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ORIGINAL ARTICLE

Impact of male fertility status on the transcriptome of the bovine epididymis

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STUDY QUESTION: Can region-specific transcriptional profiling of the epididymis from fertile and sub-fertile bulls predict the etiology of fertility/sub-fertility in males?

SUMMARY ANSWER: The highly regulated gene expression along the bovine epididymis is affected by the fertility status of bulls used for artificial insemination.

WHAT IS KNOWN ALREADY: In mammals, sperm maturation and storage occur in the epididymis. Each epididymal segment has his own transcriptomic signature that modulates the intraluminal composition and consequently governs sequential modifications of the maturing male gamete.

STUDY DESIGN, SIZE, DURATION: Epididymides from six Holstein bulls with documented fertility were used. These bulls were divided into two groups: high fertility ($n = 3$), and medium–low fertility ($n = 3$) and their epididymal transcriptomic profiles were analyzed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Bovine cDNA microarray probing and bioinformatic tools were used to identify genes that are differentially expressed in caput, corpus and cauda epididymidal tissues of bulls with the documented fertility index.

MAIN RESULTS AND THE ROLE OF CHANCE: Hierarchical clustering and principal component analysis revealed a clear separation between caput, corpus and cauda epididymides. Some transcripts characterize a particular anatomical segment, whereas others are expressed in two out of three epididymal segments. Gene ontology analysis allowed deduction of specific functions played by each epididymal segment. The transcriptional profiles between fertile versus sub-fertile conditions clustered most closely in the corpus and cauda segments, whereas the profiles in the caput segment were distinct between fertile and sub-fertile bulls. Of the differently expressed genes, 10 (*AKAP4, SMCP, SPATA3, TCP11, ODF1, CTCFL, SPATA18, ADAM28, SORD and FAM161A*) were found to exert functions related to reproductive systems and 5 genes (*DEAD, CYST11, DEFB119, DEFB124 and MX1*) were found to be associated with the defense response.

LARGE SCALE DATA: The GEO number for public access of bovine epididymis microarray data is GSE96602.

LIMITATIONS, REASONS FOR CAUTION: Further work is required to link these modulations of epididymal functions with sperm fertilizing ability in order to understand the etiology of certain cases of idiopathic infertility in livestock and men.

WIDER IMPLICATIONS OF THE FINDINGS: As fertility can be quantified in bulls used for artificial insemination, this species is a unique model to aid in the understanding of male fertility/sub-fertility in man. Our data provide a molecular characterization that will facilitate advances in understanding the involvement of epididymal physiology in sub/infertility etiology.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by a grant to R.S. from the Natural Sciences and Engineering Research Council (NSERC) of Canada. C.L., A.A., E.C. and R.S. have no conflict of interest to declare. P.B. is R&D director at Alliance Boviteq Inc., a bovine artificial insemination company.

Key words: epididymis / male fertility / infertility / microarray analysis / differential gene expression / sperm maturation / transcriptional network.

Introduction

Spermatogenesis is a developmental process by which male germ cells go through a series of differentiation steps that lead to the production mature male gametes (Hermo *et al.*, 2010a,b). This complex spermatogenic event includes chromatin rearrangement, cytoplasm elimination, acrosome formation and flagellum development (Griswold, 2016; Neto *et al.*, 2016). Following spermatogenesis, sperm maturation occurring through the epididymis is characterized by a change in lipid composition of the plasma membrane, modification of surface proteins and nuclear condensation of sperm (Cornwall, 2009; Sullivan and Mieusset, 2016). The coordinated expression of many genes with different cellular and temporal specificities are implicated in this process (Belleannee *et al.*, 2012; Sipila and Bjorkgren, 2016).

About 8–12% of couples will face fertility problems during their reproductive years. The etiology underlying fertility problems can involve the female, male or both partners, with some cases remaining unexplained. Male sterility, i.e. the inability to impregnate a female partner, can originate from anatomical, genetic, endocrine or environmental factors and is not always curable. Fertility is a concept based on the probability of conception in couples having unprotected intercourse in order to impregnate the female partner. In a normal/fertile population, the pregnancy probability is 20% per ovarian cycle with a cumulative probability of 93% over a 12-month time period (Mosher and Pratt, 1987, 1991, 1993). In couples presenting with a fertility problem, this monthly probability of conception is diminished and can be as low as 3–5%. Testicular functions in men (androgen production and secretion and spermatogenesis) can easily be evaluated by circulating hormone concentration determination and by performing semen analysis according to the WHO recommendations (WHO, 2010). It is unfortunate that even with the currently available diagnostic tools, the etiology of male infertility remains unexplained for many men (Tuttelmann *et al.*, 2011). Considering the function of the epididymis in sperm maturation, we previously hypothesized that the former can be involved in the etiology of infertility in some men presenting with a normal clinical assessment of testicular functions (Sullivan, 2004; Sullivan and Mieusset, 2016).

Many knock-out mice present with a phenotype affecting male reproductive function. The males are generally sterile in these models and this can be attributed to the critical role played by the proteins encoded by the knocked-out genes in the complex processes leading to fertilization (Ikawa *et al.*, 2010). However, there is currently no laboratory animal model available to investigate causes of infertility defined by a low probability of conception. There are some endangered species that exhibit reduced fertility due to inbreeding resulting from the limited number of individual available for breeding but this condition is difficult to extrapolate to humans (Comizzoli, 2015).

Artificial insemination is a common practice for livestock species, particularly for the dairy industry. Frozen semen samples from genetically selected bulls are widely used for artificial insemination of cattle in the dairy industry (Funk, 2006). Bull semen processing for artificial insemination is standardized and the number of sperm contained in a semen straw is generally constant. In this context, male fertility is defined as the percentage of inseminated females that are not re-inseminated after a given number of days following the first insemination: the non-return rate (NRR) (van Doormaal, 1993). This fertility index is based on thousands of inseminations using comparable frozen-thawed semen straws. The NRR is adjusted by a linear statistical model to include confounding factors such as the semen price, the season, the age of the inseminated cow and the technician performing the insemination. NRRs are converted to another fertility index, the 'SOL', where zero is the average fertility of the bovine population at a given location for a specific period. This index is a strong tool for fertility quantification in cattle (Schaeffer, 1993). This presents a unique model with which to understand the physiological conditions governing inter-individual variability of male fertility that can be applied to humans.

As a result of the evolution of the artificial insemination market, some bulls used by the artificial insemination industry for semen production are slaughtered and their epididymides can be recovered. In the present study, we combined an mRNA-based microarray study with computational bioinformatics analysis to interrogate variation in gene expression between epididymal segments from fertile and sub-fertile bulls in order to investigate the contribution of the epididymis to the etiology of male infertility.

Material and Methods

Animals and fertility data

Bovine epididymides were obtained from six Holstein bulls (Gencor, Guelph, Ontario, Canada) that had phenotypic records and pedigree information. Bulls used in this study were sexually mature and within the same age range (51–71 months). Fertility indexes for each bull were evaluated by the Canadian dairy network as the NRR. The latter is based on the number of cows that do not return to service 56 days after the first insemination as the estrus cycle in cows is 21–22 days. The NRRs in this study were based on 282–486 first inseminations depending on the bull. The fertility index was adjusted by linear statistical model according to the effects of the month of insemination, the age of the inseminated cows, the herd, the technician performing the insemination and the semen price. The NRR was converted to 'SOL', another fertility index where zero is the average fertility of the population at a given location for a specific period (Table I) (van Doormaal, 1993). Epididymides of bulls with a well-characterized fertility index were recovered from the slaughterhouse and brought on ice to the laboratory within 2 h. These bulls were selected for slaughter based on milk production performances of the F1 females. The epididymides were dissected into caput, corpus and cauda segments and small pieces of tissue were snap-frozen in liquid nitrogen and stored at −80°C until use.

Table I Fertility data for fertile and sub-fertile bulls used in the transcriptomic studies.

