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# Transcriptome Analysis of Ovarian Tissues Reveals Key Genes Involved in the Prolificacy of Ussuri Raccoon Dog

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## ABSTRACT

**Background:** Ussuri raccoon dog is an important economic animal, which brings considerable economic benefits to Hebei, China. The high fertility rate of Ussuri raccoon dog is vital for its breeding industry. However, the molecular mechanism of prolificacy traits in raccoon dog remains unclear. Therefore, it is of great importance to identify the related genes and analyze the genetic mechanism of its high reproductive traits.

**Methods:** In our study, RNA-seq was performed using ovarian tissues from six Ussuri raccoon dogs during estrus. The samples were divided into two groups based on the litter size over the last three consecutive years. We performed a standard transcriptomic analysis procedure to dig for differentially expressed genes (DEGs) and analyze gene function in detail.

**Result:** Statistical analysis revealed a total of 612 DEGs between the low-fecundity and high-fecundity group including 277 upregulated and 335 downregulated DEGs, respectively. KEGG pathway analysis shows the DEGs enriched in several reproductive related pathways, such as the Circadian entrainment pathway. A total of 8 DEGs were identified with differential alternative splicing (DAS) events and recognized as DAS-DE genes. Among the up-regulated DAS-DE genes, ABCA6 and GPAM were found to be the most important genes regulating the prolificacy trait of Ussuri raccoon dog. The DEGs and DAS-DE genes identified in this study provide a probable genetic mechanism for improving prolificacy of Ussuri raccoon dog breeding.

**Key words:** Differentially expressed gene, Fertility, RNA-seq, Ussuri raccoon dog.

## INTRODUCTION

Ussuriraccoon dog (*Nyctereutes procyonoides*) belongs to Carnivoraes, Canidae, Nyctereutes. It is a rare local species of macrocapillaria fur animals domesticated and cultivated by Chinese Academy of Agricultural Sciences. Ussuri raccoon dogs' skins are characterized by long villi, elastic, soft and flexible (China National Commission, 2012). It is the most important breeding variety in Hebei Province, which has brought considerable economic benefits to the people and become the first characteristic industry in Hebei Province, China.

However, the low reproductive number of Ussuri raccoon dogs becomes one important reason limited the development of the industry in some areas. Litter size is an important index of fertility, which is a highly complex trait determined by many complex processes and has low heritability (Ma *et al.,* 2019). Therefore, it is necessary to make use of scientific method to identify important candidate genes related to litter size. Artificial selective breeding can lead to a litter size improvement of the population (Chen *et al.,* 2015). As an important reproductive organ, the ovary determines the limit of litter size and the number of ovulation is a significant, ongoing factor of litter size (Huang, 2016). Therefore, it is feasible to conduct a research on the litter size trait of raccoon dogs using ovary as materials.

With the rapid development of high-throughput sequencing technology, RNA-seq technology has been well established. It can detect a complete set of transcripts and their quantities in specific tissues or cells during specific developmental stages or functional states (Zhang, 2019). Transcriptome sequencing is one of the effective technologies for studying gene functions and screening new genes (Sangwan *et al.,* 2013). As our knowledge, many

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researches have used transcriptome technology to study the litter trait of animals, revealing potential candidate genes, coding functional or regulatory proteins related to litter traits. For example, Dong *et al.* (2023) found that several differentially expressed mRNAs (SOCS3, POSTN, MFAP5 and DCN) in goats might play an important role in the regulation of the hypothalamus during the follicular phase Liu (2022) found that AMH and BMP15 genes might be involved in follicle development in milk goats Wen-Guang, (2017), identified eight differentially expressed genes (DEGs) that regulate ovulation and follicle development in sheep through neuroactive ligand-receptor interaction signaling pathways. Xu (2019) obtained 15 candidate genes that affect the litter size of pigs by RNA-seq and these genes were enriched in steroid hormone synthesis and ovarian steroid hormone synthesis pathways.

In this study, we focused on the ovarian tissue of low- and high-fecundity Ussuri raccoon dogs, RNA-seq was used to measure mRNA expressions to obtain a reliable transcriptomic dataset. With this dataset, we analyzed the genes and signaling pathways associated with reproductive traits and provide a basis for clarifying the regulatory mechanism of reproductive traits of Ussuri raccoon dog.

