Final Report

Identification of RNA transcripts present in chicken sperm and their relation to fertility

SR/FT/LS-147/2011

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1. Title of the project: **Identification of RNA transcripts present in chicken sperm and their relation to fertility (**SR/FT/LS-147/2011).

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4. Date of commencement: 30/07/2012

5. Planned date of completion: 29/07/2015

6. Actual date of completion: 15/08/2014

7. Objectives as stated in the project proposal:

I. **Optimization of the RNA isolation methodology for chicken sperm.**

II. **Molecular analysis of the population of RNA in chicken spermatozoa.**

III. **Expression analysis of fertility related genes in low and high fertility chickens.**

8. Deviation made from original objectives if any, while implementing the project and reasons thereof: Not applicable

9. Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams & photographs

Objective 1: Optimization of the RNA isolation methodology for chicken sperm.

Experiment 1

Sperm standard curve preparation: To estimate the sperm concentration in semen a standard curve was made using the following protocol. A 40-mL volume of pooled semen was mixed and subdivided into 2 mL micro-centrifuge tubes. Semen was centrifuged at 20°C for 20s at 15,000 × g in a centrifuge. Seminal plasma supernatants were pooled and filtered through a 0.2-μm syringe filter. Volumes of concentrated semen were pooled, and sperm cell concentration was determined with a hemacytometer. Next, sperm-free seminal plasma and concentrated semen were mixed to yield 500 μL volumes that contained 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% (vol/vol) concentrated semen. A 2 mL volume of 3% (wt/vol) sodium chloride was added to each of 50 polystyrene cuvettes. Thereafter, a 10 μL volume of semen from each was added to a cuvette (n = 5 replicates per standard). Each cuvette was covered with a small square of Parafilm, contents were mixed by inversion, and absorbance at 550 nm measured with a spectrophotometer.

Standardization of RNA isolation from sperm: Semen from five males were collected and used for RNA isolation. Before RNA isolation, semen samples were subjected to Percoll or PureSperm density gradient centrifugation to remove somatic cells followed by sperm concentration measurement. Variable numbers of sperm were further used for RNA isolation by TRIzol reagent/ RNAzol according to the manufacturer's instructions. The quantity and quality of RNA was measured using NanoDrop. Genomic DNA free RNA samples were converted into cDNA by using 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. Further gDNA and somatic cell contamination was verified by PCR using exon spanning primers of somatic cell marker genes i.e. CD4 and PTPRC.

Standardization of density gradient centrifugation: Different density gradient mediums i.e. Percoll and PureSperm were used to find out a proper gradient medium which can remove somatic cells. A discontinuous gradient was formed by using 40% and 80% Percoll. Then 1 mL semen diluted in PBS (1:1) was overlaid carefully on to the Percoll medium, and centrifuged at 1600 rpm or 2000 rpm for 30 minutes. Similarly, a discontinuous gradient medium of 45% and

90% Percoll were also used at 2000 rpm for 30 min. Two different continuous gradient medium of 40% and 80% PureSperm were also tried at 2000 rpm for 30 min to separate mature sperm from somatic cells and immature diploid spermatocytes. The efficiency of purification was confirmed by light microscopy examinations before and after purification.

RNA isolation by using varying amount of TRIzol: We were unsuccessful in isolating RNA from chicken sperm by using previously published protocols for stallion sperm RNA isolation. Hence, we optimized our protocol by using different amounts of Trizol to isolate pure RNA from sperm. We used different ratios (1:2, 1:3, 1:4 and 1:5) of semen/TRIzol in our trials.

Experiment 2

Optimization of PCR amplification protocol for GC rich protamine gene: Protamine (PRM), a marker of sperm RNA is one of the genes having very high GC content (88% in the coding region) in chicken. Due to high GC content, it was difficult to amplify PRM by PCR. In order to overcome from this limitation, we optimized a PCR amplification protocol for GC rich PRM gene.

Experimental birds: Adult male (35 weeks old) broiler breeders (IC 3) from the same hatch were used for this experiment. The birds were housed in individual cages and were maintained under uniform standard management conditions with 14 h light: 10 h dark.

Sample collection: Four birds were sacrificed and a small piece (approx. 1 cm long) of testis from each bird was collected aseptically and washed in chilled RNase free PBS. Special care was taken to remove blood from the samples. Each collected sample was divided into 2 parts: the first part was used immediately for RNA isolation while the second part was immersed in RNA later and stored at –20°C.

RNA isolation and reverse transcription: Total RNA from individual sample was extracted by RNAzol® RT (Molecular Research Centre, Inc., Cincinnati, USA) according to the manufacturer's instructions. Approximately 25 mg tissue samples were used for RNA isolation. An optional phase separation step by using 4-bromoanisole was performed during total RNA isolation to eliminate gDNA contamination. The concentrations and purities of isolated RNA samples were determined spectrophotometrically at A260 and A280 nm. For all samples, the RNA 260/280nm absorbance ratio was ≥2.0, and concentration was approx. 4500ng/µL.

Further, all the RNA samples were used for PCR with β-actin primer to check DNA contamination. DNA free total RNA samples (1 µg) were reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. The resultant cDNA was stored frozen at –20°C until used.

Protamine amplification by PCR: Exonic region of PRM gene was amplified using a pair of gene specific primer (F: 5′-CGCAGCAGGACCCGCAGCCG-3′ and R: 5′-CGGCGGCGGCGGCTCAGTAG-3′) with available three different Taq DNA Polymerases (Go Taq from Promega, USA; Taq DNA polymerase from Geneaid, Taiwan; and hot start Taq DNA polymerase from Fermentas, Canada). The polymerase chain reaction (PCR) was carried out in a total volume of 25 µL containing 4 µL of 10 times diluted first strand of cDNA, 1X PCR buffer (Geneaid, Taiwan), 1.5 mM $MgCl_2$ (2.0 mM for Geneaid Taq), 2.5 mM of dNTPs, 5 pM of each primer and 0.625 unit of Taq DNA polymerase for Go Taq and Hot Start Taq (one unit for Geneaid Taq) in presence or absence of 8% DMSO. Gradient PCR methodology was used to ascertain the optimum annealing temperature of protamine primer pair for successful amplification of PRM gene. After the initial denaturation at 96°C for 5 min, the PCR profile consisted of a denaturation step at 97°C for 20s, an annealing step from 60.6 to 67.5°C for 30s with an elongation step at 74°C for 20s in total for 34 cycles, followed by a final extension of 5 min at 74°C. The PCR product was run on 1.5 % agarose gel including EtBr in 1X TAE buffer. The gel was visualized under UV light and was photographed using automated gel documentation system. The concentration of buffer, $MgCl_2$, DMSO, template and hot / normal start were optimized for the Go Taq DNA Polymerase.

Experiment 3

Development of a new method for sperm RNA purification in the chicken

Experimental birds, semen collection and evaluation: Twenty healthy adult male broilers (Colored Synthetic Male Line) from the same hatch (about 45 weeks of age), were selected and maintained in individual cages under uniform husbandry conditions in accordance with institutional animal ethics guidelines. They were given a breeder ration and water ad libitum with 14 h light/day. Abdominal massage method (Burrows and Quinn, 1937) was employed for semen collection from birds with an interval of two days. Proper care has taken to minimize faecal and uric acid contamination during semen collection. After collection, semen was stored

on ice until processed further. Semen samples were subjected to centrifugation at 1000 g for 2 min to remove any traces of faeces and uric acid. Semen volume was measured by pipette. Crude semen from individual bird was scored for motility as described by Wheeler and Andrews (1943), and those samples having a score above 3 were selected for RNA isolation. Concentration of spermatozoa was determined spectrophotometrically at 550nm as described by Brillard and McDaniel (1985).

Somatic cell removal by density gradient centrifugation: Pooled semen of four birds was considered as a single sample, and subjected to density gradient centrifugation (DGC) by using two different density gradient mediums, Percoll (Sigma, St Louis, MO, USA) and PureSperm (Nidacon International, Mölndal, Sweden), for the removal of somatic cells and immature diploid spermatocytes. In brief, 400 µL of neat semen was mixed well with 1000 µL of 1X PBS and centrifuged at 1000 g for 5 min at RT to pellet sperm. Pellet obtained was then again suspended with 1000 µL 1X PBS, and sperm concentration was measured and adjusted. An aliquot of 1000 µL (500-800 x 10^6 sperm) from this resultant semen solution was spread over 3 mL prewarmed (37 °C) Percoll (1.5 mL each 45% / 90%) or 40% PureSperm (silanized silica particle solution) and centrifuged at 280 g for 30 min at room temperature. This density gradient centrifugation separated samples into two distinct layers; mature sperm settled at the bottom of tube, whereas somatic cells and immature spermatocytes were observed in the upper layer. The two layers were more prominent in Percoll as compare to PureSperm. The bottom layer was carefully aspirated with the help of Pasteur pipette without disturbing the upper layer, and transferred into 15 mL centrifuge tube and washed twice with 1X PBS by centrifuging at 1000 g for 5 min at room temperature. Finally sperm pellet was suspended in 1 mL 1X PBS, and an aliquot of purified sperm was examined by phase-contrast light microscopy (X 400) to check the presence of somatic cells and immature spermatocytes , whereas sperm concentration in samples after DGC was measured to calculate the loss of sperm and other cells. We observed that 40% PureSperm was better in removing all other cells from semen. The sperm concentration in purified samples was adjusted to approximately 100-500 x 10^6/mL, and used for either RNA isolation or mixed with an equal amount of RNAlater and stored at 4°C for further use. Further, somatic cell RNA contamination in purified RNA samples was examined by reverse transcriptase PCR with intron-spanning primers for CD4 and protein tyrosine

phosphatase receptor type C (PTPRC), classical somatic cell marker genes. The % sperm loss data was transformed to arc sin, and analyzed by t-test.

Total RNA isolation from sperm: RNA isolation procedure was carried out in an area dedicated to RNA work using RNase/DNase free water and RNase free glass- and plastic-ware. Total RNA was isolated from purified sperm samples by TRIzol (Invitrogen) or RNAzol (MRC) isolation medium following the manufacturer's protocol with certain modifications as given below. Additionally, attempts were made to isolate sperm RNA using RNeasy Mini Kit with spin columns (Qiagen, Valencia, CA, USA), following manufacturer's instructions.

TRIzol method: Purified sperm samples containing about 300-500 x 10^6 sperm/mL were centrifuged at 15,000 g for 5 min at room temperature to pellet sperm. TRIzol (800 µL) was added to the sperm pellet, and homogenized (Polytron PT 1600E) for not more than 2 min at 2500 rpm on ice. After homogenization, samples were incubated at 35°C for 15 min in water bath, and then 300 µL chloroform was added into samples and incubated for 5 min in the similar conditions. RNA separated to aqueous phase by spinning at 16700 g for 15 min at 4°C and processed further for RNA isolation following the manufacturer's protocol. Another modification was RNA pellet washing twice with ice-cold 75% ethanol. The RNA pellet was air dried and dissolved in 10 µL nuclease free water, and stored at −20°C until used.

RNAzol method: Purified sperm samples (about 300 x 10^6) were pelleted at 15,000 g for 5 min, resultant pellet was mixed with 1000 µL of RNAzol and homogenized (Polytron PT1600E) for not more than 2 min at 2500 rpm by keeping tubes on ice. After homogenization, 400 µL of nuclease free water was added into the tubes and mixed well, incubated for 15 min at RT, and then centrifuged at 16000 g for 15 min to separate aqueous phase. 1000µL of supernatant was transferred to new tubes in which 5 µL 4-bromoanisole (BAN) was added and kept for 4 min at RT, and then centrifuged at 16000 g for 10 min to precipitate any remaining genomic DNA. 750 µL of supernatant was transferred into new tubes and mixed with equal volume of isopropanol kept at RT for 15 min. After incubation, tubes were centrifuged at 12000g for 10min to precipitate RNA. Isopropanol precipitation was carried out at 22ºC, whereas all previous steps were at 16ºC. RNA pellet was washed twice with ice cold ethanol (75%) by centrifugation at 4ºC. First washing was done with 1000 µL ethanol at 4000 g for 5 min, whereas second washing was with 500 µL ethanol at 4000 g for 3 min. The RNA pellet was air

dried and dissolved in 10 µL nuclease free water, and stored at −20°C until used.

RNA quantity and quality assessment and cDNA synthesis: RNA quality and quantity was measured by using Nanodrop (Thermo Scientific, Wilmington, DE, USA). Additionally, the quality of RNA samples were also evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The genomic DNA contamination in purified RNA samples was examined by reverse transcriptase PCR with intron-spanning primers for CD4 gene. Samples that contained low quality RNA were removed from further use. All genomic DNA free RNA samples were reverse transcribed with random hexamer primers using the 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. A total 500 ng RNA was used for cDNA synthesis. The reverse transcription reaction was carried out in a final volume of 20 µL. The resultant cDNA was stored at −20°C until used.

CD4, PTPRC, PRM and PLCZ1 amplification by PCR: The presence of CD4, PTPRC, PRM and PLCZ1 mRNA in sperm samples were examined by using PCR with gene specific primers (Table 20). The gene specific primers were designed from NCBI reference sequences available on website (http://www.ncbi.nlm.nih.gov/pubmed?db+nucleotide) using Molecular Beacon software. The amplification was carried out in 25 µL volume containing 2.5 µL PCR buffer (10X), 0.5 µL dNTPs mix (10mM each), 0.5 µL each forward and reverse primer (10 pmol/ µL), 0.2 µL Taq polymerase (5U/ µL, Biotools, Spain), 2 µL of cDNA (1:10 diluted) and 18.8 µL nuclease free water. The PCR cycling conditions for CD4, PTPRC and PLCZ1 were: initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s; annealing (Table 20) for 30s and extension at 72°C for 30s.

For each gene of interest, negative and positive controls were included. Testes cDNA was used as template for positive control PCR reaction. Negative controls were samples in which cDNA was not added. PCR products were resolved on 2.5% agarose gels and visualized by staining with ethidium bromide

Objective 2: Molecular analysis of the population of RNA in chicken spermatozoa.

Experiment 4

Semen and testis sample preparation: Semen from ten healthy adult male broilers (IC3) of the same hatch (about 40 weeks of age) was collected by abdominal massage method (Burrows and

Quinn, 1937), and evaluated for motility as described by Wheeler and Andrews (1943). Fist few ejaculate from each male were discarded in order to obtain good quality semen. Semen samples showed a motility score above 3 were used for further processing. Concentration of spermatozoa was determined spectrophotometrically at 550nm as described by Brillard and McDaniel (1985). To eliminate variations between individuals, semen samples from all males were pooled, and subjected for density gradient centrifugation as described by Shafeeque et al. (2014). After semen collection, all ten males were euthanized; testes were removed aseptically and washed with RNase free chilled phosphate buffer saline. Approximately 1 mg of testicular tissue from each male was collected, pooled and mixed with 1 mL of RNAlater and stores at -20°C for further use.

Total RNA isolation and quantity and quality assessment: RNA isolation procedure was carried out in an area dedicated to RNA work using RNase/DNase free water and RNase free glass- and plastic-ware. Total RNA was isolated from purified sperm and testis sample using RNeasy Mini Kit with spin columns and Qiagen Rneasy Lipid tissue kit (Qiagen, Valencia, CA,USA) respectively, following manufacturer's instructions. The concentration and purity of the RNA extracted were evaluated using the Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of the extracted RNA were analysed on the Bioanalyzer (Agilent; 2100, Santa Clara, CA, USA). We considered RNA to be of good quality based on the 260/280 and 260/230 values (Nanodrop), additionally rRNA 28S/18S ratios and RNA integrity number (RIN) were also considered for testis RNA. The genomic DNA contamination in purified RNA samples was examined by reverse transcriptase PCR with intron-spanning primers for CD4 gene. Further, somatic cell RNA contamination in purified RNA samples was examined by reverse transcriptase PCR with primers for CD4 and protein tyrosine phosphatase, receptor type, C (PTPRC) gene.

RNA Labelling, Amplification and Hybridization: The RNA samples were labeled using Agilent Quick Amp Kit (Part number: 5190-0424). 500ng of total RNA was reverse transcribed using oligodT primer tagged to T7 promoter sequence. cDNA thus obtained was converted to double stranded cDNA in the same reaction. Further the cDNA was converted to cRNA by in-vitro transcription reaction by T7 RNA polymerase enzyme in presence of Cy3 dye. During cRNA synthesis Cy3 dye got incorporated into the newly synthesized cRNA strands. cRNA

obtained was cleaned up using Qiagen RNeasy columns (Qiagen, Cat No: 74106). Concentration and amount of dye incorporated were determined using Nanodrop. Specific activities of the labeled samples were determined. The labeling QC passed samples were hybridized. 1650ng of labeled cRNA were fragmented and hybridized on the array (AMADID: 49081) using the Gene Expression Hybridization kit (Part Number 5190-0404; Agilent) in Sure hybridization Chambers (Agilent) at 65º C for 16 hours. Hybridized slide was washed with wash buffer I for 1 minute at room temperature followed by wash buffer II for 1 minute at 37°C using Agilent Gene Expression wash buffers (Part No: 5188-5327). The hybridized, washed microarray slides were then scanned on a G2600D scanner (Agilent Technologies).

Validation of microarray results: RNA samples were reverse transcribed with random hexamer primers using the 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. A total 500 ng RNA was used for cDNA synthesis. The reverse transcription reaction was carried out in a final volume of 20 µL. The resultant cDNA was stored at −20°C until used. The presence of testis and sperm specific genes (PRM and PLCZ1) and two from each most and least abundant genes were examined by using PCR with gene specific primers (Table 20). The amplification was carried out in 25 µL volume containing 2.5 µL PCR buffer (10X), 0.5 µL dNTPs mix (10mM each), 0.5 µL each forward and reverse primer (10 pmol/ µL), 0.2 µL Taq polymerase (5U/ µL, Biotools, Spain), 2 µL of cDNA (1:10 diluted) and 18.8 µL nuclease free water. The PCR cycling conditions for all genes except PRM were: initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s; annealing (Table 20) for 30s and extension at 72°C for 30s. The PCR cycling conditions for PRM was similar as described earlier (Sharma, et al., 2014). Negative controls were samples in which cDNA was not added. PCR products were resolved on 2.5% agarose gels and visualized by staining with ethidium bromide.