Group	Bull	Age (months)	SOL	Insemination number
Fertile	1	62	4.1	474
	2	71	3.9	380
	3	60	3.9	376
Sub-fertile	4	61	−3.0	282
	5	55	−3.5	441
	6	60	−4.5	486

NRR, non-return rate.
Bulls were divided into two groups: fertile (SOL > 3.0; $n = 3$) and sub-fertile (−2.8 > SOL > −4.9; $n = 3$). SOL indicates the fertility solution, a normalized NRR.

RNA extraction and purification

As previously described by Belleannee *et al.* (2013), epididymis tissues from each segment were homogenized in Qiazol buffer (Qiagen, Toronto, ON, Canada) and total RNA was extracted using the RNeasy mini kit on-column DNase (Qiagen) treatment following the manufacturer's instructions. RNA quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Microarray and data analysis

Analysis of the DNA microarray was carried out with Affymetrix Bovine Gene 1.0 arrays according to the Affymetrix standard protocol. In brief, total RNA (200 ng per sample) was labeled using the Ambion WT Expression Kit, and hybridized to the arrays as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). After hybridization, the arrays were washed and stained in an Affymetrix GeneChip fluidics station 450. The arrays were scanned with the Affymetrix GCS 3000 7 G and GeneChip command Console Software (AGCC) (Affymetrix) was used to produce the probe cell intensity data. The image data were analyzed by Affymetrix Expression Console Software to perform the quality control, the background subtraction and the normalization of probe set intensities with the method of Robust Multiarray Analysis. The analysis was performed with Partek Genomics Suite 6.5 software (http://www.partek.com/partekgs). Microarray analyses were performed by the CHU de Québec Research Center (CHUL) Gene Expression Platform, Québec, Canada.

GOrilla

Gene ontology (GO) analysis was performed using a web-based (http://cbl-gorilla.cs.technion.ac.il/) application that identifies enriched GO terms at the top of a ranked list of genes using the minimum hypergeometric score as described by Eden *et al.* (2009). GOrilla also provides an exact *P*-value for the observed event and a directed acyclic graph with the representation of the significantly enriched terms.

Reverse transcription quantitative real-time PCR

Total RNA was isolated from epididymis segments using TRIzol Reagent (Invitrogen, Burlington, Ontario, Canada) followed by treatment with DNase 1. The quality of RNA samples was analyzed using Agilent Bionalyzer 2100 Chips and the quantity of total RNA was measured using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). As already described (Belleannee *et al.*, 2013), real-time PCR was performed by using SYBR Green assay on a Light Cycler (Roche, Laval, QC, Canada) according to manufacturer's instructions. In brief, 2 µl of reverse transcription templates, as well as a no-template negative control, were mixed with SYBR Green I Master Mix in addition to 0.5 µM of each primer to give a total volume of 20 µl in a capillary tube. Samples were incubated at 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. Fluorescence signals were continuously acquired at 530 nm from 65°C to 95°C at 0.2°C per second. Duplicate reactions were performed three times. A standard curve comprising five points was plotted for each mRNA. The standard curve was generated using serial dilutions of cDNA for all corresponding transcripts. Efficiency of amplifications was calculated from the slope of the standard curves according to the equation: $E = 10^{[-1/\text{slope}]}$. Relative quantification was obtained by normalization to three housekeeping genes, namely *TUBA1B*, *ACTIN* and *GAPDH*.

Statistical analysis

The numeric data from qRT-PCR were analyzed using GraphPad Prism (version 4; GraphPad Software Inc., San Diego, CA, USA) using one-way ANOVA followed by a two-tailed unpaired *t*-test. Values are presented as mean ± SEM.

Results

The Affymetrix Bovine GeneChip® was used to compare gene expression in caput, corpus and cauda epididymides from fertile and subfertile bulls. Three individuals from each group were included in this study (Table I). Among the 23 000 bovine qualifiers spotted on the chip, 14 725 (threshold applied with a log two intensity >6) were detected in the epididymis, while 14 110, 14 278 and 14 339 transcripts were in the caput, corpus and cauda regions, respectively.

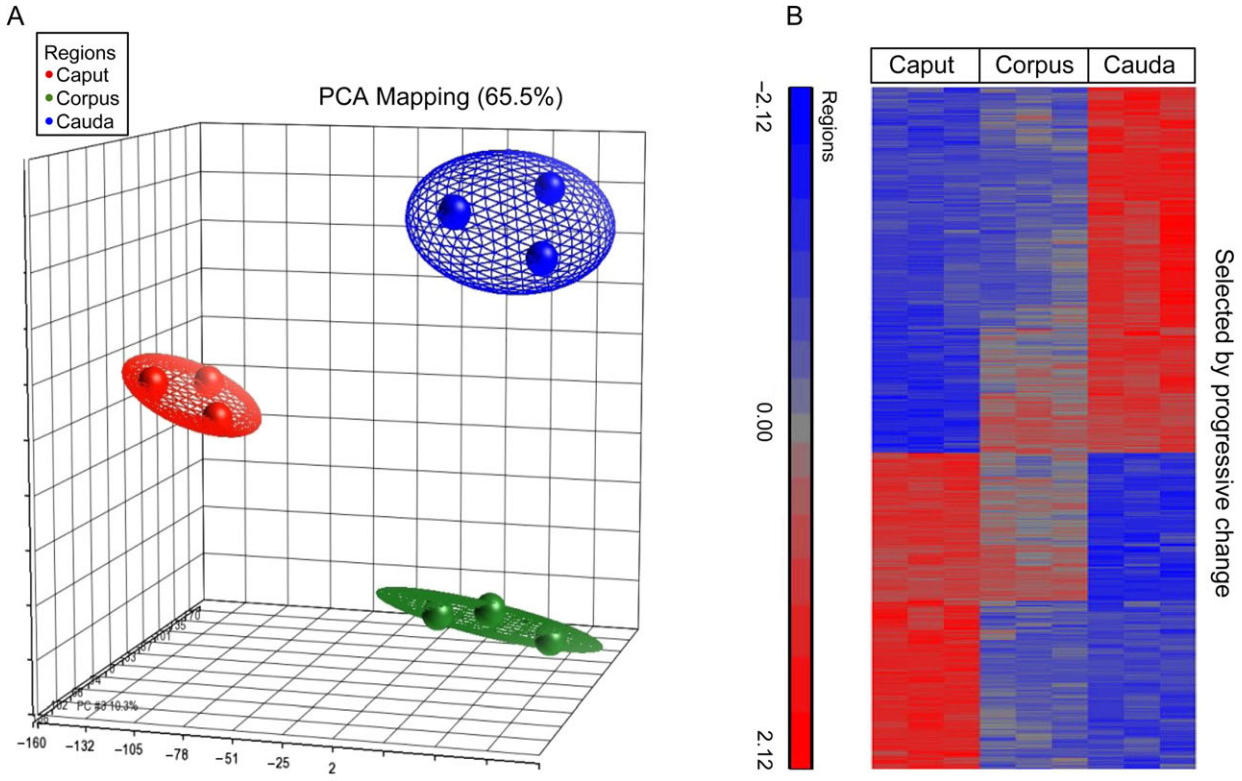
Temporal dynamics of gene expression along the epididymis of fertile bulls

A three-dimensional distribution profile known as principal component analysis (PCA) can be constructed from the variance for each gene. Genes with similar expression profiles cluster together in this space. This analysis clearly separated the data into three subgroups which clustered each epididymis segment, showing that the pattern of gene expression was different in caput, corpus and cauda sections, thereby demonstrating that the principal source of statistical variation was the epididymal segment (Fig. 1A).

Gene expression data of samples were compared by one-way ANOVA. Using stringent cut-off criteria of 2.0-fold change with a *P*-value corrected by FDR ≤0.05, a total of 2029 genes were found to be differentially expressed across the different segments of the epididymis. Among these genes, more than half ($n = 1028$) were progressively up- or down-regulated from caput to cauda segments of epididymis, as shown in Fig. 1B. The number of genes up-regulated toward the cauda was much higher than the number of genes with decreasing expression during progression from the caput to the cauda. To connect the individual gene lists to a broader functional framework, enriched biological processes (BPs) were identified by functional annotation clustering. Variations in the intensity of signal, the number of genes implicated, and the top 20 GO enriched BPs are shown in Fig. 1C. We identified a positive regulation of probe-sets involved in cilium and microtubule-based movement and a positive regulation of a large number of metabolic processes toward the caput segments, whereas cell signaling transduction, cell communication and proliferation were clearly up-regulated toward the cauda.