## MATERIALS AND METHODS

### Animals and sample collection

All samples were collected from a nucleus herd of racoon dogs cultured in captivity in the North region of Hebei Province, China. According to the litter records of breeding farms, those raccoon dogs with a litter size more than 12 for three years were defined as high-fecundity but considered as low-fecundity with a consecutive litter size no more than 7 in three years. All animals were housed outdoors individually in conventional cages in a two-row shed and were provided with high-quality diet and concentrate, with clean water available ad libitum during the experiment.

According to the litter size, 6 racoon dogs were equally divided into two groups, the low-fecundity (LF) group (n= 3) and the high-fecundity (HF) group (n= 3), respectively (Table 1). The two groups showed no significant difference in the body length, chest circumference, average weight and average age. The racoon dogs in the ovulatory period were slaughtered and ovarian tissue from both sides were obtained in each case. The tissues were cut into small pieces of 1g. The separated ovaries were quick-frozen in liquid nitrogen in a freezing tube and stored at -80°C until needed. All the experiments were conducted from April to July 2022 at Shijiazhuang Academy of Agriculture and Forestry Sciences.

### RNA isolation, library preparation and sequencing

RNAprep Pure Tissue Kit (Tiangen DP431) was used to extract the mRNA from ovarian samples of 6 raccoon dogs for RNA-seq. The RNA concentration and purity (with a standard: total RNA ≥10 μg and OD 260/280: 1.8~2.2) were measured using a NanoDropTM 2000 (ThermoScientificTM, Wilmington, DE, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

### Quality control and reference genome mapping

We used Fast-QC (https://www.bioinformatics. babraham. ac.uk/projects/fastqc/) to evaluate the quality of the raw data. The raw fastq format reads were firstly filtered by removing low-quality reads, ≥1% poly-N bases reads. Secondly, the GC contents and Q20, Q30 indexes were calculated of the preprocessed data and clean reads were obtained with a bases quality value Q20 >50%. Sequentially, HISAT2 (v2.1.0) (Kim *et al.,* 2015) was used to map the clean reads to the reference genome (GCA_905146905.1). Then, we used StringTie (v1.3.5) (Kovaka *et al.,* 2019) software to assemble the mRNA transcripts. Both known and novel mRNAs were constructed and identified by Cufflinks (v2.1.1). The fragments per kilobase per million reads (FPKM) (Trapnell *et al.,* 2010) were calculated of each mapped gene.

### Differentially expression analysis and function enrichment analyses

The feature Counts tool of the subread software was used to analysis the mRNA expression levels in both groups. We transformed the readcount to the FPKM values, which is the most common method to eliminate the effects of gene length, sequencing depth and sample difference on gene expression levels (Trapnell *et al.,* 2010). The expression levels of genes between the HF and LF groups were compared and the R package DESeq2 (v4.0.2) was used to calculate $\log_2$FC (Fold Change) values as well as p-values based on the normalized counts (Love *et al.,* 2014). mRNAs with a cutoff value of adjusted $P<0.05$ and $|\log_2 FC| > 0$ were considered as DEGs.

The clusterProfiler package in R software (v4.0.2) was used to conducted GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analyses to annotate the functions of DEGs. GO terms were classified into biological processes (BP), cellular components (CC) and molecular functions (MF). The hypergeometric distribution tests method was used to conduct enrichment analysis. Then, only the GO terms with adjusted p-value (BH-method) <0.05 were considered as significantly enriched and KEGG pathways were considered significant with a $p<0.05$.

### Novel gene identification

After mapping the clean reads to the reference genome, unannotated transcription regions were identified. The mapped reads were subsequently assembled using StringTie software (Kovaka *et al.,* 2019) to determine the novel genes. The region encodes a peptide chain longer than 50 amino acid will be defined as a new gene.

The potential protein-coding mRNAs was analyzed by screening novel isoforms against the NR, GO, KEGG, Swiss-Prot and Pfam databases with the BLASTX program using an e-value $\leq 1 \times 10^{-5}$.

### Alternative splicing analysis

AS events were identified and classified using rMATS software with default parameters (Shen *et al.,* 2014). Then, DAS events were also identified between the two groups. The rMATS software classified AS events into the following five basic categories, A3SS (Alternative 3′ splice site), A5SS (Alternative 5′ splice site), MXE (Mutually exclusive exon), RI (Retained intron) and SE (Skipped exon). The expression ratio of inclusion level (IncLevel) was applied to evaluate the AS level and the difference between the two groups was calculated to determine the changes in the splicing of all five AS events. The differential AS events were characterized based on the IncLevel and FDR <0.05.