Semi-quantitative RT-PCR was chosen to estimate the transcripts amount in testis and sperm according to Singh et al. (2011). To control the variation in the efficiencies of the RT step among different experimental samples, mRNA concentrations of GAPDH, a housekeeping gene, presumed to be expressed at constant amounts in chicken testis and sperm were also calculated, along with mRNA concentrations of targeted genes, by densitometry analysis using Image J software (NIH). Relative expression was determined as arbitrary units, defined as the

ratio of mRNA level to the corresponding β-actin mRNA level after subtraction of background intensity (value = (intensity; gene of interest - intensity; background)/(intensity; β-actin-intensity; background). Mean values of three measurements for testis and sperm bands were taken for analysis.

Microarray data analysis: Images were quantified using Feature Extraction Software (Version-11.5, Agilent). Feature extracted raw data was analyzed using GeneSpring GX software from Agilent. Normalization of the data was done in GeneSpring GX using the 50^{th} percentile shift (Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently, and computes the nth percentile of the expression values for this array, across all spots (where n has a range from 0-100 and n=50 is the median). It subtracts this value from the expression value of each entity) and normalized to Specific control Samples. Significant genes up and down regulated showing one fold (log base2) and above within the samples with respect to control sample were identified. Differential expression patterns were identified among the samples. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns. Genes were classified based on Gene ontology functional classification.

Objective 3: Expression analysis of fertility related genes in low and high fertility chickens.

Experiment 5

Experimental birds, semen collection and evaluation: Fifty healthy adult male broilers (IC3) from the same hatch (about 45 weeks of age), were selected and maintained in individual cages under uniform husbandry conditions in accordance with institutional animal ethics guidelines. They were given a breeder ration and water ad libitum with 14 h light/day. Abdominal massage method (Burrows and Quinn, 1937) was employed for semen collection from birds with an interval of two days. Proper care has taken to minimize faecal and uric acid contamination during semen collection. After collection, semen was stored on ice until processed further. Semen samples were subjected to centrifugation at 1000 g for 2 min to remove any traces of faeces and uric acid. Semen volume was measured by pipette. Crude semen from individual bird was scored for motility as described by Wheeler and Andrews (1943). Concentration of

spermatozoa was determined spectrophotometrically at 550nm as described by Brillard and McDaniel (1985).

Fertility was evaluated by artificial insemination (AI) of semen into females. After AI, eggs were collected at regular intervals and were incubated in the hatchery of the institute. After 7 day of incubation, eggs were break open to evaluate fertility. On the basis of physical parameters of semen and fertility, birds were allocated into two groups (low and high fertility).

Gene expression studies using Real-time PCR: Semen from low and high fertility birds were subjected for RNA isolation following our new developed method (Shafeeque et al., 2014a). RNA quality and quantity was measured by using Nanodrop (Thermo Scientific, Wilmington, DE, USA). The genomic DNA contamination in purified RNA samples was examined by reverse transcriptase PCR with intron-spanning primers for CD4 gene. All genomic DNA free RNA samples were reverse transcribed with random hexamer primers using the 'RevertAid First strand cDNA synthesis kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. A total 500 ng RNA was used for cDNA synthesis. The reverse transcription reaction was carried out in a final volume of 20 µL. The resultant cDNA was stored at −20°C until used. The expression of PRM and PLCZ1 genes were quantified in low and high fertility birds using SYBR green chemistry in qRT-PCR. PRM and PLCZ1 were amplified following protocol developed by Sharma et al. (2014) and Shafeeque et al. (2014b. Results were expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye (DRn) passes the significance threshold. The relative mRNA expression level of each gene was normalized against GAPDH mRNA levels. The fold expression of GOI was calculated according to Pfaffl et al. (2002).

Experiment 6

Experimental birds and semen sample preparation: Eighty healthy adult (aged 30 wk) broilers males from same hatch were used for this study. These birds were maintained under uniform husbandry conditions at 15 h light/ d with standard breeder ration and water ad libitum. Semen was collected by manual massage method from twenty males, pooled and considered as single sample. Six such samples were used for each functional analysis. Semen samples were centrifuged at 1000 g for 2 min to remove uric acid crystals and debris. Spermatozoa concentration was determined from a previously established chicken spermatozoa standard

curve and an absorbance read at 580 nm using a UV/Visible Spectrophotometer (ECL, India). Semen samples were then diluted with CARI semen extender to give a final concentration of about 500×10^6 spermatozoa/mL. A total of 17 aliquots were prepared from each pooled semen sample for incubation and evaluation. Of these 17 aliquots, 11 were used for incubation in Bisphenol-A (BPA) or dimethyl sulfoxide (DMSO) and remaining six were used for artificial insemination (AI). One aliquot incubated in BPA or DMSO was used for RNA isolation. For the fertility study, 100 healthy hens from the same hatch were also taken at random and maintained in a similar manner.

Incubation of spermatozoa in BPA: A stock solution of 100 mM BPA (≥99%, Sigma-Aldrich, USA) was prepared in DMSO, and further diluted to obtain 50, 25 and 12.5 mM solutions for treatment. Aliquots of 500×10^6 sperm/mL prepared in 1 mL CARI semen extender were incubated with 0.18, 0.37 and 0.74 mM BPA concentrations (15µL stock of each 50, 25 and 12.5 mm BPA) for 30 min at 4°C. Similarly, aliquots incubated with or without DMSO (15µL) served as vehicle control and negative control, respectively. Samples were checked for sperm motility as described by Wheeler and Andrews (1943) after 10, 20 and 30 min incubation. Following incubation, the supernatant was removed by centrifugation at 3500 g for 2 min and the sperm pellet was re-suspended in 1 mL of CARI extender or normal saline (0.9% w/v) and used for flow cytometry and AI. For flow cytometry, sperm number was re-adjusted to 50×10^6 sperm/mL after incubation in BPA or DMSO for staining and analysis.

Artificial insemination (AI): Pre-incubated semen samples were used for AI in females to evaluate fertility. Semen from each treatment was inseminated into 20 females @ 100 million sperm/bird at 15.00 h once-weekly followed by egg collection from 2 to 7 d post AI. Fertility was assessed by incubating the eggs (99.5°F temperature and 55-60% relative humidity) in an incubator. The eggs were examined after 9 d of incubation by candling to determine fertilization. The unfertilized eggs were broke open to examine any early embryonic death. The percent fertility was determined by the ratio of numbers of fertile eggs to the number of total egg set in the incubator.

Evaluation of mitochondrial function and plasma membrane integrity by flow cytometry: The lipophilic cationic 5,5',6,6'-tetrachloro-1,1',3,3' tetramethyl benzimidazolyl carbocyanine iodine (JC-1, Molecular Probes Europe) has the unique ability to label differentially

mitochondria with low and high membrane potential. In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. However, at the same excitement wavelength (488 nm) in mitochondria with low membrane potential, the JC-1 forms monomers that emit in the green wavelength (525–530 nm).

For staining, a 1.53 mM stock solution of JC-1 was prepared in DMSO. Pre-incubated aliquots of 50×10^6 sperm/mL in BPA or DMSO solutions were incubated again with 1µL of JC-1 stock solution at 37°C in darkness for 30 min before flow-cytometric analysis. In order to confirm that the JC-1 response is sensitive to changes in mitochondrial membrane potential, sperm aliquots incubated in 100µM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 10 min at 37°C were used before adding JC-1. CCCP is an inhibitor which is known to compromise mitochondrial membrane potential. In this manner, virtually all the sperm will emit green light and will be detected by flow cytometry.

Sperm membrane integrity was assessed with dual fluorescence probes, SYBR-14 and propidium iodide (PI) by Live/Dead Sperm Viability Kit (InvitrogenTM, Eugene, OR, USA). Briefly, pre-incubated aliquots of 50×10^6 sperm/mL in BPA or DMSO solutions were again incubated with 1.0 µL working SYBR-14 solution (10 fold diluted in DMSO) at 37°C for 10 min and then sperm were counterstained with 5 µL PI for 5 min before flow-cytometric analysis. A positive (sperm stained with only SYBR-14) and negative (heat killed sperm stained with only PI) control was used to validate the staining and accuracy of flow-cytometer.

Flow cytometry: Measurements were done on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer. All the fluorescent probes were excited by an Argon ion 488 nm laser. The green fluorescence of SYBR-14 and JC-1 (monomers) was detected on detector FL1, whereas red/orange fluorescence of PI and JC-1 (aggregates) was detected on detector FL3 and FL2, respectively. In order to determine the forward (FS) and side scatter (SS) of the population of chicken spermatozoa to be analysed for plasma membrane integrity, the non-sperm events were gated out based on scatter properties of unstained sperm and were not included in the analysis. Further, the accuracy of gating/cytometer was verified using two different controls: 1) heat killed sperm stained with only PI detected on FL3, and 2) sperm stained with only SYBR-14 detected on FL1 (Figure 28). Similarly, a negative control in which sperm were incubated in

CCCP before JC-1 staining was used to backgate the sperm event to quantify mitochondrial membrane potential in samples. Two replicates from each sample were analyzed and data were gathered as the logs of green and red fluorescence on 50,000 spermatozoa. Dot plots were generated and analyzed with the CellQuest 3.3 software (Becton Dickinson, San Jose, CA, USA). Each gated population appearing on the dot plots was quantified as a percentage of the total number of gated spermatozoa thus deleting doublets and non-sperm particles from the analyses. Dot plot populations for plasma membrane integrity included dead red-stained spermatozoa (P1-stained), viable green-stained spermatozoa (SYBR-14 stained), and those intermediate (moribund) spermatozoa that stained both red and green (Figure 28A, B & C). Dot plot populations for mitochondrial membrane potential included high mitochondrial membrane potential sperm (orange), low mitochondrial membrane potential sperm (green) and those intermediate (moribund) spermatozoa that stained both red and green (Figure 28 D & E). The detailed instrument setting parameters are given in supplementary file S1.

RNA isolation from sperm: Pre-incubated aliquots of 500×10^6 sperm/mL in 0.74 mM BPA or DMSO solutions were subjected for density gradient centrifugation, and then used for RNA isolation by RNAzol as described previously. The purified RNA was of poor quality and substantially less quantity, so could not be processed further for gene expression analysis.

Data analysis: All percentage data were transformed to arc sin prior to analysis. Data were analysed using statistical software package SPSS-16 for ANOVA and Duncan's multiple range tests by comparing means for significant differences at 5% level ($P \leq 0.05$). The relationships among the parameters were quantified according to the Spearman's correlation test at 5% and 1% significance level.

10. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject

Experiment 1

Sperm standard curve for sperm concentration measurement: We generated a standard curve from which sperm concentration in an ejaculate can be predicted. As shown in Figure 1, a linear relationship (R2= 0.984) was observed between sperm cell concentration and absorbance.

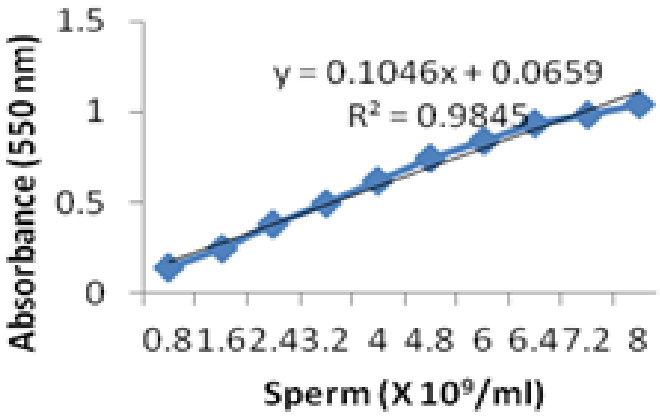


Figure 1: Sperm concentration graph.

Somatic cell removal from semen: In the present study, sperm purification with a one-layer 40% PureSperm gradient at 2000 rpm for 30 min was found to be optimal (Table 1, Fig. 2). On average, gradient purification resulted in 30–50% reduction of the initial sperm count, probably due to the loss of sperm which accumulated in the intermediate layer.

Table 1: Sperm purification by using different density gradient medium.

Density gradient Medium	Centrifugation speed (rpm) and time (min)	Observations	Somatic cells
80%+40% Percoll	1600 rpm for 30 min	No pellet	Present
80%+40% Percoll	2000 rpm for 30 min	Pelleted	Present
90%+45% Percoll	2000 rpm for 30 min	Pelleted	Very few
40% PureSperm	2000 rpm for 30 min	Pelleted	Rare
80% PureSperm	2000 rpm for 30 min	No proper pellet	Very few

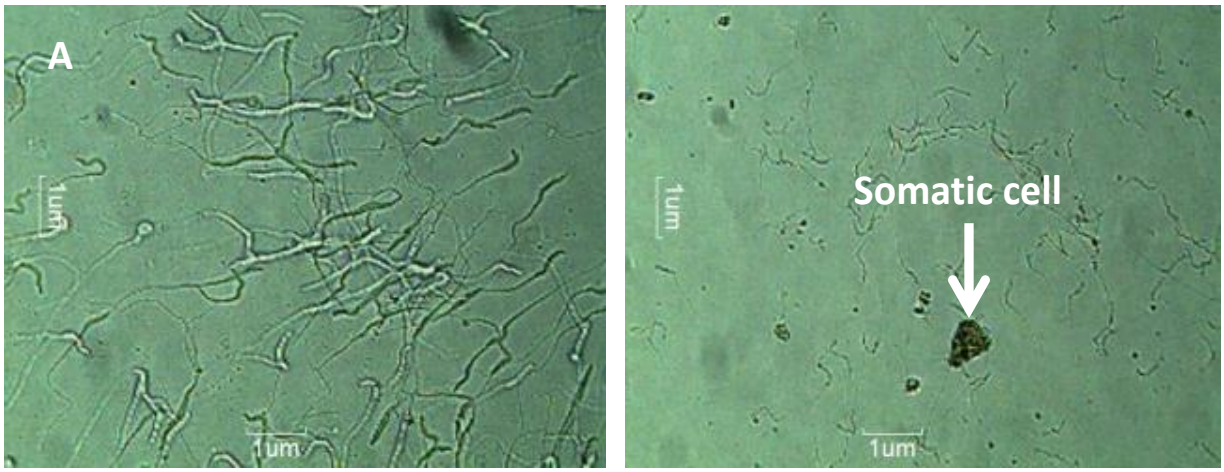


Figure 2: A) Enriched sperm after density gradient centrifugation B) Presence of somatic cell debris in semen.

RNA isolation by using TRIzol from sperm purified by Percoll and without purified: The RNA isolation was best achieved by using 1:2 semen/TRIzol without purified sperm. Further we have verified these results in 30 samples, which showed good quality and quantity. Other combinations did not yield good quality of RNA (Table 2). Therefore, we used 1:2 semen/Trizol for all further isolations.

Table 2: RNA isolation from sperm (without purified) by using different TRIzol amount.

Sample No.	Semen: TRIzol	260/280 OD	260/230 OD	RNA quantity (ng/µl)
1	1:2	1.74	1.88	544.3
2	1:3	1.57	1.16	56.2
3	1:4	1.50	1.17	59.4
4	1:5	1.55	0.31	537.4

When Percoll purified sperm were used for RNA isolation, the quality and quantity of RNA compromised (Table 3). This may probably due to very less quantity of RNA present in chicken sperm. Increasing number of spermatozoa also did not improve RNA quality and quantity probably because of high glycoprotein contamination. Therefore, we tried other strategies which are mentioned elsewhere.

Table 3: RNA isolation from sperm (purified by 45% and 90% Percoll) by using 1:2 semen/TRIzol.

Sample No.	Sperm count (10^6/ml)	260/280 OD	260/230 OD	RNA quantity (ng/µl)
1	276	1.22	0.49	20
2	424	1.24	0.56	33.3
3	800	1.52	0.24	8.8
4	980	1.75	1.27	40.6
5	980	1.80	1.32	39.8
6	1016	1.74	0.61	19.8
7	1016	1.38	0.34	43.1
8	1932	1.60	1.29	77.7
9	1932	1.55	1.25	78.3
10	2780	1.37	1.24	80

RNA isolation by using TRIzol from sperm purified by PureSperm: The results of this experiment showed very good OD values, which is a primary measure of RNA quality. However, DNA contamination was observed in these samples.

Table 4: RNA isolation from sperm (purified by 40% PureSperm) by using 1:2 semen/TRIzol.

Sample No.	Sperm count (10^6/ml)	260/280 OD	260/230 OD	RNA quantity (ng/µl)
1	1500	1.74	2.18	1061.5
2	1000	1.74	2.25	716.6
3	800	1.73	2.18	662.7
4	600	1.70	2.22	1467.0
5	400	1.76	2.32	702.5
6	<400	1.78	2.14	633.0

DNA and somatic cell contamination in RNA samples: The PCR results showed that isolated RNA by TRIzol was highly contaminated with gDNA. Somatic cell contamination was also observed in semen even after Percoll density gradient centrifugation. We tried DNA digestion by DNAse enzyme, which resulted into RNA degradation, also because of very less quantity. However, somatic cells contamination was not detected by PCR in samples that were purified by PureSperm. We also tried another reagent called 'RNAzol RT', which is known for gDNA free RNA isolation. We also tried a different method to remove somatic cells from the semen by somatic lysis.

RNA isolation by using RNAzol RT: The 260/280 OD of RNA samples isolated using RNAzol has yielded well, The quantity of RNA was also comparatively higher than TRIzol. However, 260/230 OD is still a point that require further work (Table 5). Most important, of these 4 RNA samples, 3 were free from gDNA as evident by PCR. All the samples were free from somatic cell RNA as confirmed by somatic cell marker gene PTPRC (Figure 3).

Table 5: RNA isolation from sperm (somatic cell lysis) by using RNAzol.