Profiling gene expression along the bovine epididymis

We further explored the dynamic nature of gene expression changes between three epididymal segments of fertile bulls: caput versus corpus, caput versus cauda and corpus versus cauda. A total of 1045 specific probe-sets, corresponding to 1022 bovine genes were significantly modulated between the caput and the cauda (Fig. 2A). GO analysis (GOrilla) of the modulated BPs is illustrated in Supplementary Fig. 1 and includes angiogenesis, ganglioside biosynthesis, calcium ion transmembrane transport, epithelial cilium movement and sialylation (Fig. 2B). There were 1285 probe-sets corresponding to 1250 genes



C

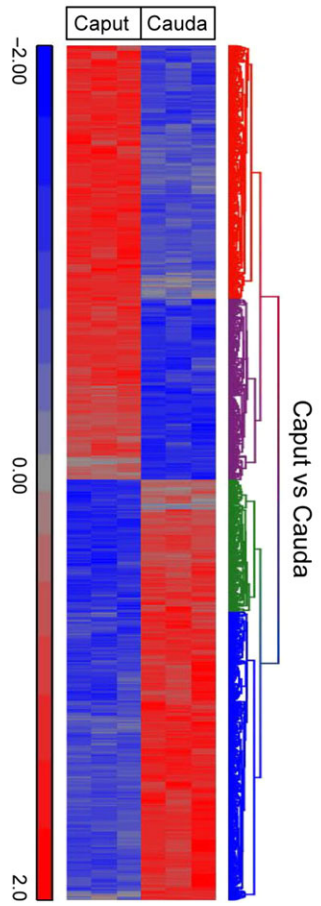
Up toward caput (biological process)	ES	# genes
cilium movement	17,029	8
microtubule-based movement	12,562	14
cellular lipid metabolic process	11,851	28
lipid metabolic process	10,892	33
amino-acid betaine transport	9,9397	3
carnitine transport	9,9397	3
nucleotide-sugar metabolic process	9,4998	5
small molecule metabolic process	9,4826	56
epithelial cilium movement	9,0266	4
anion transmembrane transport	8,5176	10
single-organism cellular process	8,5078	175
quaternary ammonium group transport	8,3649	3
microtubule-based process	8,3511	19
glycerolipid biosynthetic process	8,1072	8
alcohol metabolic process	8,0954	13
regulation of neuronal synaptic plasticity	7,9828	5
single-organism biosynthetic process	7,9044	30
organophosphate biosynthetic process	7,8899	16
lipid biosynthetic process	7,8053	16
nucleotide-sugar biosynthetic process	7,3698	3

Up toward cauda (biological process)	ES	# genes
positive regulation of biological process	27,62	163
positive regulation of cellular process	26,09	150
regulation of multicellular organismal process	24,28	92
regulation of cellular process	23,29	278
regulation of phosphate metabolic process	22,64	81
regulation of phosphorus metabolic process	22,46	81
regulation of signaling	22,23	96
regulation of cell proliferation	22,14	62
regulation of signal transduction	21,49	89
regulation of multicellular organismal development	21,24	66
anatomical structure development	21,14	96
regulation of cell communication	21,08	95
regulation of biological process	20,61	284
single-organism process	20,49	287
developmental process	20,02	142
regulation of developmental process	19,87	78
regulation of cellular component movement	19,65	37
single-organism developmental process	19,45	136
anatomical structure morphogenesis	19,42	64
negative regulation of cellular process	19,26	125

Figure 1 Microarray analysis of bovine epididymal transcriptome. (**A**) PCA plots of caput, corpus and cauda epididymide transcriptomes profiled by Affymetrix GeneChip microarray. Each blue, red and green dot represents individual samples from a fertile bull. (**B**) Hierarchical clustering of differentially expressed genes selected by progressive change along the epididymis. (**C**) GO enrichment analysis of genes selected by progressive change. PCA, principal component analysis; ES, enrichment score; GO, gene ontology.

modulated between caput and corpus epididymidis. These genes are involved in different BPs (Supplementary Fig. 2) including carboxylic acid transport, cholesterol metabolism, cellular response to cholesterol, regulation of pH and ganglioside metabolism (Fig. 3B). The corpus and cauda comparison revealed modulation of 987 probe-sets corresponding to 961 genes particularly involved in oligosaccharide and ganglioside metabolism, O-glycan processing, sialylation and response to steroid hormone (Fig. 4B and Supplementary Fig. 3).

A



B

Type	Function	p-value	Gene symbol
Biological Process	Angiogenesis	1.25E-05	*ANG, APOLD1, ARHGAP24, CALCRL, CDH13, CLIC4, COL4A2, CSPG4, CTGF, CYR61, ECSR, FGFR1, FN1, GJA5, HSPG2, ID1, ITGA5, JUN, MCAM, MFGE8, MMP14, NOV, NRP2, PARVA, PDGFRB, PIK3CB, PLCD3, PTPRB, RAPGEF3, RORA, S1PR1, SEMA3E, SERPINE1, THBS1, THSD7A, TSPAN12, VAV3*
	Ganglioside biosynthetic process	6.46E-05	*ST3GAL1, ST3GAL4, ST6GALNAC1, ST6GALNAC2, ST8SIA4, ST8SIA6*
	Calcium ion transmembrane transport	2.54E-05	*ATP2B3, ATP2B4, ATP2C2, CACNA1D, CACNA2D3, CACNG6, GAS6, GPM6A, ITPR1, ITPR2, ITPR3, JPH2, MCOLN2, MCOLN3, NCS1, PDE2A, PKD2, PRKCE, SLC24A2, SLC24A3, TMEM37, TRPA1, TRPM4, TRPM6, TRPM7, TRPV4*
	Epithelial cilium movement	8.68E-04	*CCDC103, CCDC39, DNAH1, LRRC6, NME5, ROPN1L*
	Sialylation	2.67E-05	*ST3GAL1, ST3GAL4, ST3GAL, ST6GAL1, ST6GALNAC1, ST6GALNAC2, ST8SIA4, ST8SIA6*

Figure 2 Transcriptomic differences between caput and cauda epididymides from fertile bulls. (**A**) Unsupervised hierarchical clustering analysis of genes differentially expressed between caput and cauda epididymidis. (**B**) Gene enrichment analysis of caput versus cauda epididymidis.

Comparison of epididymal transcriptomes of fertile and sub-fertile bulls

To uncover the transcriptional modification underlying the putative causes of sub-fertility in bulls, we compared the global transcriptome profiles of caput, corpus and cauda epididymides from fertile versus sub-fertile bulls.

PCA revealed that, in both the sub-fertile and fertile groups, the transcriptomic profiles are consistent from one bull to another. The transcriptional profiles between fertile and sub-fertile conditions clustered most closely in corpus and cauda segments, whereas the profiles in the caput segment were distinct between fertile and sub-fertile bulls (Fig. 5A). Two factor ANOVA analysis considering fertile conditions and caput/corpus/cauda anatomical localization revealed 530 genes with a fold change ≥ 1.2 with a *P*-value ≤ 0.05 (Fig. 5B).

Identification of common and exclusively regulated genes between the groups

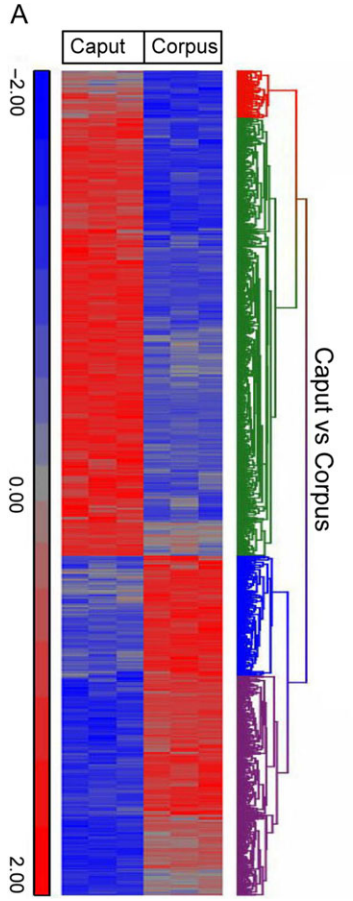
Heat maps of differentially expressed genes between fertile and sub-fertile bulls showed and heterogeneous distribution along the epididymis. Under-expressed genes in fertile bulls are less dependent on the anatomical epididymal segment in contrast to genes up-regulated in fertile bulls, which show more dependency on epididymal segment (Fig. 6A).