### SNP variant calling

Picard-tools were used to sort and remove duplicate reads of the bam files. SNP and indels were called from the bam files using the GATK software (McKenna *et al.,* 2010). We implemented "Variant Filtering" in the GATK according to the following parameters setting "QUAL< 30.0 | QD< 2.0 | MQ<40.0 | FS>60.0 | SOR>3.0 | MQ Rank Sum<-12.5 | Read Pos Rank Sum<-8.0" and "QD<2.0 | QUAL<30.0 | FS> 200.0 | ReadPosRankSum<-20.0" to filter SNPs and InDels, respectively (McKenna *et al.,* 2010).

After filtering, all the identified SNPs and InDels were further annotated using the SnpEff software (Cingolani *et al.,* 2012) and subsequently functionaled as missense, nonsense and silent variation.

## RESULTS AND DISCUSSION

### Overview of transcriptome sequencing data

Total transcriptome sequencing data was 41.28 Gb. After filtering low-quality reads, 39.63 Gb of data was obtained, with an average of 6.605 Gb per sample. The mean Q20 and Q30 for all samples was 97.415% and 92.99%. GC content of high quality reads in all samples was higher than 45.89%. All sequencing information is shown in Table 2. We mapped clean reads to the reference genome, the total mapped reads of samples ranged from 88.23%~91.55%, the unique mapped reads was 84.75%~87.61% and the multiple mapped reads were less than 4.37%. These results suggest that transcriptome sequencing data can be used for further analysis.

### Identification of mRNA in ussuri raccoon dog ovary tissue

We identified 31,149 mRNA transcripts in 6 ovarian samples, including 29,143 protein-coding mRNAs and 1,986 new mRNAs. Only 408 genes (1.40%) were highly expressed in all samples, the FPKM value exceeds 60. More than 44% of the genes were not expressed in all six samples and the FPKM value was less than 1. There were 13,886 genes (FPKM>1) co-expressed in two groups (Fig 1A). The distribution of gene number in different FPKM intervals of each sample is shown in (Fig 1B).

### Analysis of DEGs in the ussuri raccoon dog ovary

In the comparison of HF and LF group, totally 612 DEGs were identified, including 277 upregulated and 335 downregulated DEGs, respectively (Fig 2A). We performed a clustered heatmap analysis and the results. In addition, the DEGs were divided into several clusters according to the $\log_2$ (fpkm+1) level and the genes in the same cluster showed similar expression pattern under different treatment conditions. Among the significantly up-regulated genes in the HF group, BMPR2, SMAD9, SOX5, BMP5 and RIF1 were found to be related to reproduction.

### Functional analysis of the DEGs

GO and KEGG functional enrichment analysis of DEG sets were conducted and the enriched GO terms were classified into three categories. A total of 536 GO terms were enriched, of which 267, 64 and 205 terms were enriched in BP, CC and MF terms, respectively. The top 10 GO terms of each category are displayed in Fig 2B. KEGG analysis results revealed that 20 pathways were found to be enriched ($p<0.05$), including Wnt signaling pathway and osteoclast differentiation (Fig 2C). In addition, KEGG enrichment analysis also found that the Circadian entrainment pathway associated with reproduction was enriched.

**Table 1:** Information of sample group and litter size.

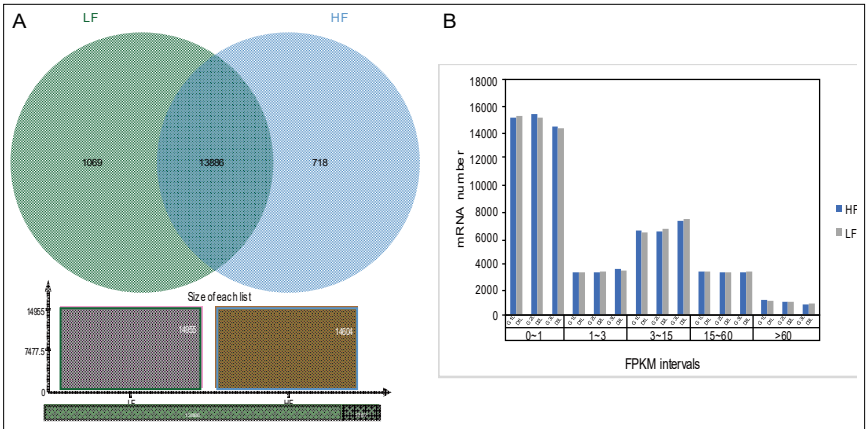
| Sample | Group | Mean litter size | Sample | Group | Mean litter size |
|---|---|---|---|---|---|
| G1L | HF | 13 | D1L | LF | 7 |
| G2L | HF | 13 | D2L | LF | 7 |
| G3L | HF | 12 | D3L | LF | 7 |