Sample No.	Sperm count (10^6/ml)	260/280 OD	260/230 OD	RNA quantity (ng/µl)
1	1300	1.70	1.23	274.3
2	1100	1.57	0.77	155.0
3	1100	1.52	0.73	98.2
4	1100	1.54	0.64	116.5

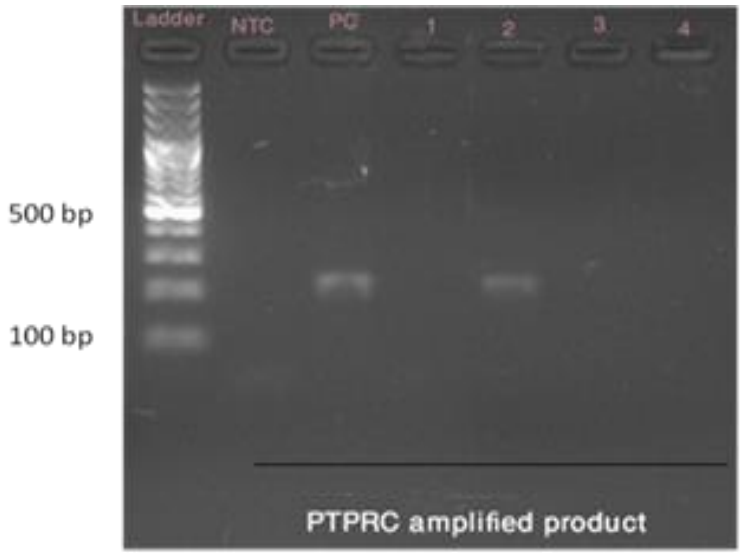


Figure 3: Amplification of PTPRC gene in cDNA of 4 different RNA samples.

Experiment 2

We have amplified PRM amplicon successfully at all the given temperatures (60.6-67.5) using 1.5 mM $MgCl_2$ and Go Taq DNA polymerase only in presence of 8% DMSO (Fig. 4.1). However, it could not be amplified productively at any given temperature by using hot-start Taq DNA Polymerase from ferments (Fig. 4.2) or Taq from Geneaid (Fig. 4.3) in presence /absence of DMSO. To find out optimal buffer concentration, separate PCR reactions were setup with addition of 1X, 0.8X, 0.6X, 0.4X, 0.2X buffer and without buffer. Results indicated that 0.8X buffer (Fig. 5) concentration was optimum for good amplification. Further, concentration of $MgCl_2$ was optimized in separate PCR reactions with addition of 1.0mM, 1.5mM, 1.8mM, 2.2mM, 2.6mM and 3.0mM $MgCl_2$ and good amplification was achieved at 1.5mM $MgCl_2$ (Fig. 6). Additionally DMSO concentration was optimized at optimum buffer and $MgCl_2$ concentration in a separate PCR reaction with addition of 4.0%, 6%, 8%, 10%, 12% and 14% DMSO. Final concentration of 10% DMSO was the only one to provide the desired amplicon yield without nonspecific amplification (Fig. 7). Furthermore, concentration of template (1-6 µL of 10 time's diluted cDNA) was also optimized in a new PCR reaction with optimum concentrations of buffer, $MgCl_2$ and DMSO. The optimum template concentration was 5 µL (Fig. 8) to obtain best PCR efficiency. Finally, effect of hot start and normal start PCR was observed by two separate reactions, one kept at cold PCR block while running the PCR and another kept directly at Hot (96°C) block while running the PCR. Results indicated that product amplified using Hotplate has very less primer dimer (Fig. 9).

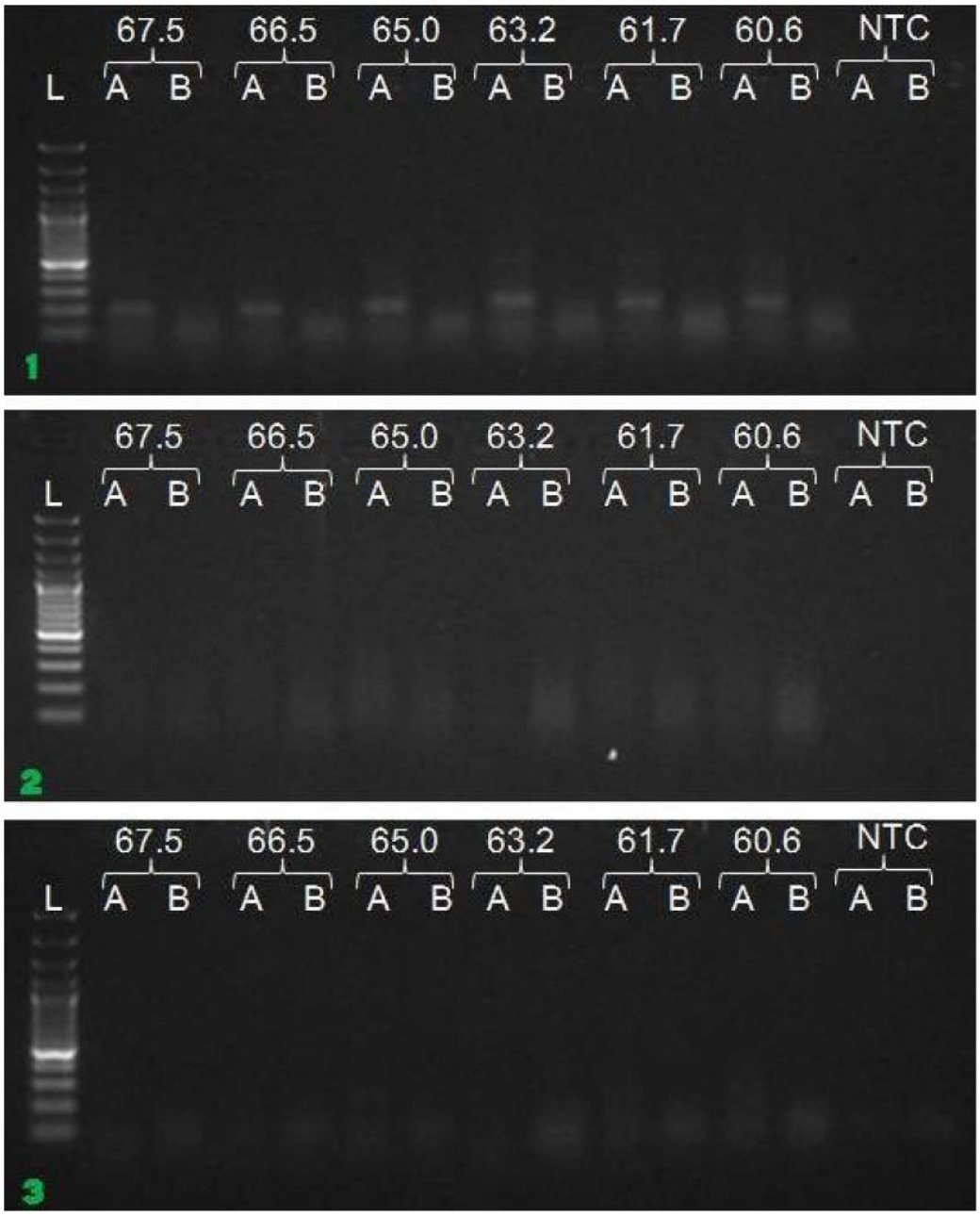


Figure 4: Amplification of PRM with Go Taq (4.1), Hot start Taq (4.2) and gene aid (4.3) (L= 100 bp DNA Ladder, A=With DMSO, B= Without DMSO).

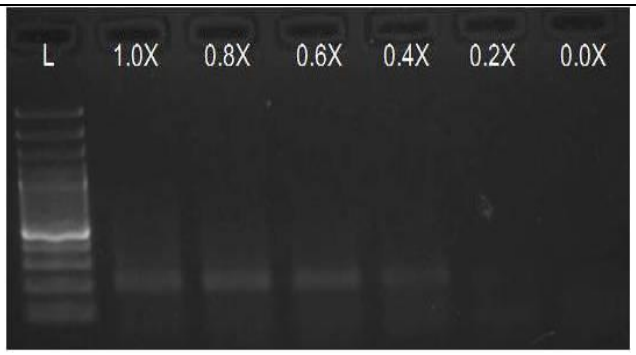


Figure 5: Amplification of PRM with Go Taq using different buffer concentrations (L= 100 bp DNA Ladder)

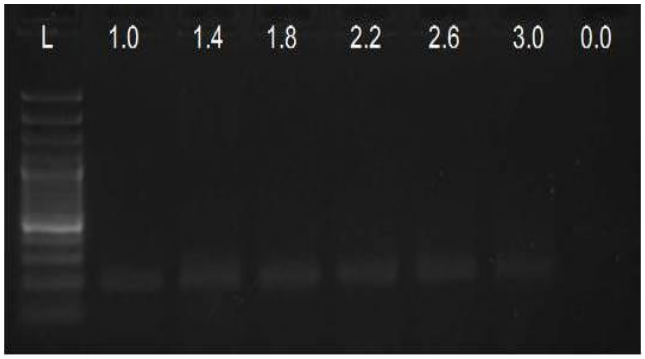


Figure 6: Amplification of PRM with Go Taq using different MgCl2 concentrations (L= 100 bp DNA Ladder)

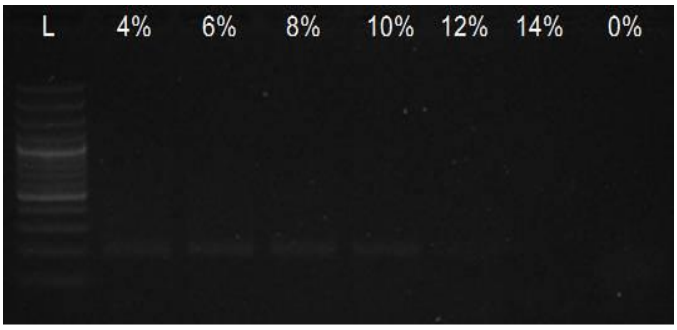


Figure 7: Amplification of PRM with Go Taq using different DMSO concentrations (L= 100 bp DNA Ladder)

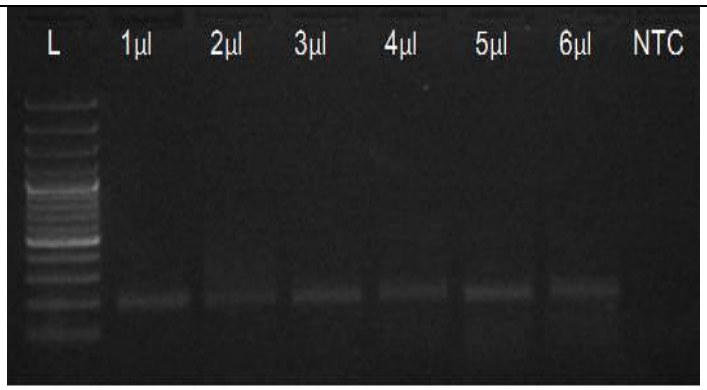


Figure 8: Amplification of PRM with Go Taq using different Template concentrations (L= 100 bp DNA Ladder)

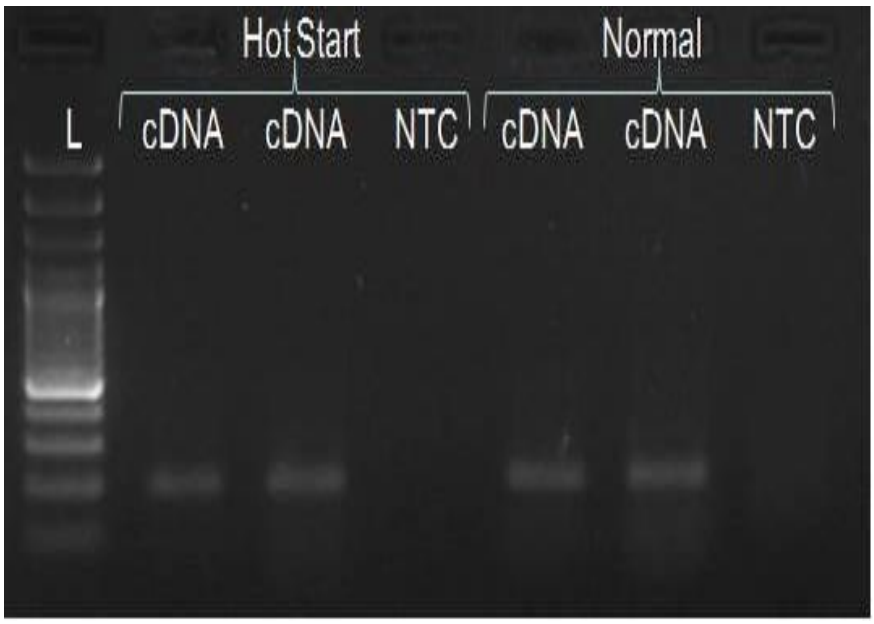


Figure 9: Amplification of PRM with Go Taq using Hot start and normal block (L= 100 bp DNA Ladder)

Experiment 3

Somatic cell removal from semen: Two density gradient mediums, Percoll and PureSperm were used to enrich normal sperm for RNA isolation. This density gradient centrifugation separated samples into two distinct layers; mature sperm settled at the bottom of tube, whereas somatic cells and immature spermatocytes were observed in the upper layer. The two layers were more prominent in Percoll as compare to PureSperm. Sperm enrichment with a one-layer 40% PureSperm density gradient medium was found to be optimal in removing somatic cells, bacteria, immature round spermatids, and diploid spermatocytes from semen samples as compared to two-layer 45% / 90% Percoll density gradient medium. Sperm loss of the initial sperm count during density gradient centrifugation was about 50% in both Percoll and PureSperm (Table 6).The light microscopy assessments before and after purification confirmed the absence of other cells in the purified samples (Fig. 10). The purification efficiency was observed higher in 40% PureSperm compared to 45% / 90% Percoll, which was further substantiated by PCR results.

Table 6: Sperm loss during density gradient centrifugation (DGC) with Percoll and PureSperm.

DGC medium	Sperm concentration before DGC (10^6)	Sperm concentration after DGC (10^6)	% sperm loss
Percoll (n=11)	2462 ± 599	1448 ± 413	50.15 ± 6.0
PureSperm (n=13)	2710 ± 452	1244 ± 252	50.26 ± 2.16

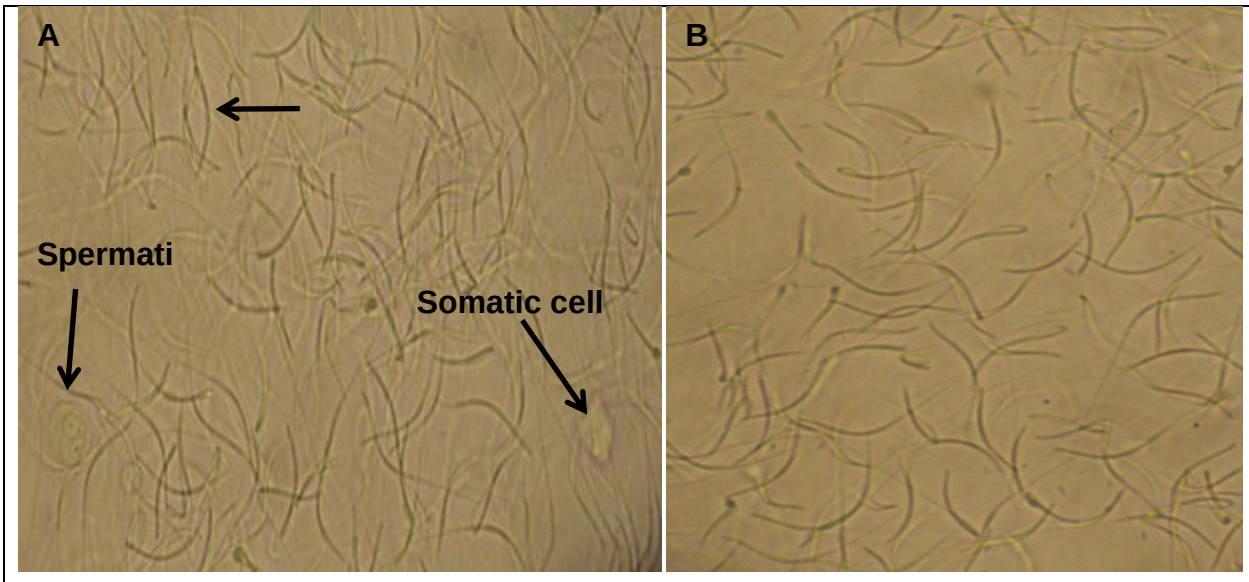


Figure 10: Photograph showing sperm purification after one-layer density gradient centrifugation with 40% PureSperm. (A) Before purification (x400); (B) After purification (x400).

RNA isolation and quality assessment: RNA from chicken sperm was isolated successfully by using all three methods (TRIzol, RNAzol and RNeasy Micro Kit with spin columns). The best results were obtained by using RNAzol or RNeasy Mini Kit. RNA isolated by TRIzol or RNeasy Mini Kit was always found contaminated with genomic DNA, thus DNAse treatment is mandatory. RNA isolated by RNAzol was devoid of any genomic DNA contamination, thus DNAse treatment was not required. The overall RNA concentration was observed higher (293 ng/µL) when isolated by using RNeasy Mini Kit as compared to RNAzol and TRIzol (Table 7).

The absorbance ratios at A260/ A280 for RNA samples isolated by RNAzol and RNeasy kit were between 1.24 –2.05 (Table 7). The absorbance ratios represented that isolated RNA samples were free from proteins and organic compounds. The Bioanalyzer profile of testes RNA showed two distinct peaks of 18S and 28S rRNA respectively (Fig. 11), at the same place as indicated in reference with a RNA integrity number (RIN) of 7.9. In contrast, sperm RNA bioanalyzer profile exhibited only a single peak at 40-50 s intervals, indicating the principle difference between testes and sperm RNA (Fig. 11). The absence of 18S and 28S rRNA peaks in sperm RNA indicated that the RNA was originated from sperm and was uncontaminated with somatic cell RNA.