A Venn diagram was constructed to illustrate the distribution of common and exclusively up- and down-regulated genes found in the epididymal segments of sub-fertile versus fertile bulls (Fig. 6B). The highest number of differentially regulated genes (365) was found in the caput of the sub-fertile group versus the fertile group. Of these 365 genes, 13 showed common differential regulation with the other two epididymal regions, whereas 352 showed exclusive differential regulation. A total of 84 genes in the corpus segment were differentially regulated in sub-fertile versus fertile group analysis, of which 15 and 69 genes showed common and exclusive differential expression with other analyzed groups, respectively. There were 77 genes differentially regulated in the cauda section between the sub-fertile versus fertile groups, with 14 genes showing common differential expression. In total, only two genes were commonly regulated all along the epididymis (Fig. 6B).

Enrichment analysis of differentially expressed genes

Along with gene clustering distribution, gene enrichment analysis of dysregulated genes was performed to uncover alterations in BPs that

A



B

Type	Function	p-value	Gene symbol
Biological Process	Carboxylic acid transport	1.98E-04	*ABCC4, ABCD1, ACSL3, ATP8B1 , CLN3, CPT1A, EMB, FOLR1, OCA2, PDZK1, SLC16A12, SLC1A4, SLC1A5, SLC22A16, SLC25A13, SLC26A2, SLC26A3, SLC26A4, SLC38A1, SLC5A12, SLC6A14, SLC6A15, SLC7A11, SLC7A2, SLCO1A2, STXBP1, LCN12*
	Cholesterol metabolic process	3.57E-04	*ABCA1, ACLY, CAT, CEL, CFTR, CH25H, CUBN, CYP11A1, CYP39A1, CYP7A1, CYP7B1, NSDHL, OSBPL5, SCARB1, TM7SF2*
	Cellular response to cholesterol	3.14E-04	*AACS, ABCA1, CYP7A1, INHBA, LRP8*
	Regulation of pH	9.75E-04	*ATP6V1C2, CA2, CFTR, CLN3, EDN1, RHCG, SLC26A2, SLC26A3, SLC26A4, SLC9A2, SLC9A4, SLC9A9, TCIRG1*
	Ganglioside metabolic process	8.24E-07	*ST3GAL1, ST3GAL5, ST6GALNAC1, ST6GALNAC2, ST6GALNAC4, ST6GALNAC6, ST8SIA4 , ST8SIA6*

Figure 3 Transcriptomic differences between caput and corpus epididymides from fertile bulls. (**A**) Unsupervised hierarchical clustering analysis of genes differentially expressed between caput and corpus epididymidis. (**B**) Gene enrichment analysis of caput versus corpus epididymidis.

occurred in sub-fertile versus fertile groups using GO statistical analyses (GOstats) and the Partek microarray analysis tool. It was observed that genes involved in locomotion, immune system processes, biological adhesion, reproduction processes and cellular processes were highly represented (Fig. 7A–C).

With relevance to reproductive physiology, we identified nine genes with higher expression and three with lower expression in the sub-fertile caput region when compared with the fertile caput region (Table II). The expression levels of six genes were up-regulated in corpus from sub-fertile bulls versus the fertile group and two genes were down-regulated (Table III). Moreover, there were two genes with higher expression and 3 with lower expression in the sub-fertile cauda region when compared with the fertile cauda region (Table IV). Of the differently expressed genes, 10 (including *AKAP4, SMCP, SPATA3, TCP11, ODF1, CTCFL, SPATA18, ADAM28, SORD* and *FAM161A*) exert functions related to reproduction activity and the expression of five genes (including *DEAD, CYST11, DEFB119, DEFB124* and *MX1*) is associated with the defense response. Differentially expressed transcripts from the caput, corpus and cauda epididymidis in fertile versus sub-fertile bulls are listed in Supplementary Table 1.

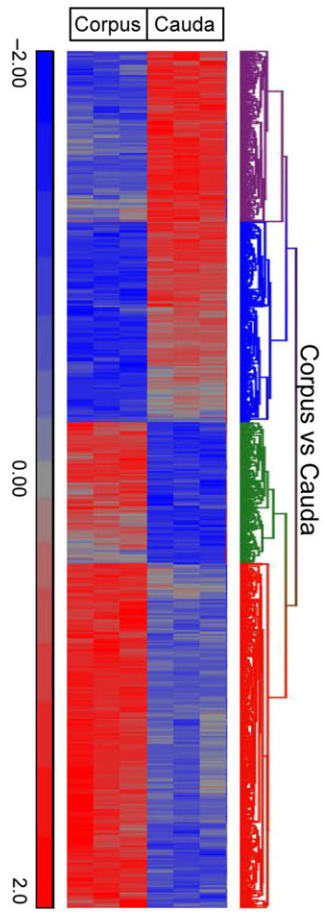
Microarray validation by qRT-PCR

Validation of differentially expressed genes in the microarray was performed by qRT-PCR. Because of the magnitude in fold change and relevance to reproductive physiology, *AKAP4, SMCP, GPX5, GSTA2, DEFB119, DEFB124* and *DEFB7* expression were detected in three individuals of each group by qRT-PCR. Figure 8 illustrates the qRT-PCR results confirming the consistency of microarray data. Of the seven genes assayed, the expression along the epididymis of five genes was similar to microarray data. Although the results were not statistically significant, the remaining two genes showed the same expression pattern between microarray and qRT-PCR.

Discussion

The involvement of the epididymis in the acquisition of sperm fertilizing ability has been recognized since the 1960s. The excurrent duct is also involved in sperm concentration and storage (Caballero *et al.*, 2010; Bedford, 2015; Sullivan and Mieusset, 2016) and has recently been shown to be involved in the transfer of epigenetic information to offspring (Sharma *et al.*, 2016). Omic technologies have been applied to

A



B

Type	Function	p-value	Gene symbol
Biological Process	Oligosaccharide metabolic process	1.36E-05	*GM2A, MAN2A2, MAN2B2, MGAM, MGAT4A, NEU1, ST3GAL4, ST6GAL1, ST6GAL2, ST6GALNAC6, ST8SIA4, ST8SIA6, ST6GALNAC2*
	Response to steroid hormone	1.50E-07	*AAC, ABCA2, ADM, ALAD, BCHE, BMP6, C3, CDKN1A, CITED1, CLDN4, CTGF, ESR1, FBXO32, FLT3, FN1, FOS, GC, GRN, IGFBP2, IL10, JUNB, KCNJ11, LOX, MAOB, PAWR, PENK, PLN, POSTN, PTGER3, PTPRU, S100B, SREBF1,STC1,TGFB2 TGFB3, THBS1*
	Sialylation	3.54E-05	*ST3GAL4, ST6GAL2, ST6GALNAC2, ST6GALNAC6, ST8SIA4, ST8SIA6,ST6GAL1*
	O-glycan processing	3.19E-04	*GALNT1, GALNT12, GALNT3, GALNT5 , GALNT8 , MUC20, ST3GAL4, ST6GAL1, ST6GALNAC2, MUC1*
	Ganglioside metabolic process	1.43E-04	*GM2A , NEU1, ST3GAL4, ST6GALNAC6, ST8SIA4, ST8SIA6, ST6GALNAC2*

Figure 4 Transcriptomic differences between corpus and cauda epididymides from fertile bulls. (**A**) Unsupervised hierarchical clustering analysis of genes differentially expressed between corpus and cauda epididymidis. (**B**) Gene enrichment analysis of corpus versus cauda epididymidis.

different mammalian species to document the molecular mechanisms involved in sperm post-testicular processing (Dacheux *et al.*, 2009; Rolland *et al.*, 2013; Drabovich *et al.*, 2014). Descriptions of epididymal transcriptome are available for the mouse (Johnston *et al.*, 2005), rat (Jelinsky *et al.*, 2007), human (Thimon *et al.*, 2007) (Zhang *et al.*, 2006; Dube *et al.*, 2007) and boar. More recently, deep-sequencing of mRNAs obtained from primary human epididymal principal cells enriched *in vitro* has completed the transcriptomic pictures of this organ from the human male reproductive tract (Browne *et al.*, 2016). From these studies, it can be concluded that gene expression is highly segmented and regulated along the epididymis of all studied mammalian species (Belleannee *et al.*, 2012).