**Table 2:** The information of RNA-seq data.

| Sample | Raw_reads | Raw_bases (G) | Clean_reads | Clean_bases (G) | Q20 (%) | Q30 (%) | GC (%) | Total mapped |
|---|---|---|---|---|---|---|---|---|
| G1L | 48330268 | 7.25 | 46449018 | 6.97 | 97.62 | 93.47 | 50.91 | 90.35% |
| G2L | 44573108 | 6.69 | 43148624 | 6.47 | 97.68 | 93.33 | 45.89 | 88.23% |
| G3L | 46622308 | 6.99 | 43957000 | 6.59 | 97.65 | 93.44 | 48.49 | 90.06% |
| D1L | 44677092 | 6.7 | 44500800 | 6.68 | 97.15 | 92.56 | 51.43 | 90.7% |
| D2L | 45118802 | 6.77 | 41304250 | 6.2 | 97.05 | 92.29 | 51.28 | 89.49% |
| D3L | 45895666 | 6.88 | 44780910 | 6.72 | 97.34 | 92.85 | 50.33 | 91.55% |

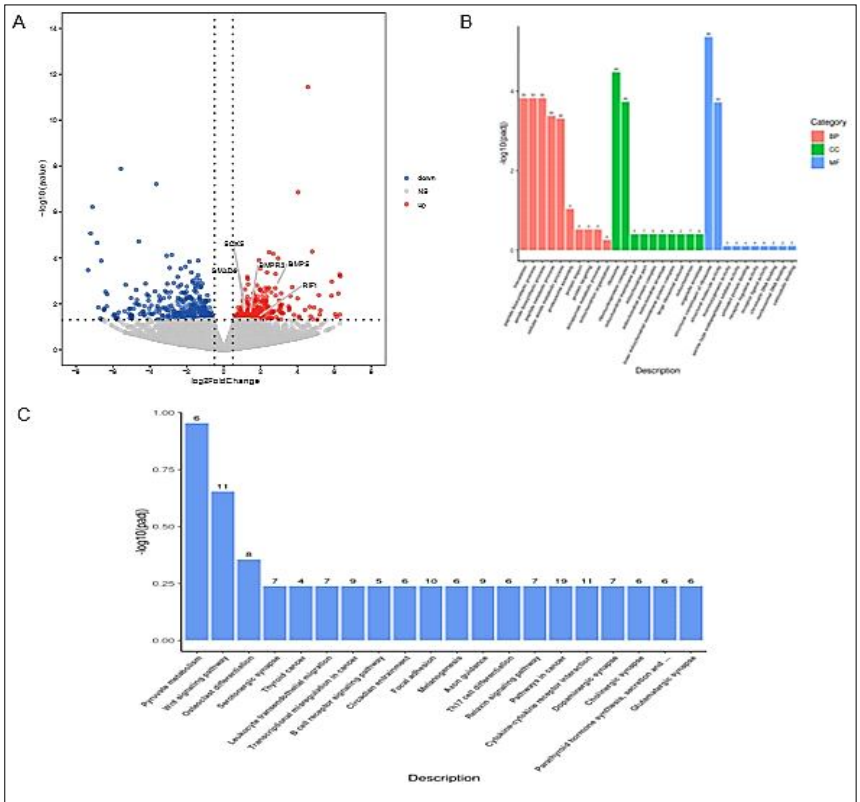
### Novel gene identification

The unannotated transcription regions were analyzed and the mapped reads were assembled to identify the novel isoforms of knowned or novel genes. A total of 1,986 novel genes were discovered in this study. We found that all the novel genes have more than one transcript. Studies have shown that protein-coding genes are usually multi-exon (Abdel-Ghany *et al.,* 2016; Pan *et al.,* 2008). The novel genes were annotated with public databases sequentially and the results showed that 208 (10.47%), 40 (2.01%) and 75 (3.78%) of them could be found in the Swiss-Prot, GO and KEGG databases, respectively. A total of 1,751




(A) The venn diagram of genes co-expressed (FPKM > 1) in both groups. (B) The distribution of gene numbers in different FPKM intervals of each sample.