Table 7: RNA yield and other parameters of sperm RNA samples

RNA isolation method	Sperm used for RNA isolation ($x10^6$)	Absorbance at 260/280	RNA concentration (ng/µL)	DNA contamination
RNAzol (n=23)	300	1.62-2.05 Mean=1.76	30-337 Mean=146	No
TRIzol (n=12)	300	1.24-1.69 Mean=1.53	34-392 Mean=106	Yes
RNeasy Micro Kit (n=4)	100	1.83-1.89 Mean=1.85	136-556 Mean=293	Yes

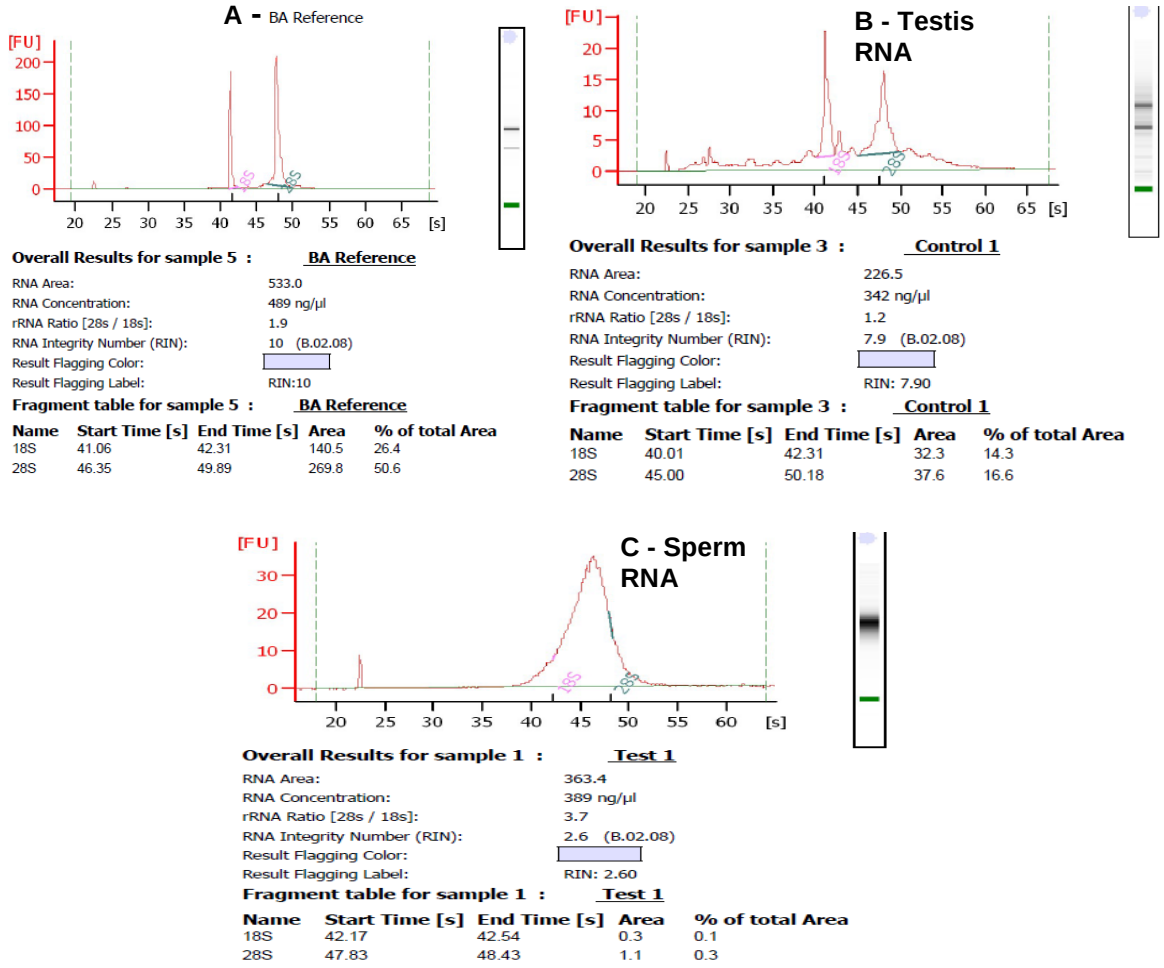


Figure 11: Representative image showing microarray electropherogram of chicken RNA sample isolated by RNAzol. (A) 18s and 28s rRNA reference; (B) Total testis RNA; (C) Total sperm RNA isolated by RNAzol.

PCR amplification of PRM and PLCZ1: All the RNA samples were used for PCR with CD4, PTPRC, PRM and PLCZ1 gene specific primers. CD4 and PTPRC genes were used to check somatic cell RNA contamination in isolated sperm RNA samples. All the sperm RNA samples were found negative for CD4 and PTPRC genes, indicating that sperm RNA samples were free from any somatic cell RNA, whereas testes RNA samples were positive for CD4 and PTPRC genes (Fig. 12 a & b). The genomic DNA contamination in RNA samples was checked by an intron spanning primer of CD4 gene, which exhibited no amplification in sperm samples. These results showed that all the sperm RNA preparations were free from genomic DNA. Further, PRM and PLCZ1 genes are known as positive marker for both sperm and testis. All sperm and testes RNA samples were found positive for PRM and PLCZ1 transcripts, indicated that chicken sperm do have PRM and PLCZ1 mRNA (Fig. 12 c & d).

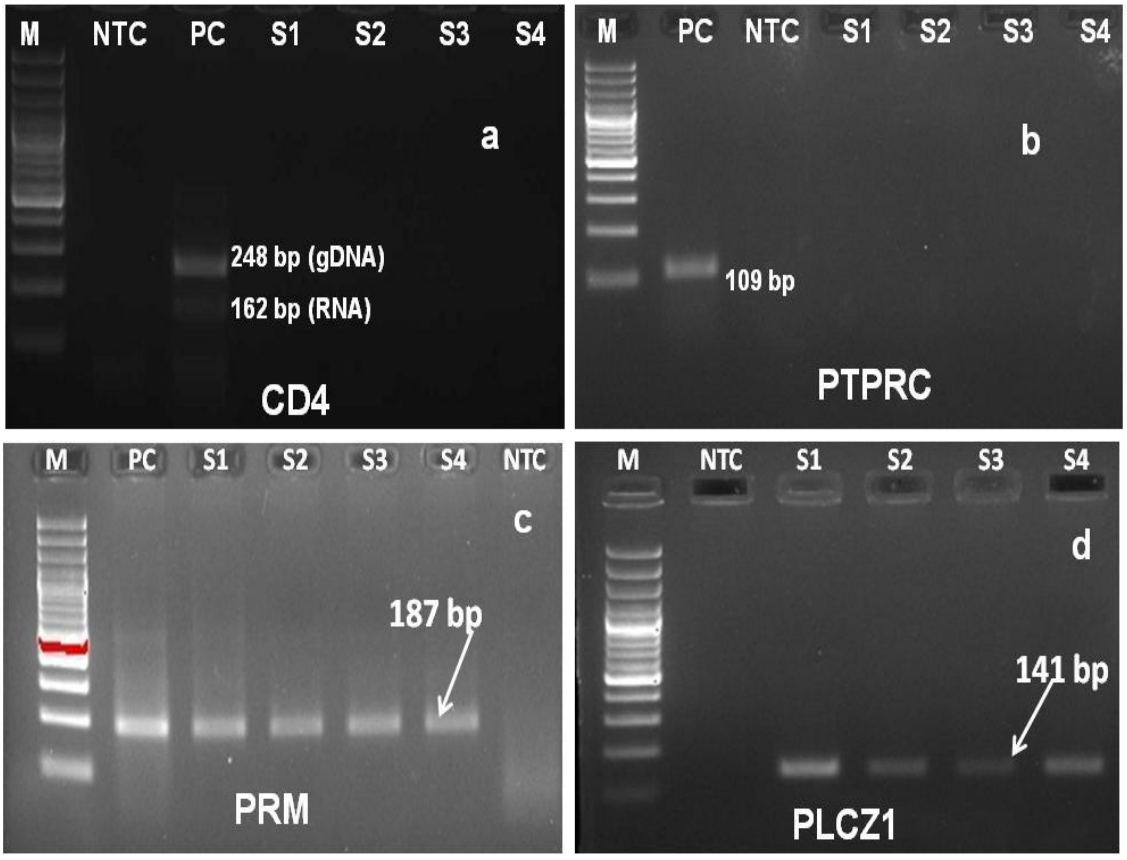


Figure 12: Agarose gel image showing PCR results in sperm RNA samples. (a) Amplification with CD4; (b) Amplification with PTPRC; (c) Amplification with PRM; and (d) Amplification with PLCZ1. M-100bp marker; NTC-non template control; PC- testis RNA sample; S1-S4-four representative sperm RNA samples isolated by RNAzol.

Experiment 4

Sperm RNA quality: The absorbance ratios at A260/ A280 and A260/230 for testis and sperm RNA samples isolated were between 1.86 –2.10 and 1.70–2.31, respectively (Table 8). The Bioanalyzer profile of testis RNA showed two distinct peaks of 18S and 28S rRNA respectively (Fig. 13), at the same place as indicated in reference with a RNA integrity number (RIN) of 7.9. In contrast, sperm RNA bioanalyzer profile exhibited only a single peak at 40-50s intervals (Fig. 13). The absence of 18S and 28S rRNA peaks in sperm RNA indicated that the RNA was originated from sperm and was uncontaminated with somatic cell RNA. Further, PTPRC and CD4 genes were targeted to detect the traces of somatic cell RNA in sperm samples. The primers for CD4 gene were intron-spanning in order to check genomic DNA contamination in RNA samples. Sperm RNA sample was found negative for PTPRC and CD4, whereas testis sample was positive for PTPRC and CD4 (Fig. 14 & 15). No genomic DNA contamination was detectable in the samples used to prepare the microarray probes (Fig. 15).

Table 8: RNA quality and yield of RNA samples used for microarray analysis.

Sample name	Absorbance value 260/280	Absorbance value 260/230	RNA concentration ng/µl
Testis RNA	2.10	2.31	1643
Sperm RNA	1.86	1.7	231

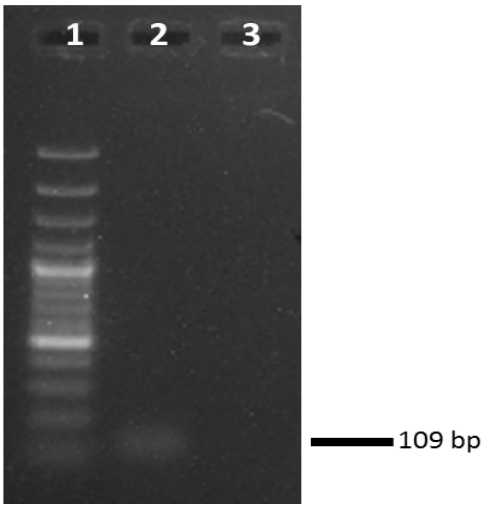


Figure 14: Image showing the absence of PTPRC RNA in sperm RNA (Lane 1: 100 bp marker; Lane 2: testis RNA and Lane 3: sperm RNA).

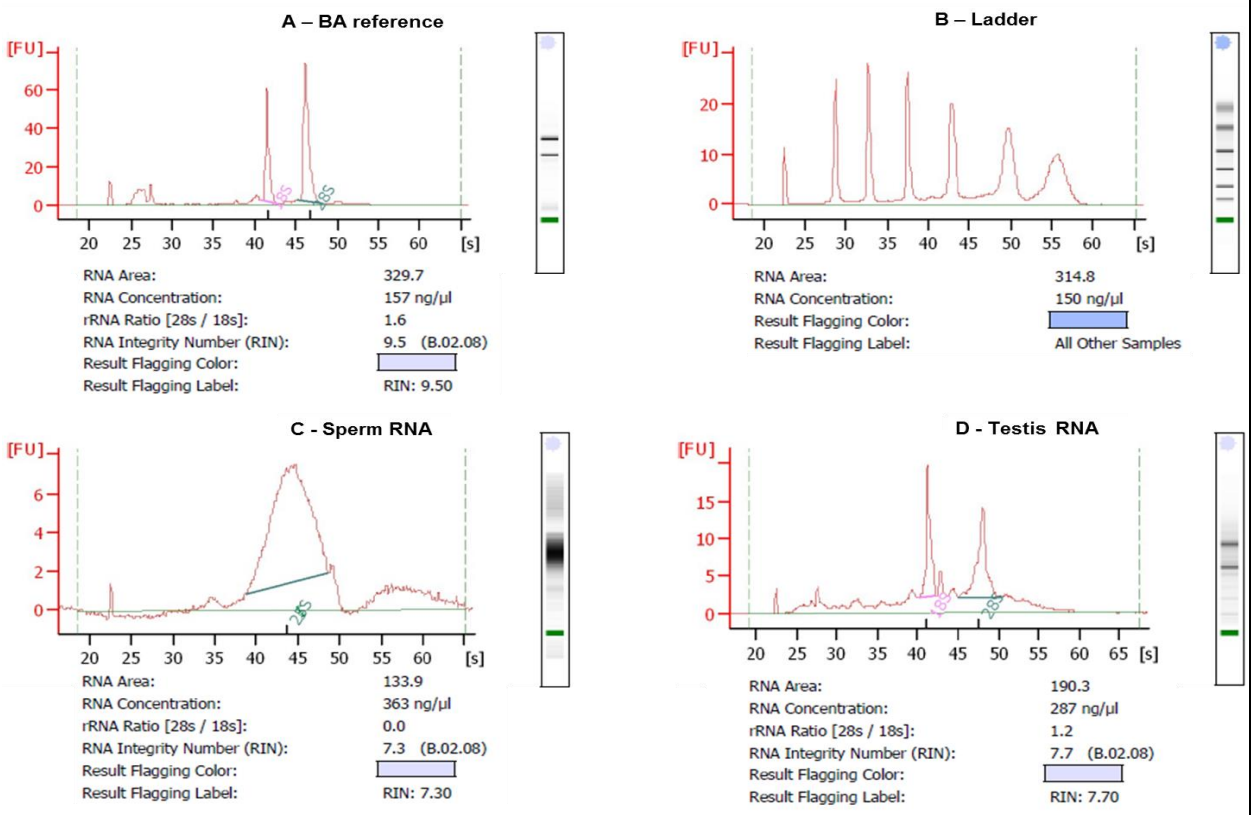


Fig. 13: Image showing microarray electropherogram of testis and sperm RNA samples. (A) 18S and 28S rRNA reference; (B) Ladder; (C) Total sperm RNA; and (D) Total testis RNA.

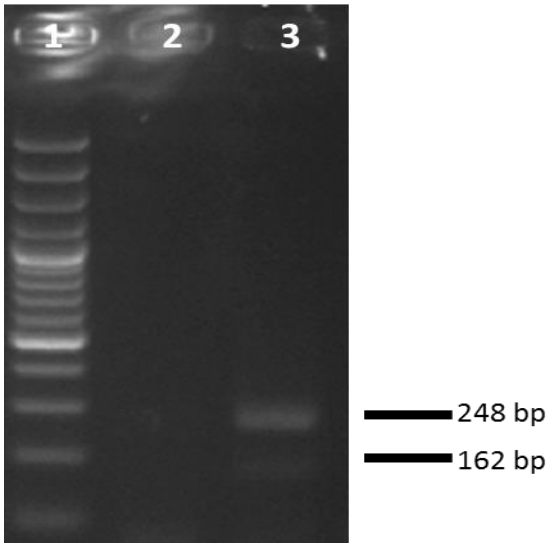


Figure 15: Image showing the absence or presence of CD4 RNA and gDNA in sperm or testis samples. A intron-spanning CD4 primer set was used to detect the presence of genomic DNA contamination. Lane 1: 100 bp ladder. Lane 2: sperm RNA. Lane 3: testis RNA spiked genomic DNA to produce the amplicon of 248 bp corresponding to genomic DNA and an amplicon of 162 bp corresponding to CD4 cDNA.

Microarray QC: The quality of Cy3 dye labelled cRNA samples were evaluated by NanoDrop. The specific activity of both the samples was 14.11 to 18.47 (Table 9), which is considered to be good. It is recommended that specific activity greater than 8 is good for hybridization. The hybridization images were manually verified and found to be devoid of uneven hybridization, streaks, blobs and other artefacts. Hybridization across the slides was good based on number of features such as spot finding of the four corners of the array, spatial distribution of all outliers on the array, negative control and net signal statistics and histogram of signal plot (Fig. 16).

Table 9: NanoDrop analysis of labelled cRNA.

Sample	Dye	Pmol/µl	Concentration (ng/ µl)	260/280	Specific activity
Testis_1	Cy3	3.43	243.12	2.28	14.11
Testis_2	Cy3	2.55	177.90	2.30	14.33
Sperm_1	Cy3	11.93	645.76	2.32	18.47
Sperm_2	Cy3	4.73	295.58	2.16	16.00

The overall mRNA expression levels within a single hybridization were presented in scatter plot where each point represent the expression values of a gene in biological replicates, one plotted on the x-axis and other on the y. The scatter plot showed linear relation in expression of genes in both the biological replicates (Fig. 17). These results were further substantiated by measuring the correlation coefficient based on the intensity values of expression (Table 10) and intra-array gene expression clustering (Fig. 18).