The epididymis is classically divided into three segments; the caput, corpus and cauda (Cooper, 1998). A proximal initial segment has been described in small rodents and is thought to play a major role in sperm physiology. In rodents, and possibly in other mammalian species, the epididymis is divided into segments by septa that have recently been proposed to be involved in the regulation of gene expression (Turner, 2008; Domeniconi *et al.*, 2016). As these septa have not yet been described in cattle, our study was based on the classical anatomical division of the excurrent duct into caput, corpus and cauda segments (Goyal, 1985). PCA revealed that as in other species, the bovine epididymis expresses thousands of genes; some transcripts characterize a particular anatomical segment, while others are expressed in two out of three epididymal segments. GO analysis allowed deduction of specific functions played by each epididymal segment. GOrilla views clearly illustrated the divergence of functions along the bovine excurrent duct, with the caput being specialized in cilium movement, angiogenesis and calcium ion transmembrane transport. The corpus plays a major role in pH regulation, which is known to affect sperm quiescence (Breton *et al.*, 1996; Bernabo *et al.*, 2016), in addition to cellular and metabolic processes regulated by cholesterol (Saez *et al.*, 2011; Bernabo *et al.*, 2016). With knowledge of the roles of pH, calcium and cholesterol in sperm physiology, it is suggested that the caput and corpus are involved in sperm maturation as described in other species. Our results showed that the cauda segment is possibly involved in post-translational modifications such as protein glycosylation (sialylation and oligosaccharide processing such as O-glycosylation) (Cornwall, 2014). The distal epididymis seems to be more responsive to steroid hormones. Thus, the bovine model is another

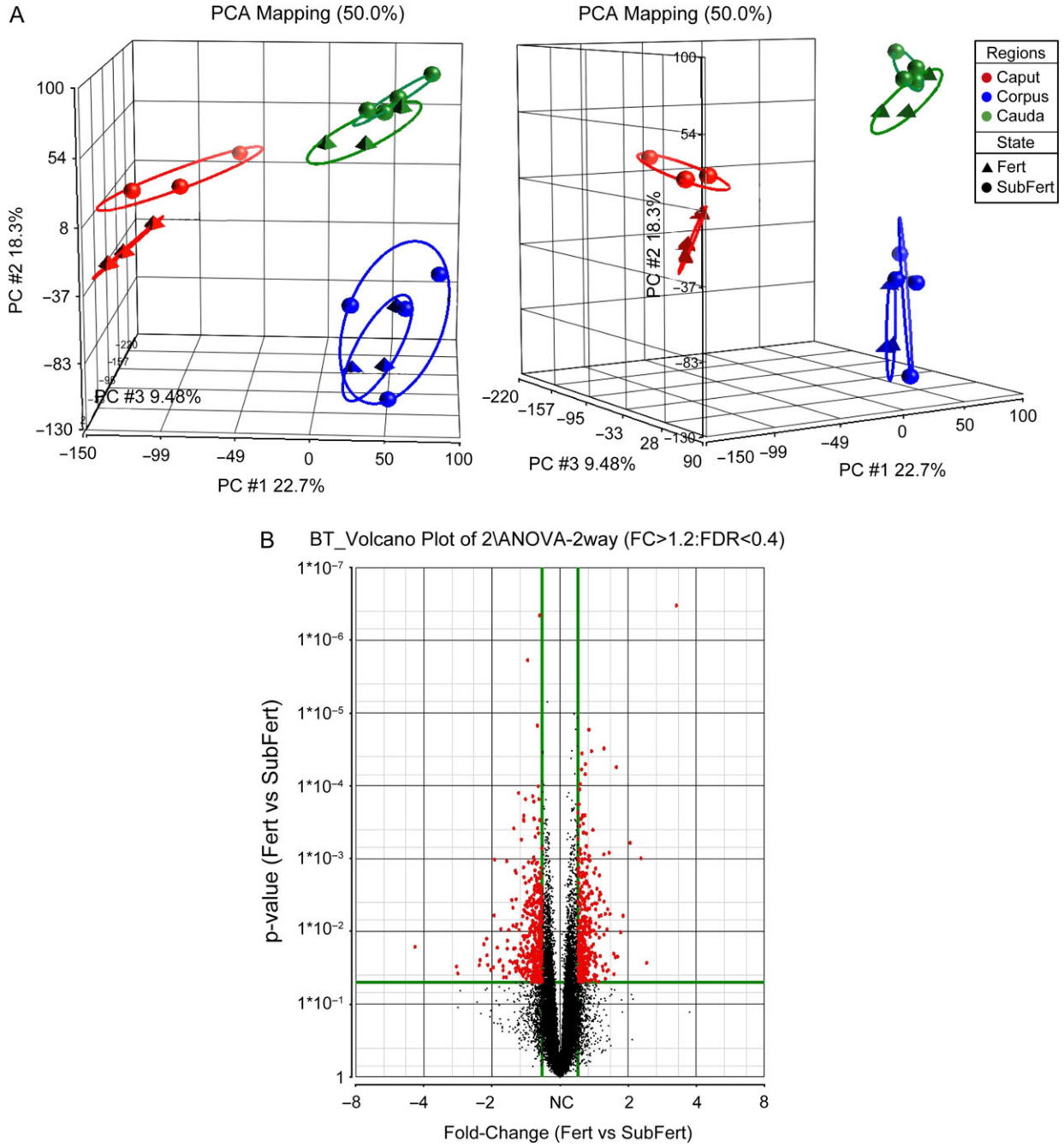


Figure 5 Comparison of global epididymis gene expression between fertile and sub-fertile bulls. (**A**) PCA of microarray data. Dots in blue and red represent individual samples from fertile and sub-fertile bulls, respectively. The two panels illustrate the same PCA from two different angles. (**B**) Volcano plot based on fold change ≥1.2 and *P*-value ≤ 0.05 of all transcripts.

species with highly modulated gene expression from one epididymal segment to the next.

Transcriptomic studies on the epididymis have focused on segregation of gene expression into anatomical regions (caput, corpus and cauda) or segments as defined by septa in rodent epididymis (Domeniconi *et al.*, 2016). By applying highly stringent analysis criteria to transcriptomic analysis of epididymal regions of fertile bulls, we identified more than a thousand genes that are progressively up- or down-regulated along the organ. GO analysis revealed multiple functions associated with these genes regulated in a progressive manner all along the bovine epididymis. Thus, gene expression along the epididymis is not only segregated according to the anatomical epididymal segments, an important cluster of genes is gradually up- or down-regulated along the length of the organ, adding another level of

