bioinformatic analysis, the results were validated by assessing transcriptional abundance of selected genes using qPCR in spermatozoa from bulls with different fertility ratings. On the other hand, spermatozoa from bulls (n=6) with different fertility ratings (CR >50 &<40) were exposed to non-luteal stage oviductal fluid in *in-vitro* conditions for 4 h and sperm functional attributes and transcriptional abundance of selected capacitation associated genes were assessed.

The major findings are summarized below:

- A total of 13,563 transcripts were observed in crossbred bull spermatozoa.
- After total hit normalization, a total of 776 transcripts were detected, of which 524 transcripts were common to both high- and low-fertile bulls, while 84 sperm transcripts were unique to high-fertile bulls and 168 transcripts were unique to low-fertile bulls.
- Among the co-expressed genes, 176 genes were upregulated while 209 genes were down regulated in low-fertile bulls.
- Biological process down-regulated in low fertile bull spermatozoa included multicellular organism development and spermatogenesis.
- Ribosomal pathway was upregulated whereas, oxidative phosphorylation was down-regulated in low fertile bull spermatozoa.
- Sperm transcripts unique to high fertile bulls were involved in oxidative phosphorylation pathway and transcripts unique to low fertile bulls were involved in ribosomal pathway.
- *TPT1, RPL14, PFN1, DDX39B, RPL3; ZNF706, PICK1, LUZP1, ANKRD9, RUNDC3A* were the top 10 differentially expressed transcripts between high- and low- fertile bull spermatozoa.
- *TPT1, PFN1, ZNF706, CRISP2, MDB4, TNP2, ADIPOR1, TNP1* transcripts were validated using qPCR out of which the expression of *ZNF703, CRISP2, MDB4, TNP2, TNP1* genes differed significantly ($p<0.05$) between high- and low-fertile bulls and were positively correlated with conception rate ($p<0.01$).
- Proportion of viable spermatozoa with intact acrosome was significantly ($p<0.05$) higher in high- compared to low-fertile bulls after 4 h incubation with NL-ODF.

- On the other hand, the proportion of viable spermatozoa with protein tyrosine phosphorylation and high intra-cellular calcium concentrations were also significantly ($p<0.05$) higher in above- compared to below-average bulls after 4 h incubation with NL-ODF.

- Transcriptional abundance of all the genes related to sperm capacitation (*CATSPER1, PEBP1, CRISP2, ADIPOR1, ADIPOR2* and *IQCF1*) were upregulated in low fertile bulls.

- Conception rate was positively correlated ($p<0.01$) with sperm viability, live high intra-cellular calcium and live acrosome intact at 4 h incubation with NL-ODF.

Conclusion

- Oxidative phosphorylation pathway is the most affected pathway in low-fertile bull spermatozoa.

- Sperm transcriptional abundance of *ZNF706, CRISP2, MDB4, TNP2* and *TNP1* genes was significantly related to bull fertility and thus these genes could serve as potential candidate markers for fertility in crossbred bulls.

- Ability to undergo capacitation upon exposure to NL-ODF varied with individual bulls and significantly related to bull fertility.

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Language skills **:** Tamil (Writing and speaking)
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Academic credentials:

Examination/ Degree passed	University/ Board	Year of completion	Marks obtained (%) / OGPA	Division/ Class
Ph.D.	ICAR-National Dairy Research Institute, Karnal, Haryana	2020	8.7	First

M.V.Sc.	ICAR-National Dairy Research Institute, Karnal, Haryana	2014	8.5	First
B.V.Sc. & A.H.	Madras Veterinary College, TANUVAS, TN	2011	7.46	First
HSC	Anderson Higher Secondary School, Kanchipuram, TN	2006	7.84	First
SSLC	Anderson Higher Secondary School, Kanchipuram, TN	2004	9.1	Distinction

Experience (Research)

1. M.V.Sc thesis on "Behavioural adaptation and concentrate feeding strategies of crossbred cows In computerized automatic feeding station for optimizing Productivity"

I do hereby declare that the particulars furnished by me above are correct to the best of my knowledge and belief.

Place: Bengaluru

Date: 24th Oct, 2019

(M. Arul Prakash)