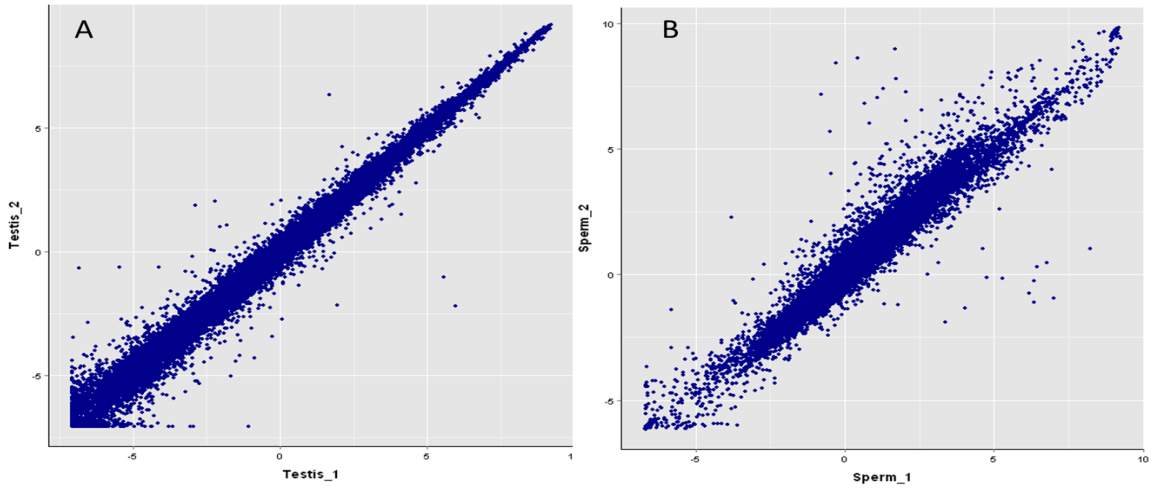
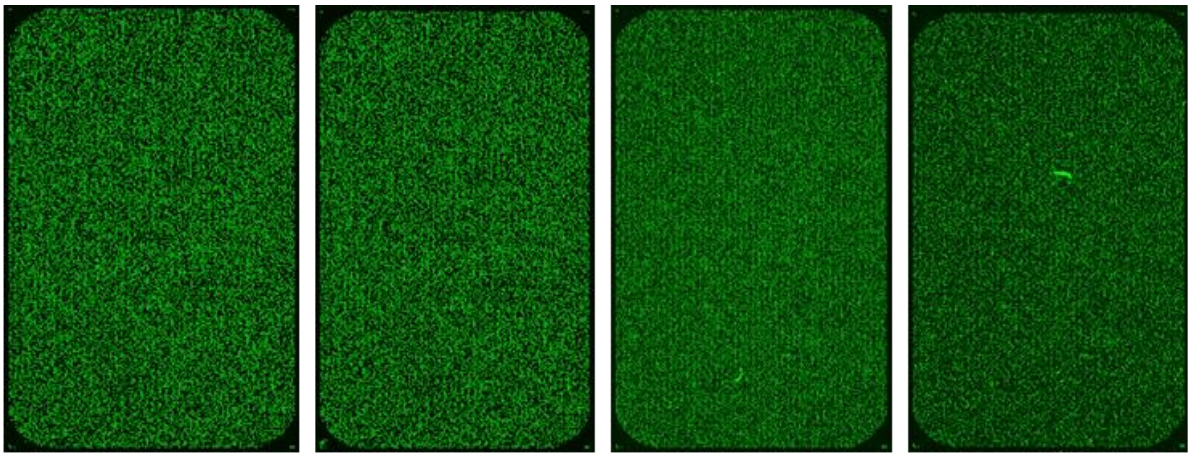
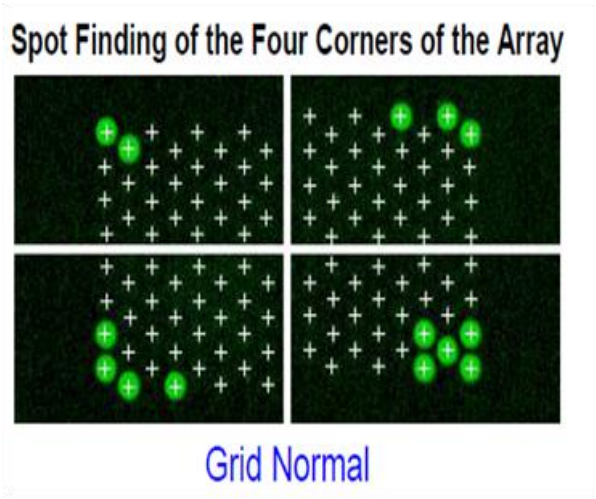
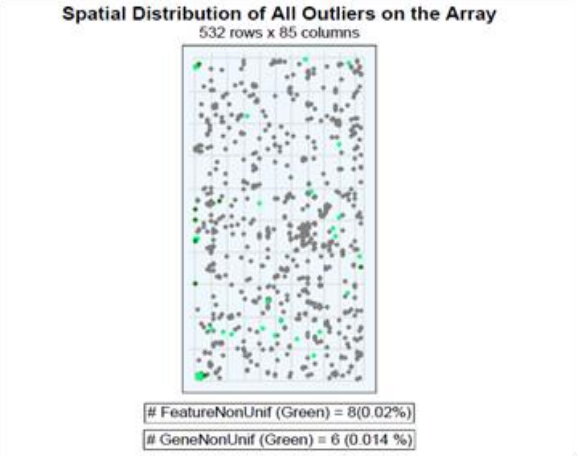


Figure 17: The scatter plots of gene expression values in two biological replicated showing linear relationship (A: testis replicates; B: sperm replicates)



Testis Testis Sperm Sperm





Negative Control Stats

	Green
Average Net Signals	5.82
StdDev Net Signals	0.71
Average BG Sub Signal	-1.13
StdDev BG Sub Signal	0.66

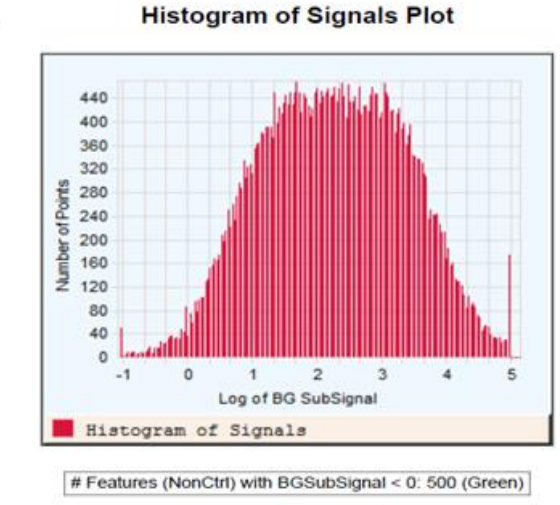


Figure 16: Hybridization images of sperm and testis samples along with spot finding of the four corners of the array, spatial distribution of all outliers on the array, negative control and net signal statistics and histogram of signal plot.

Table 10: Correlation coefficient based on the expression intensity values in testis and sperm samples.

Array Name	Testis_1	Testis_2	Sperm_1	Sperm_2
Testis_1	1			
Testis_2	0.995499	1		
Sperm_1			1	
Sperm_2			0.916439	1

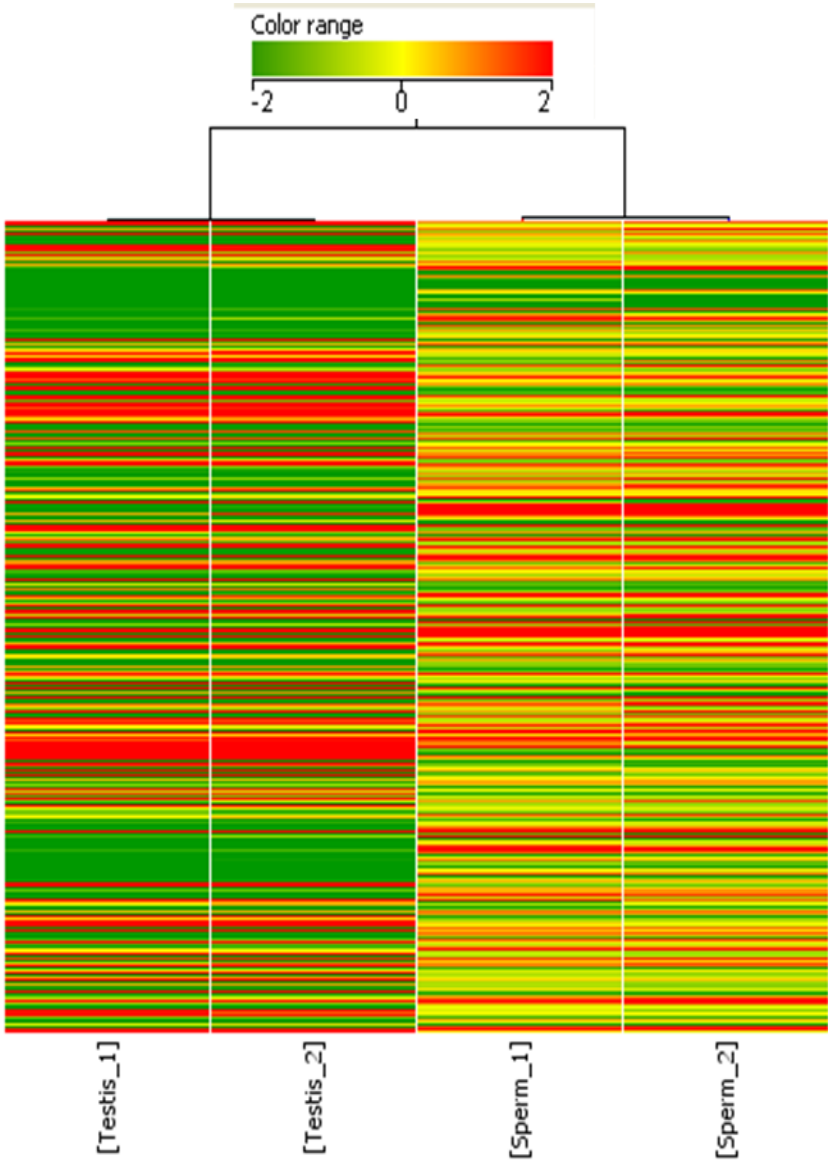


Figure 18: Image showing clusters of genes for intra-array quality control. Intra-array normalization deals with variability within a single array. In intra array normalization, g-processed signals (dye normalized background subtracted signal intensity) were log transformed and then for each of the array the 75th percentile value was calculated separately. In each sample the log transformed intensity values for each probe was subtracted by the calculated 75th percentile value of the respective array and expression values were obtained.

Sperm transcript profile: Gene expression microarray analysis revealed 17542 probes in chicken sperm and 21639 in the testes. The majority (87.3%) of the sperm transcripts were shared with the testes, while surprisingly, 12.7% transcripts were detected (raw signal intensity greater than 50) only in the sperm and not in the testes (Fig. 19). The chicken sperm transcript contains predominantly nuclear-encoded mRNAs, including 13 mitochondrial-encoded RNAs. The differential analysis revealed 11785 genes up and 9946 genes down regulated compared to testis at signal to noise ratio ≥ 2.

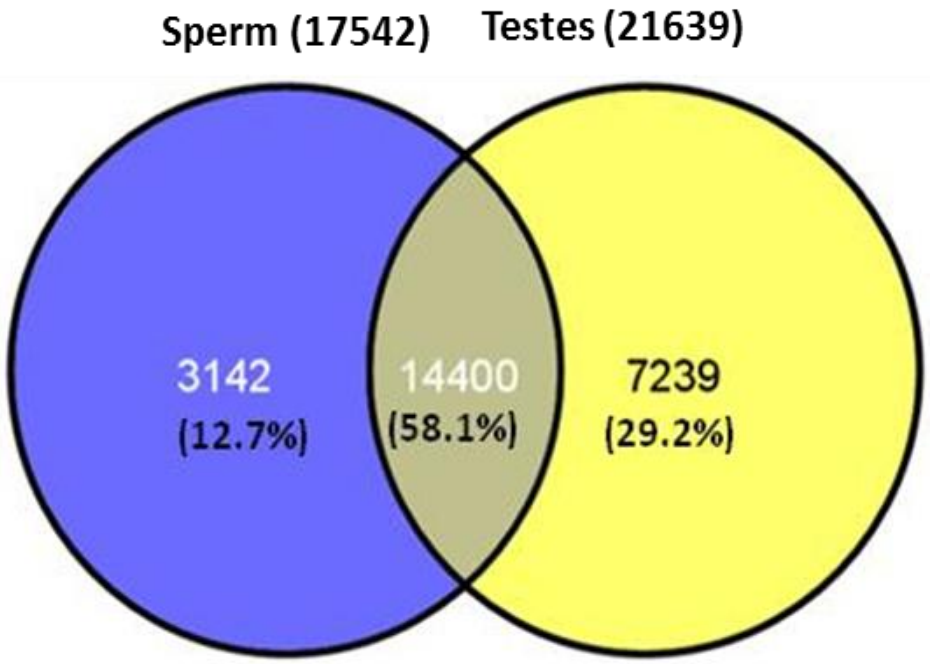


Figure 19: Venn diagram of the proportion of specific or common transcripts between testis and sperm with raw signal intensity greater than 50 by microarray analysis.

Gene ontology annotations grouped sperm transcripts into different groups as shown in table 11. The maximum transcripts were related to nucleic acid binding (1744) followed by signal transduction (1598) and transcription (1318). The maximum numbers of up regulated transcript were responsible for signal transduction (63.20%) followed by embryonic development (56.76%) and cell structure (56.25%). The lowest numbers of up regulated transcripts were associated with spermatogenesis (11.62%) and fertilization (18.18%) (Figure 20 & 21). All transcripts (110) related to ribosomal machinery was down regulated in sperm indicated translation was predominantly impaired (Table 12).

Table 11: Gene ontology functional groups of sperm up and down-regulated transcripts.

Function name	Total genes found	Upregulated genes/%	Downregulated genes
Nucleic acid binding	1744	645 (36.98)	1099
Apoptosis	471	226 (47.98)	245
Oocyte	167	52 (31.13)	115
Cell cycle	384	98 (25.52)	286
Embryonic development	377	214 (56.76)	163
Transcription	1318	623 (47.26)	695
Steroid	195	104 (53.33)	91
Fertilization	11	2 (18.18)	9
Protein modification	855	388 (45.38)	467
Cell structure	16	9 (56.25)	7
Signal transduction	1598	1010 (63.20)	588
Spermatogenesis	43	5 (11.62)	38

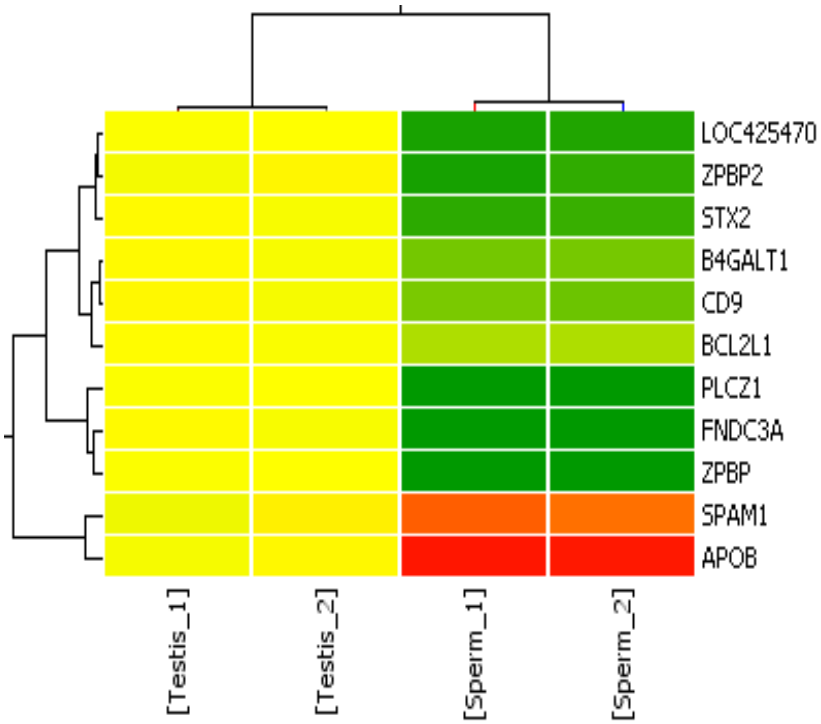


Figure 20: Heat map of genes that has role in fertilization. Hierarchical clustering of genes is based on similar expression profiles in sperm samples vs. testis samples. Clustering analysis was performed using GeneSpringGX Software using Average Linkage rule with Pearson Uncentered Distance Metric. Red color in the cluster indicates up regulation and green color in the cluster indicates down regulation in Sperm cells when compared to Testis sample. The Fold expression values represented in the clusters are in terms of log base 2.

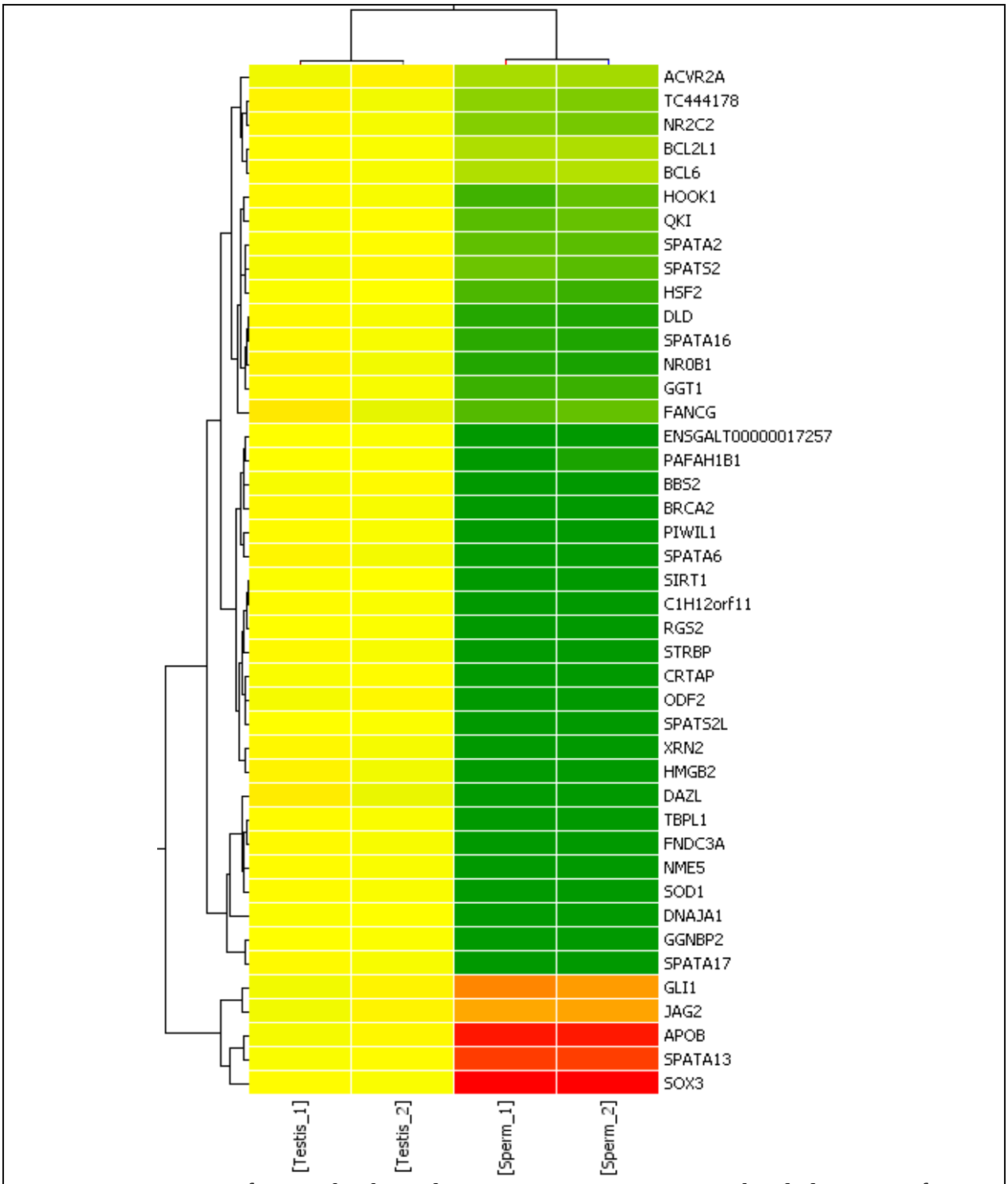


Figure 21: Heat map of genes that has role in spermatogenesis. Hierarchical clustering of genes is based on similar expression profiles in sperm samples vs. testis samples. Clustering analysis was performed using GeneSpringGX Software using Average Linkage rule with Pearson Uncentered Distance Metric. Red color in the cluster indicates up regulation and green color in the cluster indicates down regulation in Sperm cells when compared to Testis sample. The Fold expression values represented in the cluster are in terms of log base 2.