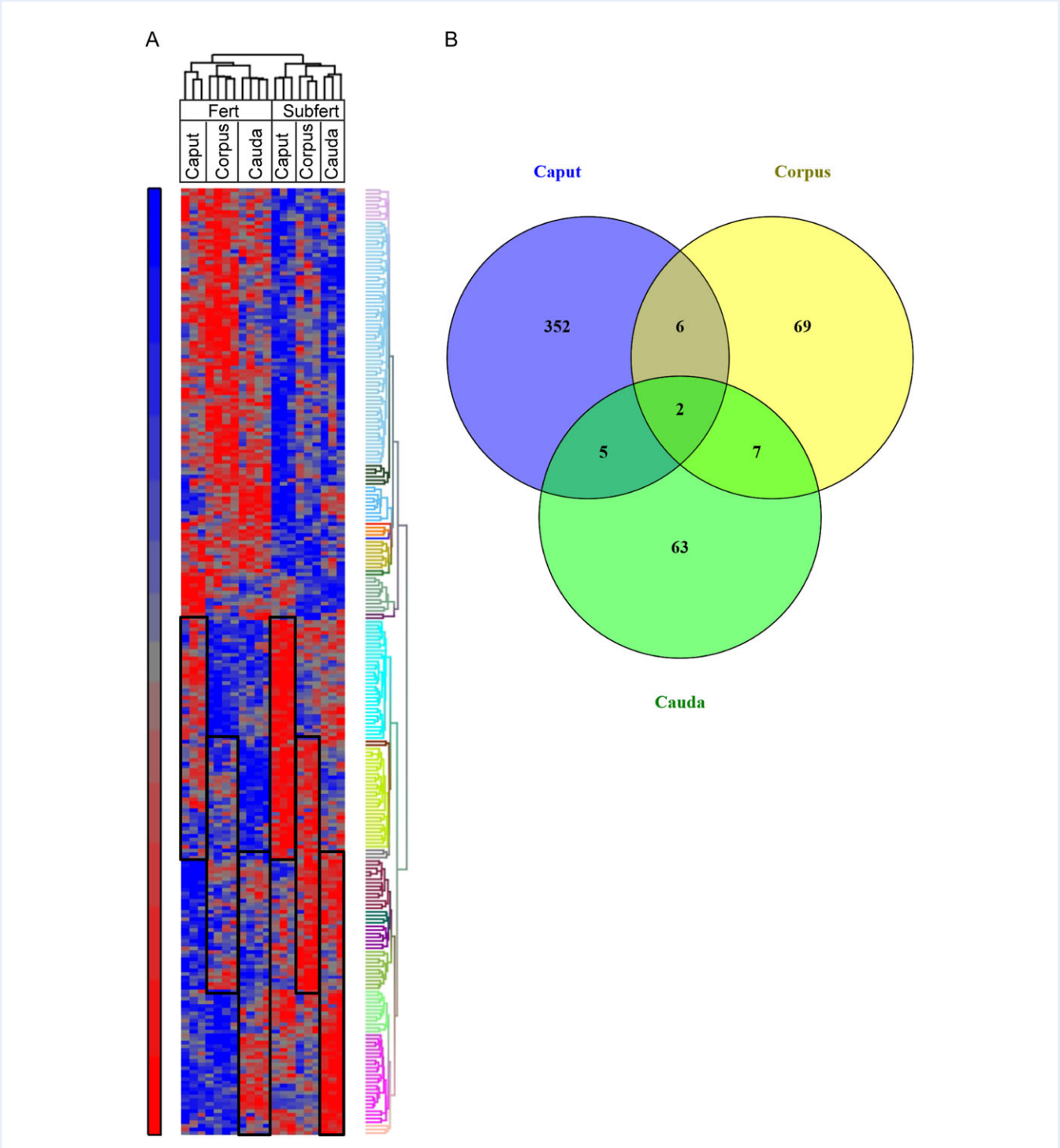


Figure 6 (**A**) Unsupervised hierarchical clustering analysis of genes differentially expressed among caput, corpus and cauda epididymides from fertile and sub-fertile bulls. The color key under the heatmap represents the different expression levels. Red represents up-regulated genes and blue represents down-regulated genes. (**B**) Venn diagram of the significantly differentially up- and down-regulated (FC ≥1.2, *P*-value ≤ 0.05) and overlapping genes found in the epididymal segments of sub-fertile versus fertile bulls.

complexity to the control of gene expression regulating epididymal functions and sperm physiology (Guyonnet *et al.*, 2011). To the best of our knowledge, this is the first report of progressive up- or down-regulation of major gene clusters along the bovine epididymal tubule.

The main objective of this transcriptomic analysis of epididymal microarray data sets was to compare the epididymal transcriptomes of fertile and sub-fertile bulls in order to document the etiology of inter-individual male fertility variability. Proteomic tools have been

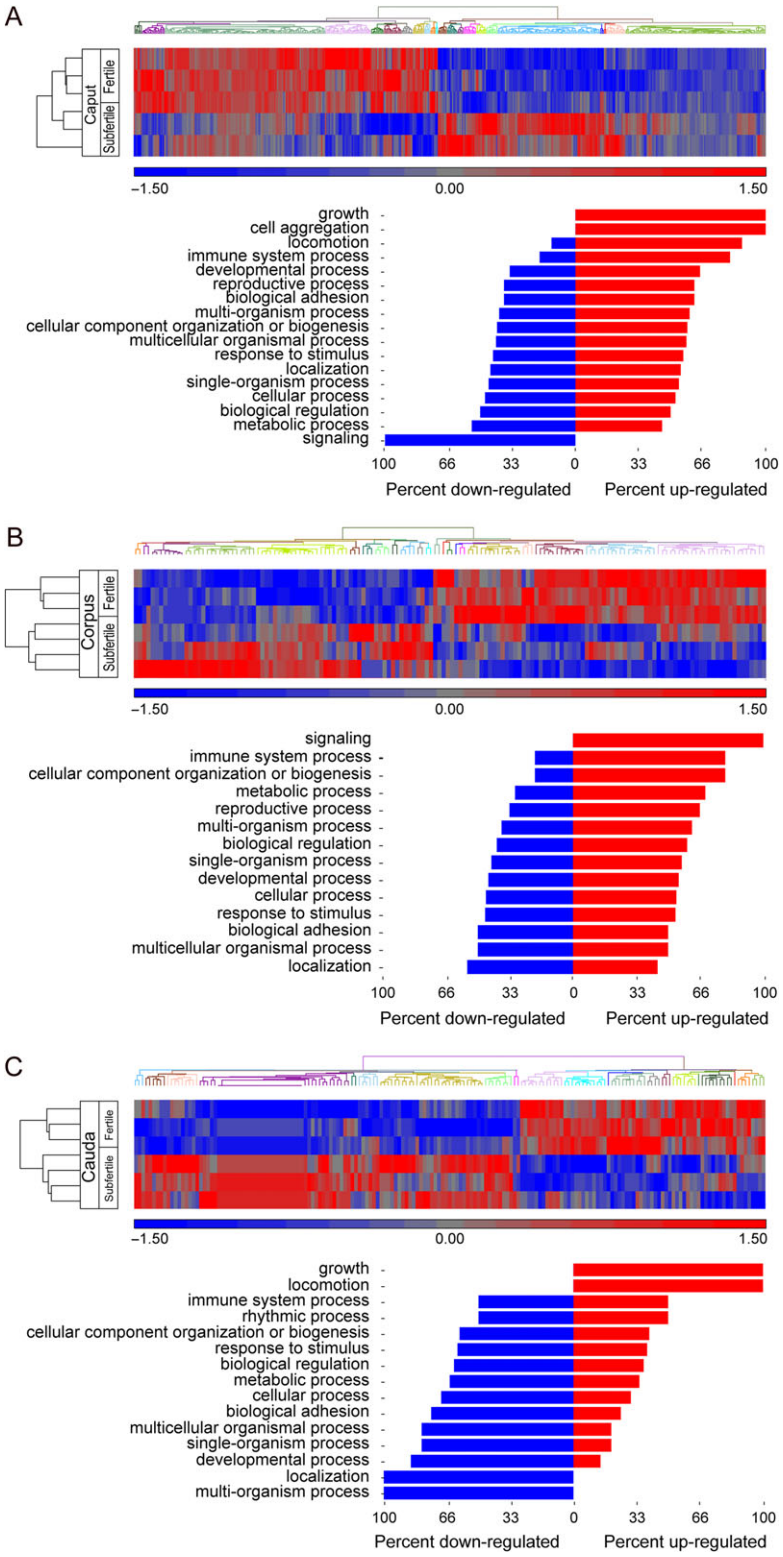


Figure 7 Clustering and GO gene annotation analysis of epididymal segment microarray data. Unsupervised hierarchical clustering analysis of genes differentially expressed among caput (**A**), corpus (**B**) and cauda (**C**) epididymides from fertile and sub-fertile bulls (top panel). The forest plot shows the percentage of genes associated with each BP that are up-regulated (red) or down-regulated (blue) (low panel). *P*-value ≤ 0.05. BP, biological process.