**Fig 1:** Expression of genes in high-fecundity group and low-fecundity group.




(A) The volcano plot presents the mRNA expression variations between the HF and LF groups; Blue points refer to down-regulated genes and red points refer to up-regulated genes. The DEGs related to the reproduction process were labeled. (B) Top 10 enriched Gene ontology (GO) terms of DEGs. (C) Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of DEGs.

**Fig 2:** The differentially expressed genes (DEGs) and their functional annotation.

(88.17%) isoforms were not found in any of the protein databases. The results suggested that the proportion of protein-coding transcripts in the 1,986 novel isoforms was relatively low.

**Alternative splicing events**

A total of 19,200 AS events were obtained by alternative splicing analysis and two of the five basic types were covered (Fig 3A). Among the basic AS types, 17,457 SE events and 1,743 MXE events were identified respectively. In the comparison of the two groups, 247 AS events were recognized as DAS events (FDR ≤0.05 ), including 143 MXE and 94 SE events which in turn corresponding to 135 and 94 genes, respectively. Notably, there are 9 genes that have both identified DAS types simultaneously (Table 3).

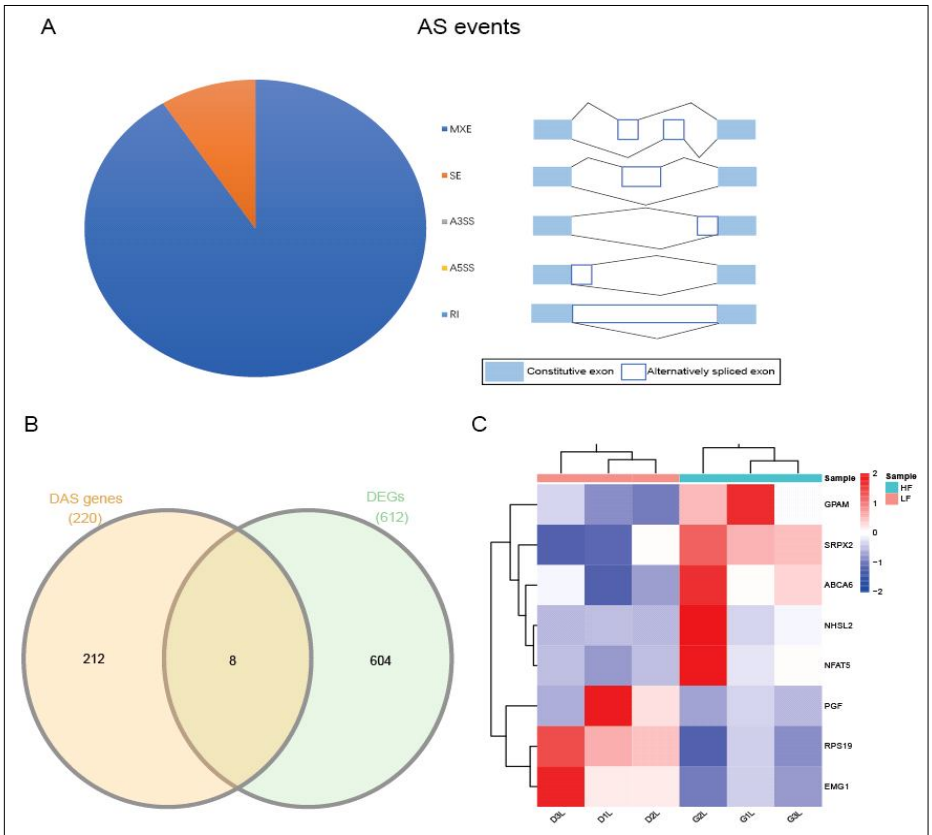
**Integrated analysis of DAS events and DEGs**

To further evaluate the effect of DAS genes in HF and LF groups, we found an intersection between the DEG list and the DAS gene list. Among the DEGs, 8 are DAS genes, of which 5 are upregulated and 3 are downregulated in HF compared to LF (Fig 3B, C).