Table 12: A list of down regulated transcripts related to ribosomal machinery in sperm.

S. No	Gene Name	Fold Expression	P-Value
1	RPL30	-8.97	5.12E-05
2	RPLP1	-8.85	1.46E-05
3	RPL37	-8.80	3.76E-04
4	RPL26L1	-8.74	6.22E-06
5	RPL9	-8.66	8.56E-05
6	ENSGALT00000035726	-8.65	2.12E-05
7	RPL32	-8.51	2.60E-05
8	RPL35	-8.51	5.03E-05
9	RPL29	-8.46	3.18E-04
10	RPS20	-8.44	1.12E-04
11	RPL24	-8.33	2.15E-04
12	RPS27A	-8.32	1.43E-04
13	RPL5	-8.28	2.95E-05
14	RPS4X	-8.25	5.22E-05
15	RPS6	-8.19	4.82E-05
16	RPS8	-8.18	1.06E-04
17	RPS17L	-8.14	4.06E-05
18	RPL13	-8.06	1.89E-05
19	RPS12	-8.03	3.06E-05
20	RPL4	-7.97	3.53E-05
21	RPL37A	-7.91	7.16E-05
22	MRPS17	-7.87	1.84E-04
23	RPL35A	-7.83	4.50E-04
24	RPS23	-7.79	1.65E-05
25	RPL18A	-7.73	9.22E-04
26	RPS3	-7.71	9.65E-05
27	RPS11	-7.63	2.11E-05
28	RPL36	-7.61	7.45E-05
29	RPS7	-7.53	4.40E-04
30	MRPS28	-7.45	5.98E-06
31	RPL11	-7.44	2.24E-04
32	RPS14	-7.43	3.03E-05
33	RPSA	-7.43	3.57E-05
34	RPL19	-7.42	2.59E-04
35	MRPL18	-7.39	1.20E-04
36	RPL10A	-7.39	5.14E-04
37	RPS29	-7.31	1.29E-03
38	RPS15	-7.29	5.11E-04

39	ENSGALT00000002410	-7.22	3.11E-04	
40	RPL7	-7.22	1.17E-04	
41	RPL27	-7.21	2.27E-04	
42	ENSGALT00000035955	-7.09	4.63E-05	
43	MRP63	-7.07	1.70E-04	
44	RPL3	-7.06	4.10E-04	
45	RPL21	-7.02	9.09E-05	
46	MRPL23	-6.87	3.41E-04	
47	UBA52	-6.71	5.55E-05	
48	MRPL44	-6.54	2.89E-04	
49	RPS13	-6.43	4.45E-04	
50	MRPL27	-6.27	1.05E-04	
51	MRPL50	-6.23	5.19E-04	
52	ENSGALT00000033073	-6.02	2.46E-03	
53	MRPL39	-6.00	1.50E-04	
54	MRPL30	-5.90	5.15E-04	
55	RPLP2	-5.87	1.79E-04	
56	RPL7L1	-5.82	1.73E-03	
57	MRPS23	-5.81	8.71E-04	
58	MRPS12	-5.78	2.47E-04	
59	MRPL53	-5.70	1.96E-03	
60	MRPS25	-5.68	3.96E-03	
61	MRPL21	-5.65	5.26E-04	
62	MRPS5	-5.65	2.44E-04	
63	MRPL12	-5.58	2.18E-05	
64	MRPL16	-5.30	5.73E-04	
65	RRP15	-5.30	8.73E-05	
66	MRPL15	-5.17	8.48E-04	
67	MRPL38	-5.13	2.37E-04	
68	MRPS26	-5.13	1.50E-03	
69	MRPS36	-5.11	1.92E-03	
70	MRPL48	-5.08	3.23E-04	
71	MRPS30	-5.06	1.39E-03	
72	RSL24D1	-5.06	3.72E-04	
73	MRPS18A	-5.00	1.08E-03	
74	RRP7A	-4.97	8.83E-04	
75	MRPS22	-4.88	2.32E-04	
76	MRPL3	-4.85	1.86E-03	
77	MRPS14	-4.69	2.32E-03	
78	MRPL37	-4.65	2.54E-04	
79	MRPL24	-4.63	2.38E-03	

80	ZCCHC17	-4.59	8.67E-04
81	MRPL45	-4.42	2.02E-03
82	MRPL33	-4.34	1.75E-04
83	MRPS35	-4.34	4.96E-04
84	ENSGALT00000002763	-4.33	9.95E-05
85	MRPL51	-4.19	1.21E-03
86	MRPL28	-4.16	8.10E-03
87	MRPL32	-4.04	5.23E-04
88	MRPS10	-3.99	2.56E-03
89	DAP3	-3.98	4.90E-03
90	MRPS21	-3.85	7.29E-04
91	TC409229	-3.64	1.33E-03
92	RSL1D1	-3.50	5.90E-04
93	ENSGALT00000035573	-3.47	1.69E-03
94	MRPL2	-3.24	1.06E-02
95	NPM1	-3.23	1.71E-03
96	MRPS33	-3.10	2.61E-02
97	MRPL19	-3.05	1.79E-03
98	RRNAD1	-2.80	1.00E-03
99	MRPS6	-2.76	1.66E-02
100	MRPL22	-2.69	6.31E-04
101	MRPS2	-2.56	9.10E-03
102	MRPS27	-2.39	2.41E-03
103	MRPL1	-2.32	1.05E-02
104	RRP1B	-2.28	2.06E-01
105	RPS6KC1	-2.27	6.96E-03
106	RPS6KA2	-2.20	4.77E-03
107	MALSU1	-1.89	6.28E-03
108	TC399074	-1.75	1.92E-03
109	RPS6KA6	-1.41	6.62E-02
110	MRPL10	-1.34	3.96E-02

A preliminary survey of the chicken sperm transcript profile for previously reported sperm RNA candidates identified several transcripts common in bovine, human, porcine and chicken (Table 13). These transcripts showed differential expression pattern in chicken spermatozoa. A list of twenty most and least abundant RNAs in chicken sperm is present in table 14 and 15. Of these 20 most abundant transcripts, 19 were uncharacterized, whereas least abundant genes were mostly associated with ribosome. Further, the mapping of differentially regulated genes on genome revealed the presence of maximum genes on chromosome 1 (up regulated-391,

downregulated-679; Fig. 22), and least on chromosome W (up regulated-1, downregulated-3; Fig. 22).

Table 13: A list of previously reported candidate sperm transcripts identified in the chicken sperm transcript profile.

Transcript name	Accession no.	Expression	Species	REFERENCE
PRM	M28100	Up	Human, Bovine, Chicken	Ziyyat et al., 1999; Gilbert et al., 2007; Shafeeque et al., 2014
CRISP2	ENSGALT00000026918	Up	Bovine	Arangasamy et al., 2011; Zhao et al., 2006
SPATA20	XM_420103	Up	Bovine	Gilbert et al., 2007
H2AFZ	NM_001031374	Down	Bovine	Gilbert et al., 2007
EEF1A1	NM_204157	Down	Bovine	Laalancette et al., 2008; Zhao et al., 2006
ACTG1	NM_001007824	Down	Bovine	Gilbert et al., 2007
PLCZ1	NM_001039273	Down	Human, chicken	Hamatani et al., 2012; Shafeeque et al., 2014
MYCBP	ENSGALT00000041046	Down	Human	Lambard et al., 2004; Kumar et al., 1993
PEBP1	NM_001198642	Down	Bovine	Bissonette et al, 2009; Arangasamy et al., 2011
SPAG4	XM_424281	Down	Bovine	Gilbert et al., 2007
CCT8	NM_001004389	Down	Bovine	Arangasamy et al., 2011;
PPIH	ENSGALT00000007781	Down	Bovine	Gilbert et al., 2007
STRBP	NM_001030680	Down	Bovine	Gilbert et al., 2007
CHMP5	ENSGALT00000021491	Down	Human	Zhao et al., 2006; Lalancette et al., 2008
CLGN	ENSGALT00000015978	Down	Porcine	Ostermeier et al., 2006; Wang et al., 2004; Kempisty et al., 2008
EIF2B2	NM_001006481	Down	Bovine	Gilbert et al., 2007
CTTN	NM_205468	Down	Stallion	Das et al., 2013
ARID5B	NM_001031220	Down	Stallion	Das et al., 2013
ATG12	ENSGALT00000003696	Down	Stallion	Das et al., 2013
GSTA1	NM_001001777	Down	Stallion	Das et al., 2013
PRPSAP1	ENSGALT00000002885	Down	Stallion	Das et al., 2013

Table 14: Tewnty most abundant Probes based on the fold Regulation (NA-not available).

Gene name	Accession number	Fold change	P value	Chromosome position	Pathway
CR389478	BU441709	9.98	1.13E-03	NA	Unknown
L48903	L48903	10.37	1.12E-03	NA	Unknown
ENSGALT00000014963	ENSGALT00000014963	10.38	9.47E-04	25	Unknown
CR407005	CR407005	10.56	8.66E-04	NA	Unknown
NP9665240	NP9665240	10.72	3.07E-06	NA	Unknown
NP9669230	NP9669230	10.41	5.76E-04	NA	Unknown
NP9668656	NP9668656	10.63	3.29E-04	NA	Unknown
NP9669152	NP9669152	10.57	6.11E-04	NA	Unknown
NP9665913	NP9665913	9.93	9.26E-04	NA	Unknown
ENSGALT00000029799	ENSGALT00000029799	11.39	1.25E-03	NA	Unknown
ENSGALT00000033125	XM_422036	9.67	2.17E-04	7	RIG-I-like receptor signaling pathway
NP9668902	NP9668902	10.87	1.61E-03	NA	Unknown
BX934641	TC442112	11.04	7.35E-04	NA	Unknown
NP9669128	NP9669128	9.76	1.83E-04	NA	Unknown
NP9668791	NP9668791	9.39	1.32E-03	NA	Unknown
NP9666246	NP9666246	11.05	1.76E-03	NA	Unknown
NP9665148	NP9665148	9.50	1.37E-03	NA	Unknown
NP9668920	NP9668920	9.12	1.06E-02	NA	Unknown
TC429602	TC429602	9.44	1.06E-04	NA	Unknown
SCARF1	XM_001234822	10.32	3.25E-03	19	Unknown

Table 15: Twenty least abundant Probes based on the fold Regulation (NA-not available).

Gene name	Accession number	Fold change	P value	Chromosome position	Pathway
RPLP1	NM_205322	-8.85	1.46E-05	2	Ribosome
LOC768497	XM_001231376	-8.60	3.55E-04	NA	Unknown
RPL26L1	ENSGALG00000002868	-8.74	6.22E-06	13	Ribosome
RPL30	NM_001007967	-8.97	5.12E-05	2	Ribosome
COX4I1	DR410805	-8.52	1.02E-04	11	Oxidative phosphorylation
LOC770729	ENSGALT00000018624	-8.53	5.81E-05	4	Unknown
RPL32	NM_001252255	-8.51	2.60E-05	12	Ribosome
LOC420220	ENSGALT00000030843	-9.04	1.49E-03	2	Unknown
RPL38	ENSGALT00000002220	-8.58	3.60E-04	18	Ribosome
RPL35	NM_204273	-8.51	5.03E-05	17	Ribosome
TC435313	XM_422851	-9.05	3.85E-04	NA	Unknown
PTTG1	ENSGALT00000002291	-8.78	9.28E-05	13	Cell cycle
BX932642	BX950575	-8.72	1.54E-04		Unknown
ENPP4	NM_001006422	-8.45	3.11E-05	3	Catalytic activity
PABPC1	NM_001031597	-8.95	3.29E-05	2	Nucleotide binding
RPS17L	NM_204217	-8.14	4.06E-05	10	Ribosome
RPS6	NM_205225	-8.19	4.82E-05	Z	Ribosome
CO635742	XM_001233012	-8.31	1.04E-03	NA	Unknown
AKAP14	ENSGALT00000032302	-8.48	1.77E-04	4	Unknown
GGNBP2	NM_001001899	-8.46	3.00E-04	19	Spermatogenesis

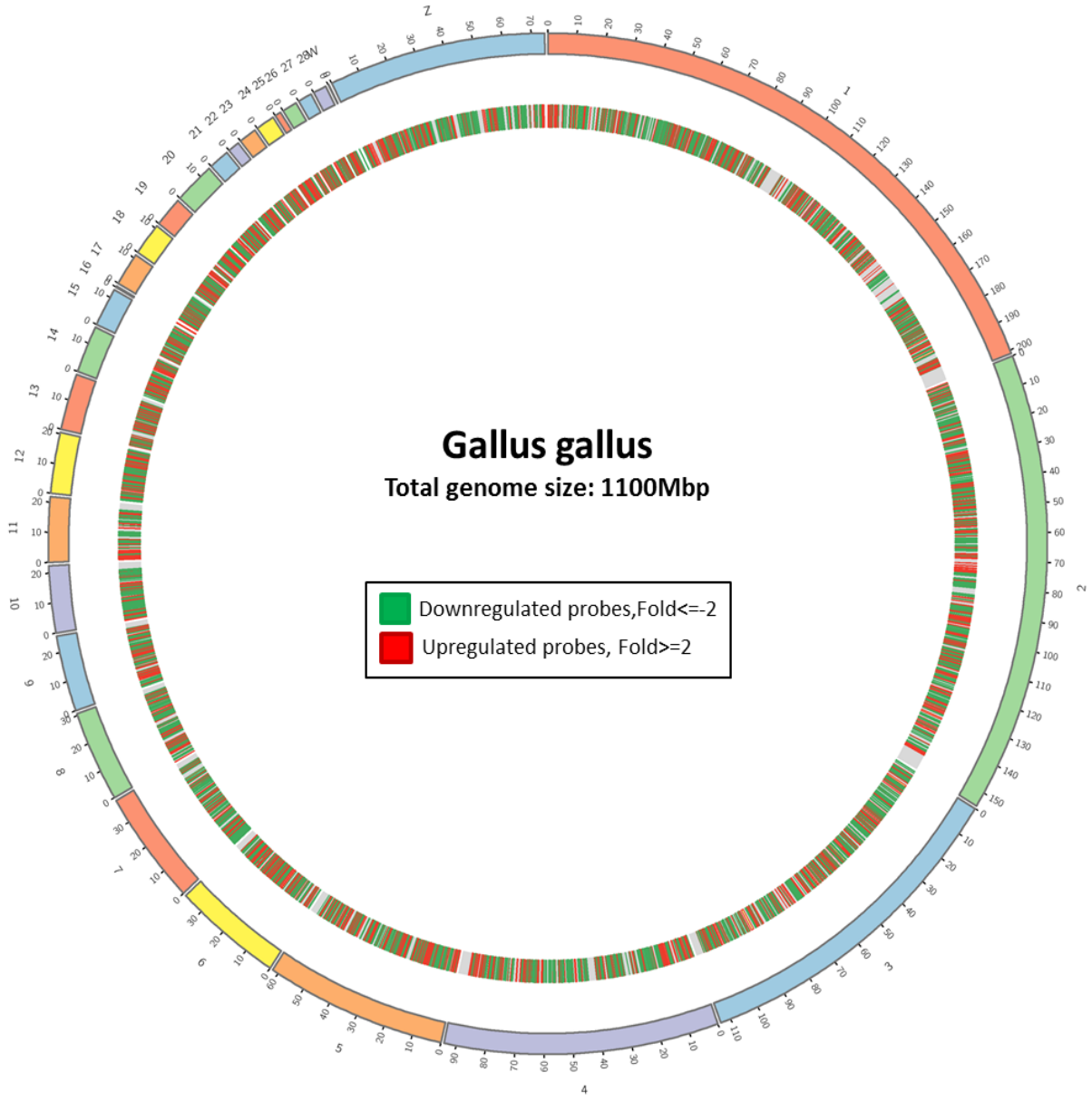


Figure 22: Integrated view of sperm transcripts on genome. Outer ring represents the various chromosomes of Chicken genome. Inner ring represent the distribution of differentially regulated genes based on the fold values. One unit=10 kb of nucleotide base pair.

Validation of microarray results: For microarray results validation, specify candidate genes were selected according to their abundance in chicken sperm. Two known sperm specific-genes (PRM and PLCZ1) were also included to validate microarray results. The RT-PCR results showed up-regulation of PRM, PLK2 and ENS1 gene, whereas down-regulation of PLCZ1, PABPC1 and RPL26L1 in sperm compared to testis, which are according to the microarray results (Fig. 23 & 24).