Table II Annotations of selected altered genes identified in caput from sub-fertile compared with fertile bulls.

Gene cymbol	Fold change	*P*-value	Gene assigment	Function
UP-regulated				
AKAP4	2.26153	0.0140419	A kinase (PRKA) anchor protein 4	Major structural component of sperm fibrous sheath. Plays a role in sperm motility
SMCP	2.15603	0.0465674	Sperm mitochondria-associated cysteine-rich protein	Involved in sperm motility. Localizes to the capsule associated with the mitochondrial outer membranes and is thought to function in the organization and stabilization of the helical structure of the sperm's mitochondrial sheath
SPATA3	2.13261	0.00359619	Spermatogenesis associated 3	Play a role in the antiapoptotic process or spermatocyte but its function as yet remains unclear
SPZ1	2.07514	0.0194219	Spermatogenic leucine zipper 1	May play an important role in the regulation of cell proliferation and differentiation during spermatogenesis
TCP11	2.05113	0.00447944	t-complex 11 homolog (mouse)	May play an important role in sperm function and fertility
ODF1	2.01084	0.0536174	Outer dense fiber of sperm tails 1	Component of the ODFs of spermatozoa. May help to maintain the passive elastic structures and elastic recoil of the sperm tail
CTCFL	2.0077	0.0329747	CCCTC-binding factor (zinc finger protein)-like	Testis-specific DNA binding protein responsible for insulator function, nuclear architecture and transcriptional control, which probably acts by recruiting epigenetic chromatin modifiers. Plays a key role in gene imprinting in male germline. Seems to act as tumor suppressor
CRISP2	1.91782	0.0544513	Cysteine-rich secretory protein 2	May regulate some ion channels' activity and thereby regulate calcium fluxes during sperm capacitation. Presence in varicoceles patients
SPATA18	1.7408	0.00884795	Spermatogenesis associated 18 homolog (rat)	
Down-regulated				
CATHL4	−1.60927	3.48E-05	Cathelicidin 4	Potent microbicidal activity
SSTR2	−1.76654	7.23E-04	Somatostatin receptor 2	May play a crucial role as a local inhibitor of FSH action on GCs apoptosis and steroidogenesis.
GSTA2	−1.81979	7.03E-02	Glutathione S-transferase alpha 2	Protecting the cells from reactive oxygen species and the products of peroxidation.

Fold change ≥1.2 corresponds to over-expression in sub-fertile bull. Values ≤ −1.2 corresponds to under-expression in sub-fertile bull. Microarray data of transcripts listed in red are those validated by qRT-PCR. ODFs, outer dense fibers.

Table III Annotations of selected altered genes identified in corpus from sub-fertile compared with fertile bulls.

Gene symbol	Fold change	*P*-value	Gene assigment	Function
Up-regulaled				
GPX5	7.40656	1.63E-03	Glutathione peroxidase 5	Play a role in protecting the membranes of spermatozoa from the damaging effects of lipid peroxidation and/or preventing premature acrosome reaction
CST11	4.62593	1.70E-03	Cystatin 11	Has antibacterial activity may play a role in sperm maturation and fertilization
ADAM28	3.76343	7.28E-03	ADAM metallopeptidase domain 28	May be involved in sperm maturation
MX1	2.906	1.99E-05	Myxovirus (influenza virus) resistance 1	Participates in the cellular antiviral response
DEFB124	2.35286	3.73E-03	Defensin. beta 124	Has antibacterial activity
LCN9	2.20753	2.37E-03	Lipocalin 9	Epididymis luminal protein ligands and transport them to specific cells
Down-regulated				
DEFB7	−1.74825	9.71E-04	Defensin beta 7	Has antibacterial activity
DEFB119	−1.87926	5.31E-04	Defensin. beta 119	Participate in immune defense

Fold change ≥1.2 corresponds to over-expression in sub-fertile bull. Values ≤ −1.2 corresponds to under-expression in sub-fertile bull. Microarray data of transcripts listed in red are those validated by qRT-PCR.

Table IV Annotations of selected altered genes identified in cauda from sub-fertile compared with fertile bulls.

Gene symbol	Fold change	*P*-value	Gene assignment	Function
Up-regulated				
EDDM3B; HE3b	1.88676	9.54E-02	Epididymal protein 3B	Possible function in sperm maturation has been found up-regulated in epididimis of nonobstructive azoospermic men
TSPAN13	1.73761	1.56E-03	Tetraspanin 13	Play a role in the regulation of cell development, activation, growth and motility. Up-regulated in prostate cancer
Down-regulated				
SORD	−1.51125	1.87E-03	Sorbitol dehydrogenase	Part of the polyol pathway that plays an important role in sperm physiology. May play a role in the sperm motility by providing an energetic source for sperm
FAM161A	−2.035	1.00E-03	Family with sequence similarity 161. member A	
MUC1	−3.05341	1.09E-03	Mucin 1. cell surface associated	May provide a protective layer on epithelial cells against bacterial and enzyme attack

Fold change ≥1.2 corresponds to over-expression in sub-fertile bull. Values ≤ −1.2 corresponds to under-expression in sub-fertile bull.

previously applied to spermatozoa of bulls presenting with different NRRs. DIGE, LC–MS/MS proteomic techniques have been used to compare seminal plasma, and total or sub-fractions of spermatozoa in order to identify protein markers of fertility (Moura *et al.*, 2006a; Rolland *et al.*, 2013; Drabovich *et al.*, 2014). Proteins from cauda epididymal fluids (Moura *et al.*, 2006b), from seminal plasma (Moura *et al.*, 2006c) and from sperm protein extracts (Parent, 1999) have been associated with fertility data. Other studies have used regression models to correlate bull fertility with two or more quantified proteins detected in sperm extracts from bulls presenting with different fertility status (D'Amours *et al.*, 2010). The involvement of the epididymis in sperm fertility is a well-established concept, but the availability of epididymal tissues and fertility data from the same individuals of a given mammalian species has limited the investigation of the involvement of the epididymis in the physiopathology of male infertility.

The availability of epididymides from bulls with robust fertility data sets, allowed PCA of bovine transcriptome variability in accordance with fertility. PCA clearly demonstrated that the principal source of variations of the epididymal transcriptomic profiles resides in the caput segment showing differences between the fertile and sub-fertile groups. The segregation of the corpus and the cauda data sets was not as affected as the caput segment by the fertility status of the six investigated bulls. With the knowledge that the proximal regions of the epididymis are the most active epididymal segments in sperm maturation, it makes sense that inter-individual variability in the caput epididymal transcriptome impacts the fertility status. Corpus and cauda segments were also affected under the sub-fertile condition, but to a lesser extent; of the 441 epididymal transcripts affected by the sub-fertility status, 365 were expressed in the caput segment. The microarray data sets were consistent between the three fertile or sub-fertile individuals.

Multiple BPs are affected by fertility status and their relative importance from one segment to another varies along the epididymis. Overall, a lower number of BPs are affected in the cauda epididymides when compared to the two other segments. How these affected genes impact sperm functions, and thereby fertility status, remains to be demonstrated. Cholesterol metabolism, regulation of pH, post-translational modifications and calcium ion transmembrane transport, are examples of epididymal biological functions that are affected in sub-fertile individuals and that impact sperm processing during epididymal transit. Examples of these modulated genes and their relationships to sperm physiology are listed in Tables II–IV. Some of these transcripts affected by fertility are known to interact with spermatozoa and to be involved in sperm maturation such as *TCP11, CRISP2, CST11, ADAM28 and LCN9*. Thus, a correlation between these transcriptomic signatures of bulls presenting with fertility variability and sperm functionality can be established.