Of all the up-regulated DAS-DE genes in HF group, we found the ABCA6 gene, which is an important lipid transporter, ABCA6 expression was detected in human placenta and increased with the progression of pregnancy (Imperio *et al.,* 2019). In our results, ABCA6 was significantly overexpressed in HF groups (P<0.05) and the MXE events were identified. Considering its critical role in the regulation of immunological responses, steroidogenesis and placental barrier function and integrity, the difference in expression and transcription may be the fundamental for a more productive pregnancy. In addition, we also discovered another gene that regulates lipid metabolism, GPAM, which is an enzyme that catalyzes the synthesis of triglycerides (Igal *et al.,* 2001). Lipid metabolism plays an important role

**Table 3:** Nine genes occurred two types of DAS events.

| Gene ID | Log2FC | Gene description | Tf_ family |
|---|---|---|---|
| gene-NYPRO_LOCUS26489 | -0.202 | NDUFV3 | - |
| gene-NYPRO_LOCUS19243 | 0.291 | ITPRIP | - |
| gene-NYPRO_LOCUS11205 | -0.142 | EIF4G3 | - |
| gene-NYPRO_LOCUS1231 | -0.313 | GOLGA2 | - |
| gene-NYPRO_LOCUS1537 | 0.108 | EXOC7 | Exo70 |
| gene-NYPRO_LOCUS21559 | 0.447 | PCNX1 | - |
| gene-NYPRO_LOCUS3157 | 0.189 | RAD52 | - |
| gene-NYPRO_LOCUS7278 | 0.371 | CCDC62 | - |
| gene-NYPRO_LOCUS8680 | 0.623 | N4BP2 | Smr |




(A) Distribution of five basic AS events and the schematic plot of the five AS events.(B)Venn diagram of DEG list and DAS gene list. (C)Heatmap of DE-DAS genes.

**Fig 3:** Identification of DEGs with differential alternative splicing (DAS) events.

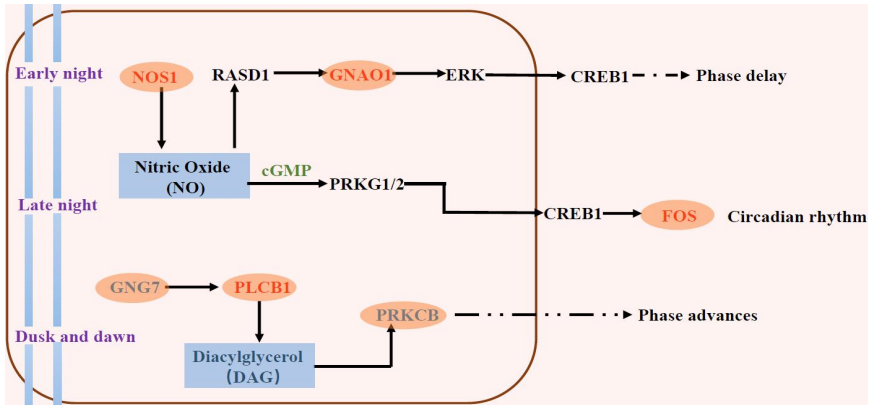
during early pregnancy. Lipid metabolism in the ovary and uterus affects the microenvironment in which oocytes and embryos develop and is critically important for blastocyst development potential (Lain and Catalano, 2007; Ye *et al.,* 2021). Maternal lipid metabolism before and during early pregnancy has profound impacts on oocyte quality and embryo survival, as well as the ovarian and uterine micro-environments, which determines the subsequent growth and development trajectories of offspring. In our analysis, GPAM was significantly up-regulated in HF groups ($P<0.01$) and the SE events occurred. The findings may suggest that GPAM plays an important role in the high reproduction pregnancy of raccoon dogs.

In this study, RNA-seq and transcriptome analysis with reference genome were performed systematically on ovulatory ovarian samples from raccoon dogs with high and low fecundity. The results of the base quality and composition analysis indicating the high-quality of libraries and sequencing.