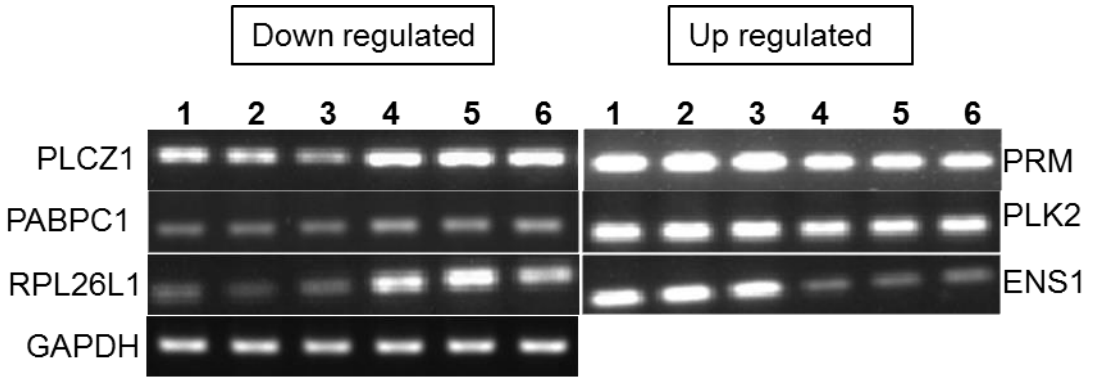


Figure 23: Amplification of selected candidate genes for validation of microarray results by RT-PCR. Lane 1, 2 and 3 contains PCR products amplified with sperm cDNA, whereas lane 4, 5 and 6 contains PCR products amplified with testis cDNA. Gel electrophoresis was performed for all the genes in identical conditions.

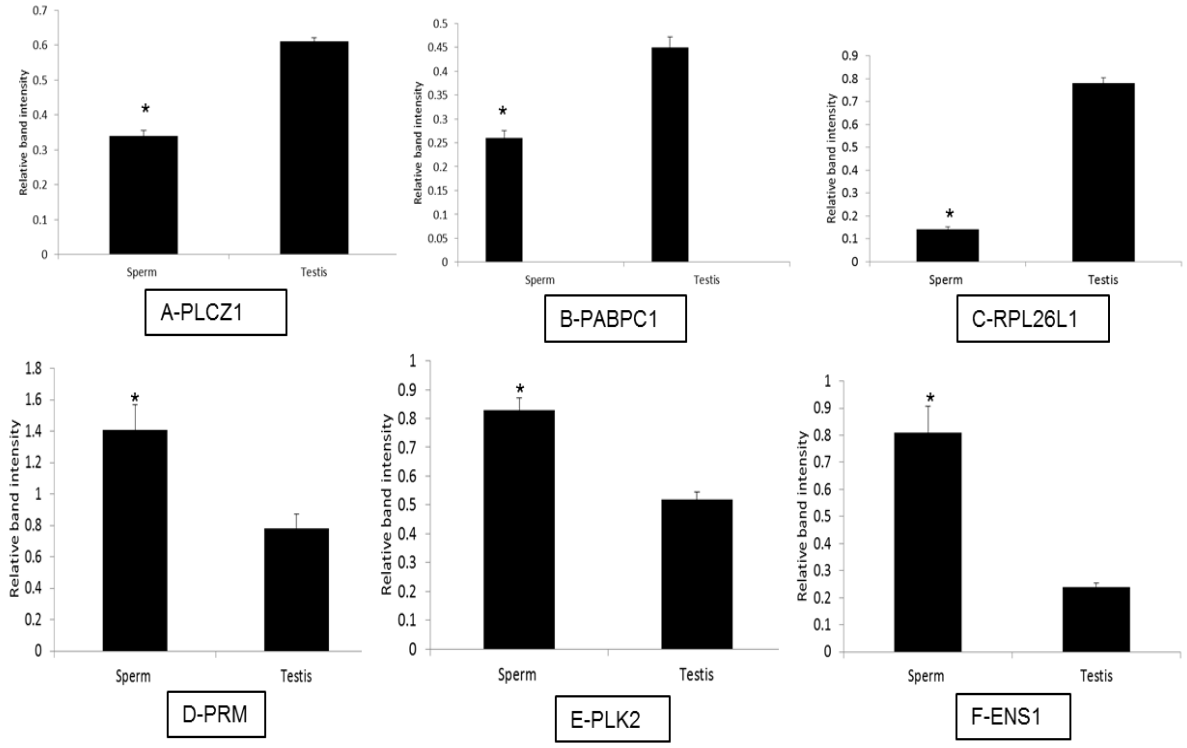


Figure 24: RT-PCR analysis of selected transcripts in sperm and testis cDNA. Graph represent the mean of the normalized band density for each mRNA band obtained by densitometric analysis (mean±SE; n = 3). Normalization was performed by dividing each band density value by the value of β-actin band density in the same sample. Asterisk (*) indicates a significant difference ($p<0.05$) between mRNA expression.

Experiment 5

Semen characteristics and fertility: Semen characteristics such as semen volume, concentration, motility and fertility data is presented in table 16.

Table 16: Semen characteristics of broiler breeder males (n=6; Mean ± SEM)

Bird no.	Semen volume (µL)	Motility (%)	Concentration (10^9/ml)	Fertility (%)	Fertility rank
5	1163 ± 44	59 ± 9.21	5.26 ± 0.35	100	High fertility (70%)
8	676 ± 82	75 ± 4.10	5.45 ± 0.35	100	
11	1163 ± 46	71 ± 7.16	4.84 ± 0.57	100	
20	813 ± 65	78 ± 3.05	4.90 ± 0.37	100	
21	413 ± 80	71 ± 7.57	3.87 ± 0.31	100	
34	1118 ± 101	76 ± 4.39	4.87 ± 0.18	100	
18	705 ± 97	65 ± 7.87	2.49 ± 0.79	100	
43	660 ± 146	72 ± 6.74	5.47 ± 0.61	100	
4	1103 ± 58	44 ± 7.53	5.26 ± 0.37	97 ± 03	
1	651 ± 65	58 ± 7.70	4.57 ± 0.36	95 ± 05	
10	761 ± 154	68 ± 5.22	3.95 ± 0.14	90 ± 11	
17	738 ± 27	83 ± 1.22	2.45 ± 0.11	90 ± 08	
44	760 ± 119	56 ± 15.28	0.72 ± 0.36 #	90 ± 11	
45	786 ± 116	43 ± 10.55	1.39 ± 0.89 #	90 ± 06	
47	776 ± 85	63 ± 11.46	4.73 ± 0.42	90 ± 11	
41	603 ± 60	54 ± 9.42	4.15 ± 0.75	87 ± 08	
22	511 ± 90	71 ± 9.45	2.81 ± 0.83	86 ± 09	
13	993 ± 84	71 ± 5.94	5.01 ± 0.16	84 ± 14	
2	1111 ± 143	29 ± 6.06	5.23 ± 0.25	83 ± 15	
6	1395 ± 58	44 ± 8.05	5.68 ± 0.50	83 ± 11	
3	990 ± 44	74 ± 4.10	5.60 ± 0.41	82 ± 11	
9	347± 116	61 ± 8.24	4.39 ± 0.27	82 ± 08	
28	808 ± 79	75 ± 4.77	4.86 ± 0.22	82 ± 08	
49	760 ± 97	61 ± 5.22	4.06 ± 0.15	82 ± 14	
15	870 ± 96	69 ± 6.98	4.83 ± 0.28	80 ± 07	
35	254 ± 75	48 ± 15.31	0.90 ± 0.33 #	80 ± 24	
46	718 ± 70	70 ± 9.10	3.58 ± 0.55	80 ± 14	
40	696 ± 71	60 ± 12.56	1.86 ± 0.28 #	80 ± 11	
14	896 ± 59	77 ± 6.12	5.34 ± 0.07	79 ± 12	
25	843 ± 61	65 ± 4.69	5.14 ± 0.46	79 ± 06	
24	743 ± 97	65 ± 5.47	4.31 ± 0.31	78 ± 12	
23	540 ± 35	81 ± 1.15	4.28 ± 0.36	76 ± 12	
7	1070 ± 88	41 ± 4.39	5.04 ± 0.34	75 ± 08	
19	580 ± 60	28 ± 8.63	0.13 ± 0.10 #	75 ± 12	
37	408 ± 87	49 ± 12.08	1.16 ± 0.78 #	75 ± 09	
38	735 ± 72	80 ± 4.47	3.81 ± 0.44	72 ± 34*	Low fertility (30%)
42	1010 ± 47	78 ± 3.05	4.59 ± 0.23	68 ± 09*	
31	548 ± 129	52 ± 10.93	2.95 ± 0.91	67 ± 33*	
27	653 ± 63	60 ± 4.0	3.86 ± 0.46	67 ± 13*	
30	1080 ± 90	75 ± 4.47	5.78 ± 0.38	66 ± 20*	
60	493 ± 113	83 ± 1.15	3.01 ± 0.36	61 ± 25*	
36	770 ± 128	58 ± 9.69	4.17 ± 0.11	60 ± 11*	
16	1390 ± 57	48 ± 12.13	4.52 ± 0.31	58 ± 21*	
32	943 ± 94	64 ± 9.63	4.44 ± 0.23	58 ± 08*	
39	805 ± 137	82 ± 1.22	3.52 ± 0.46	57 ± 20*	
26	1036 ± 72	82 ± 1.22	3.14 ± 0.28	54 ± 05*	
48	388 ± 54	11 ± 3.41	0.07 ± 0.07 #	50 ± 08*	
33	1006 ± 98	68 ± 9.45	4.00 ± 0.51	50 ± 08*	
12	651 ± 62	55 ± 7.53	4.38 ± 0.40	49 ± 23*	
29	603 ± 72	75 ± 5.29	3.63 ± 0.69	41 ± 10*	

#birds produced watery semen. *denotes significance

A wide variation in semen volume was observed. Semen volume varied from 300 to 1200 µL. Similarly motility and concentration also varied. The sperm motility varied from 11 to 90%,

whereas concentration varied from .07 to 5.78 million/mL. Of these 50 birds, seven (14%) were found producing watery semen with compromised characteristics. However, fertility in those birds was unaffected. Fertility varied from 41 to 100%. It is interesting to note that fertility was not correlated with any of the semen characteristics. Of these 50 birds, 15 (30%) showed significantly lesser fertility compared to others. Ten birds from each having high (100%) and low (40-60%) fertility potential were used for semen collection and expression analysis.

Selection of stable housekeeping gene for data normalization: In order to find out a suitable and stable housekeeping gene for data normalization, sperm cDNA from three different breeds (White Leg Horn, Kadaknath and Broiler) were used to quantify the expression of GAPDH, ACTB and 28S mRNA. A mean expression level lower than the maximum expression level subtracted with 2 standard deviation (SD) was considered a prerequisite for a candidate housekeeping gene. Our results showed that GAPDH was the most stable gene according to calculations made with both NormFinder and BestKeeper (Fig. 25).

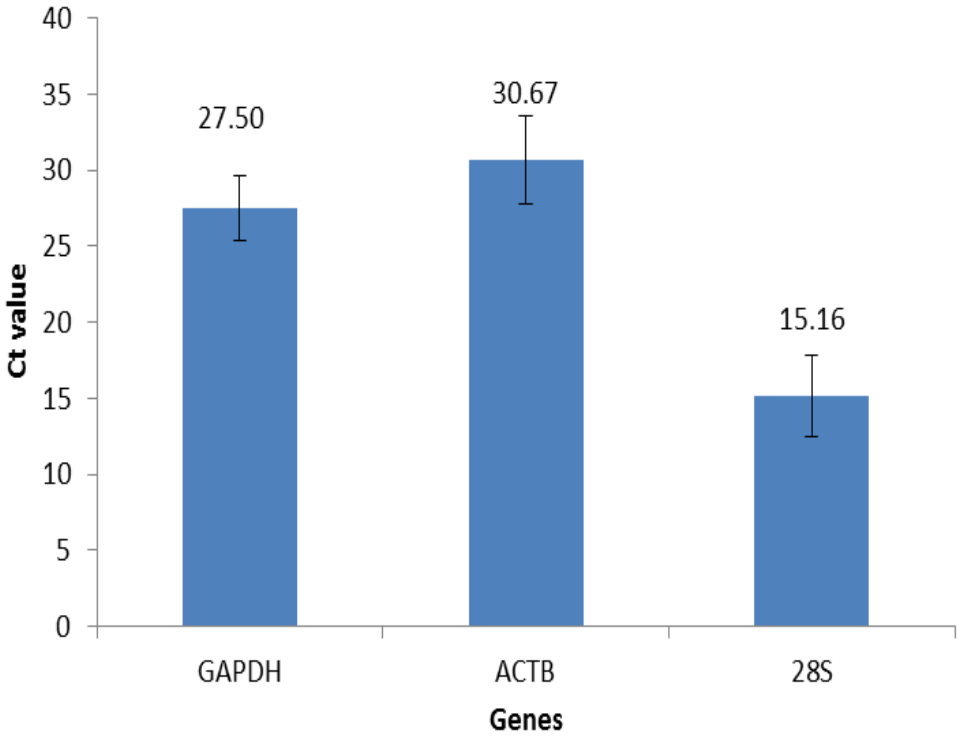


Figure 25: qRT-PCR analysis of selected genes in sperm cDNA. Graph represent the mean of the Ct values obtained by using gene specific primers (mean ± SD; n = 12).

PRM and PLCZ1 expression in low and high fertilization potential birds: Quantify of PRM and PLCZ1 mRNA was measured in sperm cDNA of low and high fertilization potential birds. Our results demonstrated higher abundance of PRM and PLCZ1 mRNA in sperm of high fertility birds when compared to low fertility birds. The PRM abundance was approximately three fold higher ($p<0.05$), whereas PLCZ1 was approximately two fold in high fertility birds (Fig. 26).

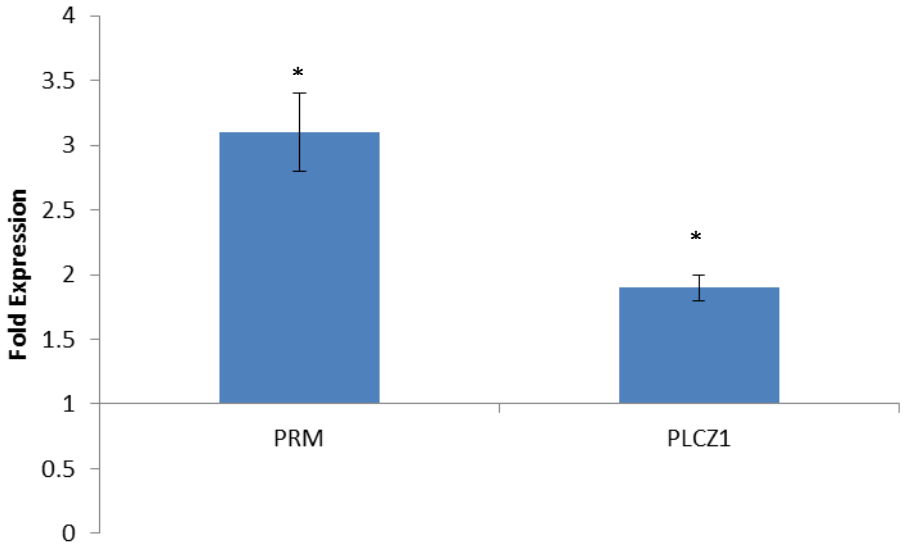


Figure 26: Relative expression of RPM and PLCZ1 in high fertility birds in relation to low fertility birds.

Experiment 6

Effect of in vitro BPA on sperm motility: Sperm motility was determined after 10, 20 and 30 min exposure to BPA in vitro. The 0.18 and 0.37 mM BPA treatments differed significantly from the negative and vehicle controls and from each other ($P\leq0.05$) after 20 and 30 min exposure and from the controls after 10 minutes (Figure 27). Maximum reduction (approximately two fold) in sperm motility was recorded after 30 min incubation in 0.74 mM BPA compared to negative and vehicle controls. Sperm motility in negative control and vehicle control remained same after 10, 20 and 30 min exposure to BPA (Figure 27). Sperm motility was also significantly correlated with fertility ($r = 0.73$, $P\leq0.01$), % live sperm ($r = 0.64$, $P\leq0.01$), % moribund sperm ($r = -0.56$, $P\leq0.01$) and high $\Delta\psi m$ ($r = 0.44$, $P\leq0.01$) (Table 17).

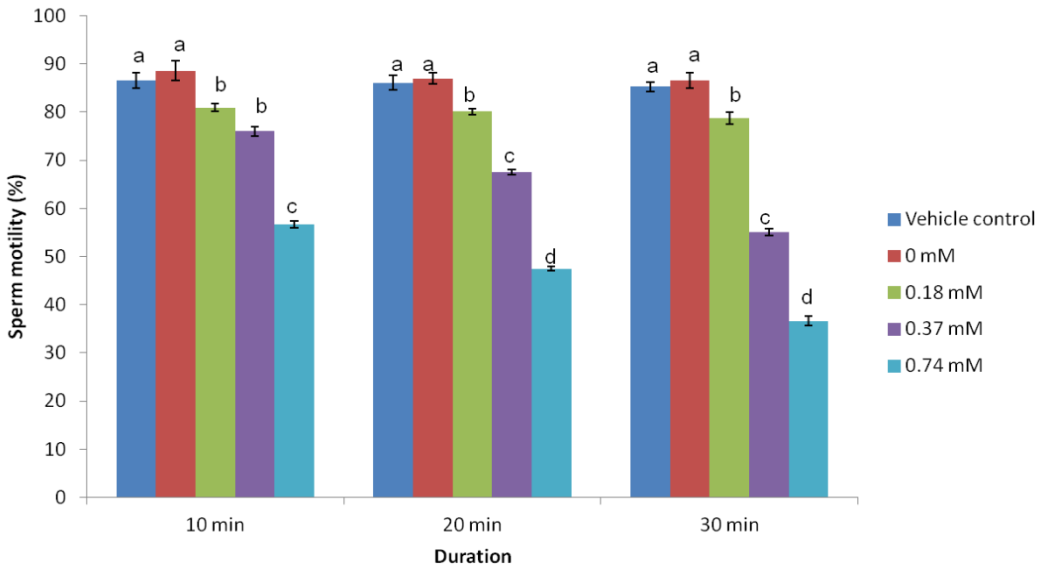


Figure 27: In vitro effect of bisphenol A (BPA) on fertility in chicken. Data are presented as mean ± SEM (n=6). The mean values in chart columns bearing different superscripts differed significantly (P≤0.05).