The majority of the epididymal transcripts up-regulated in the caput are associated with spermatogenesis and sperm ultrastructure; these include *AKAP4, SMCP, SPATA3, SPZ1, ODF1, CTCFL* and *SPATA18*. Considering that spermatozoa are present in the epididymal tissues processed for mRNA extraction, the detection of these transcripts suggests that their presence in the caput transcriptomes of sub-fertile bulls may be due to the fact that these mRNAs remain associated with sperm cells with incomplete spermiogenesis (Hermo *et al.*, 2010b). The possibility that these mRNAs remain associated with sub-optimal sperm in the caput segment can also be considered. It has been hypothesized that the epididymis is equipped with mechanisms allowing elimination of defective spermatozoa (Jones, 2004; Cornwall *et al.*, 2007; D'Amours *et al.*, 2012). Detection of transcripts associated with spermiogenesis in the caput epididymis of sub-fertile individuals could be the consequence of sub-optimal mechanisms of elimination of defective sperm cells in the proximal epididymis.

Numerous components of the immune system, in particular those involved in the innate defense, have been described in the epididymis (Cooper, 1999; Guiton *et al.*, 2013). Two defensin genes (particularly DEFB126/BBD126), have been shown to be expressed in the bull reproductive tract (Fernandez-Fuertes *et al.*, 2016; Narciandi *et al.*, 2016) and defensin expression is linked to fertility data (Dorin and Barratt, 2014). The immune system is one of the most affected biological systems when comparing epididymis transcriptomes of sub-fertile bulls with fertile bulls. In contrast to genes involved in spermatogenesis and sperm ultrastructure affected by the fertility status in the caput, these genes involved in the immune defense are dysregulated in the three epididymal segments: *CATGL4* and *GSTA2* in the caput (Table II), *GPX5, MX1* and *DEFB124* in the corpus (Table III) and

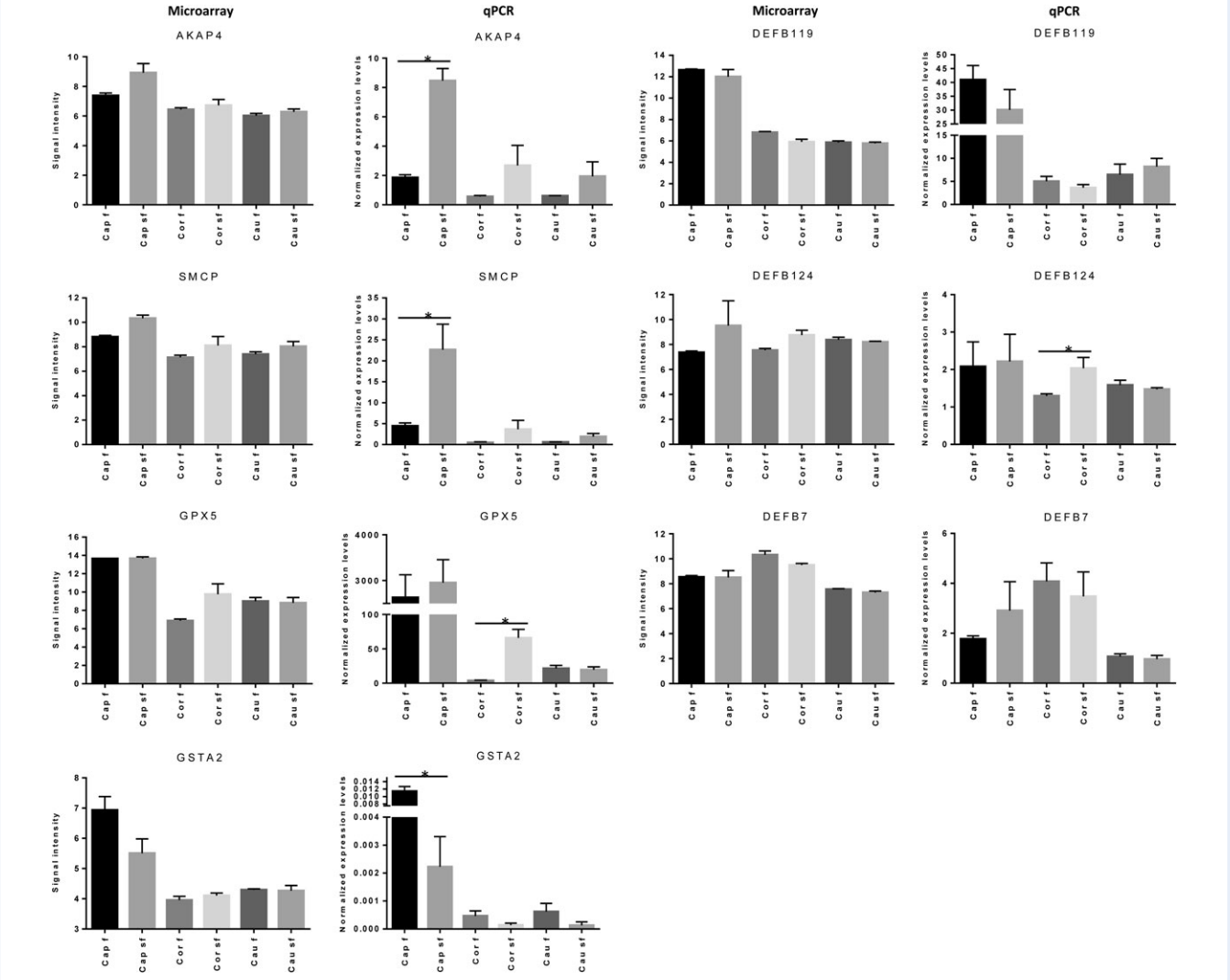


Figure 8 Validation of microarray results by qRT-PCR quantification. Expression of the genes *AKAP4*, *SMCP*, *GPX5*, *GSTA2*, *DEFB119*, *DEFB124* and *DEFB7* was evaluated by microarray and qRT-PCR experiments. In microarray experiments, values are represented by $\log^2$-transformed gProcessed signal. qRT-PCR data are shown as means and standard deviations of results obtained from $n = 3$ biological samples per group after normalization to three housekeeping genes, namely *TUBA1B*, *ACTIN* and *GAPDH*. Data were analyzed by unpaired *t*-test with 95% confidence intervals using the GraphPad Prism 5 program. $^*P \leq 0.05$.

DEFB7 and *DEFB119* in the cauda segment. It can be hypothesized that a sub-optimal immune defense system may be another factor affecting sperm functionality in sub-fertile individuals.

We have previously compared the epididymal transcriptome of normal and vasectomized men and hypothesized that the epididymis following vasectomy can represent a model of epididymal sequelae affecting fertility in men (Sullivan and Mieusset, 2016). We demonstrated that the percentage of overlap between human and bovine epididymis was observed at the anatomical level, by a coherent similarity of functional compartmentalization and also by the similarity of changes observed in sub-fertile bulls and in the epididymis after vasectomy-induced sequelae. Interesting correlations can be found between ontology terms lists, protein–protein interaction networks, upstream regulators or pathways. We found at least 32 significantly enriched BP terms in both studies: bull sub-fertility and human vasectomy-induced sequelae (data not shown). These terms highlight three main groups of BPs: intermediate filament organization, negative regulation of glucocorticoid bio-synthetic process and ion transmembrane transport. These correlations are currently under study.

In conclusion, gene expression along the bovine epididymis is highly regulated as the caput, corpus and cauda segments have their own transcriptomic signatures. The gene expression profile is affected in sub-fertile bulls when compared with bulls with a high SOL. The caput appears to be the most affected region when bull fertility is sub-optimal. GO analysis suggests that sperm maturation, immune defense systems and elimination of defective spermatozoa may be the most affected functions of the epididymis in sub-fertile bulls. Further work is required to link these modulations of epididymal functions with sperm

fertilizing ability in order to understand the etiology of certain cases of idiopathic infertility in livestock and men.

Supplementary data

Supplementary data are available at *Molecular human reproduction* online.

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Authors' roles

All authors contributed to the manuscript. C.L. and R.S. designed and supervised the project. C.L., A.A., E.C. were responsible for data collection and analysis, P.B. provided bovine fertility data and biological material. C.L. and R.S. prepared the manuscript and figures.

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Conflict of interest

C.L., A.A., E.C. and R.S. have no conflict of interest to declare. P.B. is R&D director at Alliance Boviteq Inc., a bovine artificial insemination company.

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