The DEGs were enriched in the Circadian entrainment pathway which related to the reproduction process. The circadian entrainment pathway has been reported to be closely related to the secretion of reproductive hormones (Alvord *et al.,* 2022). A study also found that fertility in middle-aged mice could be improved or reduced according to differences in the light-dark cycle (Takasu *et al.,* 2015). Six genes involved in this pathway were significantly differentially expressed in our results. NOS1, PLCB1, FOS and GNAO1 genes were up-regulated, meanwhile, PRKCB and GNG7 genes were down-regulated in HF group (Fig 4). GNRH (Gonadotropin-releasing hormone) is the master regulator of fertility and reproduction. The release pattern of GnRH by the hypothalamus includes both pulses and surges. Virginia Delli *et al* review present the idea of a Kisspeptin-nNOS-GnRH network that is responsible for generating the “GnRH pulse” and “GNRH surge”. Nitric oxide synthase I (NOS1) gene mediates the “OFF” signal on GnRH secretion while the kisspeptin a product of KISS1 gene provides the “ON” signal, promoting GnRH release (Delli *et al.,* 2021). Furthermore, PLCB1 was also a critical downstream gene of the KISS1/GPR54 signaling pathway (Zhu *et al.,* 2022). The differential expression of these key genes in the Circadian entrainment pathway may be the important factor for improving the reproductive performance of ussuri raccoon dog.

Among the genes that were significantly up-regulated in the HF group, we also found several other genes related to reproduction. BMPR2 is a member of the bone morphogenetic protein family. Studies have found that BMPR2 plays biological function through BMP/SMAD signaling pathway (Canty-Laird *et al.,* 2010; Tan *et al.,* 2017). This pathway is widely believed to be closely related to mammalian ovarian granulosa cell proliferation, reproductive hormone synthesis and secretion, oocyte maturation and ovulation and other physiological activities (Lain and Catalano, 2007). BMPR2 mainly regulates the ovulation activity of animals by affecting the synthesis and secretion of reproductive hormones such as estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Regan *et al.,* 2017; Sirard, 2016). We also found SMAD9 and BMP5 genes belonging to the BMP/SMAD pathway among the elevated genes in HF group. The high expression of these genes may play an important regulatory role in follicular growth and multiple pregnancy of raccoon dogs. SOX5 plays an important role in germ cell development and gender differentiation decisions, Manfred Schartl *et al*. (2018) found SOX5 is an evolutionarily conserved regulator of germ-cell number in medaka fish.

In the integrated analysis of DAS events and DEGs, we found key genes related to lipid metabolism and transport, suggesting that these genes could be used as crucial markers to identify high-fertility traits in raccoon dogs. ABCA6 and GPAM genes, involved in lipid transport and metabolic regulation, are significantly highly expressed in the ovarian tissues of high-fecundity raccoon dogs. Their increased expression and the occurrence of variable AS




The genes highlighted in the orange oval are differentially expressed genes (DEGs) in this study; Red color indicates the up-regulated genes and blue color refers to the down-regulated genes.

**Fig 4:** The schematic illustration of the Circadian entrainment pathway.

events may be the fundamental for more fetal pregnancies in raccoon dogs. Furthermore, the GPAM gene shows a missense variant (c.234T>A, p.Phe78Leu) of amino acids in all three samples of the HF group according to SNP calling. Further molecular biological experiments are needed to explore the possible effect on the function of GPAM with the protein structural changes.

## CONCLUSION

In summary, transcriptomic research of ovary tissues revealed differential regulation of mRNAs associated with low and high fecundity in Ussuri raccoon dog at the ovulatory phase. Based on the KEGG analysis results, DEGs in the ovary of Ussuri raccoon dog enriched in a variety of biological processes associated with prolificacy traits, such as Circadian entrainment pathway, Oxytocin signaling pathway, PI3K-Akt and MAPK signaling pathways. Moreover, we analyzed the alternative splicing events of all the annoted genes and performed a integration analysis with DEGs to discuss the possible roles of DAS-DE genes. A PPI network of DEGs in HF groups was constructed and several genes associated with prolificacy in high-fecundity Ussuri raccoon dog were screened, which provided important resources for candidate markers in the ovary tissue. These DEGs and DAS-DEs expression profiles provide a molecular mechanism for improving the prolificacy traits of Ussuri raccoon dog breeding.

## ACKNOWLEDGEMENT

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### Data availability

The raw transcriptome sequence data have been deposited in the Sequence Read Archive (SRA) database of National Center for Biotechnology Information (NCBI) with Bioproject number PRJNA983862.

### Conflict of interest

The authors declare that there is no conflict of interest.

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