Table 17: Correlation coefficients among the parameters measured in sperm after 30 min exposure to bisphenol-A (BPA). The t-test is used to establish significance of the correlation between pairs of parameters.

	Motility	Fertility	% Live Sperm	% Moribund Sperms	High Δψm
Motility	1				
Fertility	0.73**	1			
% Live Sperm	0.64**	0.62**	1		
% Moribund Sperms	-0.56**	-0.64**	-0.62**	1	
High Δψm	0.44**	0.66**	0.30	-0.66**	1

**Correlation is significant at the 0.01 level (2-tailed).

Effect of in vitro BPA on sperm viability: In order to assess the sperm viability, sperm were stained with dual fluorescence probes, SYBR-14 and PI, and analysed in flow cytometer. We observed 99.39% sperm stained with PI+ in Q1 in heat killed samples (Fig. 28A), whereas 99.74% sperm were found positive for SYBR-14 in Q4 in sperm stained only with SYBR-14 (Figure 28B), indicating the accuracy of measurement. The sperm viability in controls and BPA treated sperm showed dose-dependent response. The sperm viability was observed significantly

lesser (P≤0.05) in 0.37 and 0.74 mM BPA compared to 0.18 mM BPA, negative and vehicle controls (Table 18). No difference was observed among 0.18 mM BPA, negative and vehicle controls. Percent moribund sperm were higher (P≤0.05) in 0.74 and 0.37 compared to 0.18 mM BPA, negative and vehicle controls (Table 18). Percent live sperm were also significantly correlated with moribund sperm (r = -0.62, P≤0.01) (Table 17).

Table 18: In vitro effect of bisphenol A (BPA) on percent live and moribund sperm population (Mean ± SEM, n=6).

Treatment (mM BPA)	% Live sperm	% moribund sperm
Vehicle control	74.99±1.33[a]	15.49±1.07[a]
0	77.85±1.77[a]	16.24±2.60[ab]
0.18	72.47±1.65[a]	17.03±1.65[ab]
0.37	65.58±3.04[b]	20.32±1.55[c]
0.74	60.00±2.11[b]	22.49±1.13[c]

The mean values in columns bearing different superscripts differed significantly (P≤0.05)

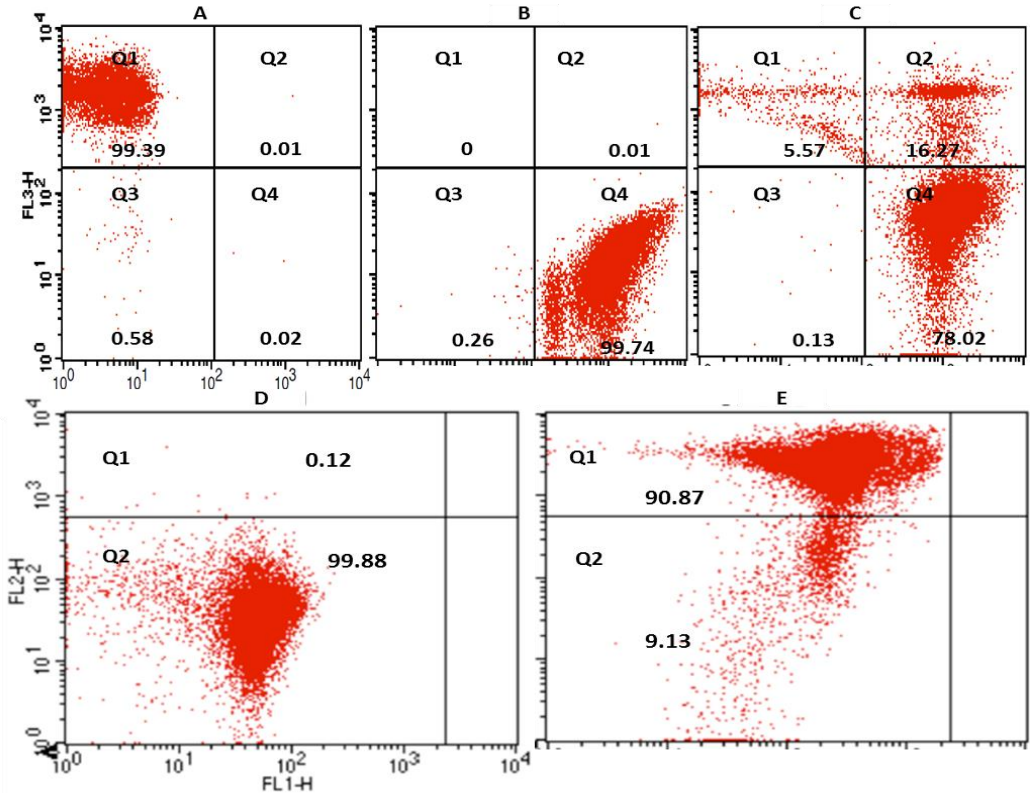


Figure 28: Flow cytometric detection of heat killed (A), fresh sperm stained with SYBR-14 (B), fresh sperm stained with SYBR-14 and PI (C) and CCCP treated and normal sperm stained with JC-1 (D & E). Q1, Q2, Q3 and Q4 quadrants containing dead sperm (PI+), moribund sperm (SYBR+PI+), debris and live sperm (SYBR-14+), respectively in figure A, B and C. Q1 and Q2 quadrants contain sperm with high and low Δψm, respectively in figure D and E. Values mentioned in each quadrant represent percent sperm population.

Effect of in vitro BPA on sperm Δψm: Sperm Δψm was assessed by JC-1 staining following quantification by flow cytometery. Sperm treated with CCCP (a mitochondrial membrane potential inhibitor) showed 99.88% sperm in Q2 (Figure 28D) with low Δψm (green fluorescence; JC-1 monomers) compared to negative control where 90.87% sperm were in Q1 (Figure 28E) with high Δψm (orange fluorescence), indicating the sensitivity of JC-1 staining and the accuracy of measurement. Sperm with high Δψm were lesser (P≤0.05) in 0.74mM BPA compared to negative control. No difference was observed in Δψm in other treatments compared to negative control and vehicle control (Table 19).

Table 19: In vitro effect of bisphenol A (BPA) on sperm mitochondrial membrane potential (Mean ± SEM, n=6)

Treatment (mM BPA)	% sperm with high Δψm
Vehicle Control	81.39±3.20ab
0	84.46±1.49b
0.18	75.90±4.28ab
0.37	75.04±3.70ab
0.74	72.20±2.70a

The mean values in columns bearing different superscripts differed significantly (P≤0.05)

Effect of in vitro BPA on sperm fertilizing ability: Fertilizing ability of sperm incubated with BPA was assessed by artificially insemination of the treated semen into females. The percent fertility values in vehicle control, negative control, 0.18, 0.37 and 0.74mM BPA were 77.46±1.38, 81.12±1.97, 73.12±1.38, 71.49±3.00 and 42.50±1.38, respectively. Fertility was significantly (P≤0.05) reduced in females in which semen was incubated in 0.74mM BPA prior to insemination as compared to other treatments and controls (Figure 29). Sperm fertilizing ability was also significantly correlated with % live sperm (r = 0.62, P≤0.01), % moribund sperm (r = -0.64, P≤0.01) and high Δψm (r = 0.66, P≤0.01)(Table 3).

Effect of in vitro BPA on sperm RNA: The quality of RNA isolated from sperm incubated with 0.74 mM BPA was poor. The absorbance ratios at A260/A280 for RNA isolated from sperm incubated with 0.74 mM BPA, vehicle control and negative control were 1.1±0.24, 1.7±0.12 and 1.7±0.28, respectively. The concentrations of RNA isolated from sperm incubated with 0.74 mM BPA, vehicle control and negative control were 10±3.0, 35±6.0 and 43±8.0 ng/µl. The absorbance ratio of RNA at A260/A280 of sperm incubated with BPA indicated its poor quality, thus could not be analyzed further for gene expression studies.

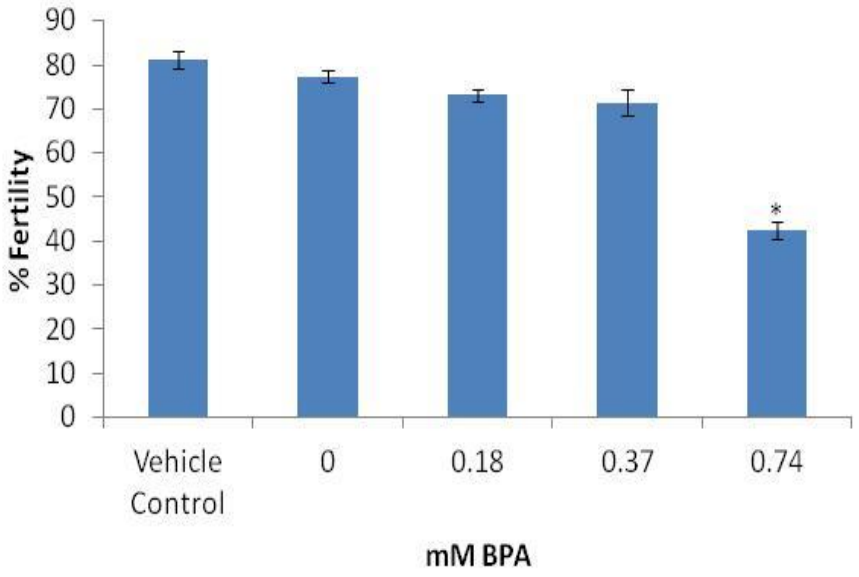


Figure 29: In vitro effect of bisphenol A (BPA) on sperm motility in chicken. Data are presented as mean ± SEM (n=6). Asterisk (*) indicates a significance ($p < 0.05$).

Table 20: Primer pairs used for PCR.

Gene name	Accession number	Primer Sequence (5'-3')	Annealing Temp. (°C)	Product size
GAPDH	NM_204305	F-CGTGTTGTGGACTTGATGG R-AACTGAGCGGTGGTGAAG	58	120
PLCZ1	AY843531	F-CGTAGTAGACAAGTATGC R-ATTGGTGAAGTAAGAAGTT	47.8	141
PRM	M28100/ L38713	F-CGCAGCAGGACCCGCAGCCG R-CGGCGGCGGCGGCTCAGTAG	66	187
CD4	DQ202315	F-GAAAGATGGGACCTGTACTTGG R-TCCAGGCTCAAGTCTGACAC	58	RNA/cDNA=162 Genomic DNA=248
PTPRC	NM_204417	F- ACTATTGACCTTACTCCAT R- GCCCATTTGTAGAACTTT	49.7	109
PLK2	XM_424739	F-TAGCCAAGCCAGGAGCATTAC R-TCCAGACATCCACGCAATACC	53.7	166
ENS-1	NM_001080873	F-CCTTGTGCTTGCTATCTTCTC R-CCTGTCCATCAGTGTCCTC	53.7	191
PABPC1	NM_001031597	F-AGAGAATGGCGAGTGTAAG R-TTGGAATGGATGAGGTCTG	59.5	197
RPL26L1	NM_001277099	F-TCAGACCGCAGCAAGAAC R-GACAACCTGGACTTCATCATC	53.7	150

11. Conclusions summarising the achievements and indication of scope for future work

Research on sperm RNA is at slow pace because of lack of suitable species-specific sperm RNA isolation protocol. RNA isolation from sperm presents several challenges such as low RNA quantity (22–45 times less RNA) and removal of somatic cells. In addition, sperm are highly condensed cells, and there are differences among species in sperm attributes and chromatin packaging which makes cellular contents isolation further challenging. In this project a protocol for RNA isolation from chicken sperm and removal of somatic cells from semen is developed. This new protocol does not require DNase treatment. Further, we encountered a problem in Protamine (PRM) amplification because of its high GC (88%) content. Protamine is expressed only in male germ cells and known as signature of sperm RNA. Therefore, a method for PCR amplification of PRM is developed during this study. The new method provides flexibility to user to use either specific Taq Polymerase or normal Taq polymerase in combination with DMSO. A protocol to check genomic DNA and somatic cell RNA contamination is developed using CD4 intron-spanning primers. The new method is very effective and economic in order to screen sperm RNA samples for genomic DNA and somatic cell RNA contamination in a single reaction. The RNA isolation protocol from chicken sperm ensures high quality genomic DNA free RNA preparations for microarray and whole transcriptome analysis to conduct functional genomics studies of fertility. Further, a simple PCR method to amplify high GC rich PRM gene would help in studying its role in fertility and in genome imprinting.

A catalog of chicken sperm RNA is developed using microarray technique. A total of 3142 unique sperm enriched RNAs are found in chicken sperm. A preliminary survey of the chicken sperm transcript profile for previously reported sperm RNA candidates identified several transcripts common in bovine, human, porcine and chicken. These transcripts showed differential expression pattern in chicken spermatozoa. Twenty least abundant probes based on the fold regulation in sperm RNA showed non-functional translational machinery in sperm. However, nineteen most abundant probes based on the fold regulation in sperm RNA are non-characterized. Therefore, it is imperative to make further attempts to characterize sperm RNAs by using more robust method such as deep RNA sequencing. Semen characteristics such as volume, concentration and motility are not associated with fertility. In a normal broiler breeder

flock approximately 30% male birds are of inherent low fertilization potential, which is huge in terms of production loss. The important point is that low fertilization potential birds simply cannot be identified by evaluating conventional semen characteristics. Our results demonstrate an association of sperm PRM and PLCZ1 RNA quantity with fertilization potential. Birds with high fertilization potential have three times more PRM and two times more PLCZ1 mRNA in their sperm compared to sperm of low fertilization potential birds. To the best of our knowledge this is the first report of an association of PRM and PLCZ1 transcripts quantity with fertilization potential in birds. There two transcripts may be exploited as fertility bio-markers in birds for captive breeding programs. However, these results are based on ten birds each of high and low fertilization potential; therefore, we strongly recommend replication of these results in large flocks of different breeds in order to use them as fertility bio-markers. Furthermore, we tried to verify these bio-markers in an *in-vitro* study in which sperm were incubated with bisphenol A for 30 minute prior to functional evaluation and artificial insemination in order to compromise sperm functions. Results demonstrate reduction in fertility and sperm characteristics as a result of compromised mitochondrial membrane potential which promotes higher incidences of apoptosis in sperm. Therefore, we could not isolate RNA from those sperm because of excess apoptosis.

It is essential to conduct future studies focused on the characterization of chicken sperm RNA by next generation RNA sequencing in order to conduct functional genomics studies of fertility. In addition, efforts should make towards understanding the role of sperm enriched transcripts in fertilization, early embryonic development and epigenetic modification.

12. S&T benefits accrued:
i. List of Research publications

S No	Authors	Title of paper	Name of the Journal	Volume	Pages	Year
1	R.P. Singh, C.M. Shafeeque, S.K. Sharma, N.K. pandey, R. Singh, J. Mohan, G. Kolluri, M. Saxena, B.	Bisphenol A reduces fertilizing ability and motility by compromising mitochondrial function of sperm	Environmental Toxicology and Chemistry	In press		2015

	Sharma, K.V.H. Sastry, J.M. kataria, P.A. Azeez					
2	C.M. Shafeeque, R.P. Singh, S.K. Sharma, J. Mohan, K.V.H. Sastry, G. Kolluri, V.K. Saxena, J.S. Tyagi, J.M. Kataria, P.A. Azeez	Development of a new method for sperm RNA purification in the chicken	Animal Reproduction Science	149	259-265	2014
3	S.K. Sharma, C.M. Shafeeque, J. Mohan, P.A. Azeez, R.P. Singh	PCR amplification protocol for GC rich protamine gene from chicken testis cDNA	Advances in Animal and Veterinary Sciences	2(11)	599-605	2014
4	C.M. Shafeeque, S.K. Sharma, K.V.H. Sastry, J. Mohan, R. P. Singh	Sperm RNA: a new class of fertility biomarkers for birds	Advances in Animal and Veterinary Sciences	2(3)	155-158	2014
5	C.M. Shafeeque, R.P. Singh, S.K. Sharma, J. Mohan, K.V.H. Sastry, G. Kolluri, V.K. Saxena, J.S. Tyagi, J.M. Kataria, P.A. Azeez	Chicken sperm transcriptome profiling by microarray	Submitted to Reproduction			2015
6	C.M. Shafeeque, S.K. Sharma, K.V.H. Sastry, J. Mohan, J.S. Tyagi, R.P. Singh	Protamine transcripts in chicken sperm: Bio-marker for fertility prediction	In: Proceedings of XXX Conference & National Symposium of Indian Poultry			2013

			Science Association 22-23 November, 2013, CARI, Izatnagar-243 122 (UP) INDIA			

ii. Manpower trained on the project

a) Research Scientists or Research Associates: Nil
b) No. of Ph.D. produced: Nil
c) Other Technical Personnel trained: One JRF

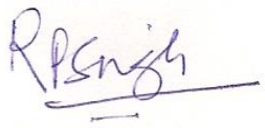
iii. Patents taken, if any: Nil

13. Financial Position:

No	Financial Position/ Budget Head	Funds Sanctioned	Expenditure	% of Total cost
I	Salaries/ Manpower costs	1800000	319838	15.23
II	Equipment		0	0
III	Supplies, Materials & Analytical charges		481826	22.94
IV	Contingencies		87109	4.15
V	Travel		163337.44	7.78
VI	Overhead Expenses	300000	300000	14.28
VII	Others, if any			
	Total	2100000	1352110.44	64.39

14. Procurement/ Usage of Equipment: Nil

Name and Signature
Date:



(Dr. R. P. Singh)
Principal Investigator