Article

PLA2G4A, SOX30 and *GPX5* genes mediate the reproductive adaptation of *Eothenomys miletus* to high altitude

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Abstract: The same species inhabiting different regions often develop different survival adaptation strategies to adapt to the local environment. In the present study, the genome of 66 *Eothenomys miletus* from Deqin (DQ, high altitude) and Ailao Mountains (ALS, low altitude) regions was simply sequenced by selective elimination method. The results showed that the obtained genes were enriched with GO and KEGG. Three genes were screened in DQ region, namely *PLA2G4A, SOX30, GPX5*. Through further analysis, it was found that these three genes were mainly related to the reproduction of male *E. miletus*, *PLA2G4A* was mainly related to the production of luteinizing hormone (LH), which can stimulate leydig cells to produce testosterone and other male hormones; Androgens were associated with the expression of *GPX5*, which was mainly aimed at avoiding oxidative damage to sperm and reducing the incidence of malformations in young offspring and miscarriage in female individuals. *SOX30* is mainly involved in sexual reproduction and spermatogenesis. By measuring the expression content of three genes, the results showed that the content in DQ was significantly higher than that in ALS, which further confirmed that *E. miletus* in DQ increased their fecundity by increasing the expression products of these three genes. Moreover, the current study expanded the research on high-altitude adaptation strategies of small mammals, providing a theoretical basis for animals' high-altitude migration.

Keywords: *Eothenomys miletus; PLA2G4A; SOX30; GPX5*; Reproduction; Adaptation strategy

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1. Introduction

A few of the many species on Earth have evolved adaptive traits to live in extreme environments with harsh abiotic conditions (Wang et al., 2023). Adaptation is the core of Darwin's evolution, and species need to adapt to the changing environment through Natural selection (Lai et al., 2019). Adaptation is the foundation of evolutionary biology and the protection of biodiversity is crucial (Rudman et al., 2022). Different regions have different spatial environments, and populations in different spatial environments need to adapt to the local biological and abiotic environments, therefore, improving the adaptation of different spatial environments is particularly important in population reproduction (Savolainen et al., 2013). Environmental variation leads to correlated variations in average population traits, as over evolutionary time, natural selection may be conducive to the occurrence of different phenotypes by acting on the heritable variation in the population (Botero et al., 2015; Franch et al., 2018; Tufto, 2015), and also affect the biological Reaction rate of organisms, thus generating biological adaptation at all levels of biological tissues (Rodriguez et al., 2017). Adaptive matching of organisms in specific environments can improve their reproductive success rate and survival rate. Organisms

that do not match environmental conditions usually have to pay a certain price. For example, birds that breed under unstable food conditions have poor growth and reproduction of their offspring (McKinnon et al., 2012; Reed et al., 2013). In research on *Tamiasciurus hudsonicus*, it was found that females who gave birth to larger offspring in years with relatively low spruce cone production reduced the likelihood of mismatch errors in years with higher spruce cone production. However, their offspring grew slowly and rarely survived the first winter, making them unable to contribute to population reproduction (Petrulo et al., 2023).

Hengduan Mountains is the widest and longest north-south mountain system in China. Different habitats in different regions of the Hengduan Mountains usually have different environmental conditions, with specific geological and geographical driving factors, combined with human influence and interaction of abiotic and biological factors, which is a hot spot in the present study of animal adaptation strategies (Zhang et al., 2019). *E. miletus* is a special species of Hengduanshan Mountain, and produces complex and diverse distribution patterns, which provides us with excellent materials for studying animal adaptation strategies (Musthafa et al., 2021). *E. miletus* is widely distributed in Hengduan Mountains. The adaptation of *E. miletus* distributed in different regions of Hengduan Mountains to these climates may play an important role in forming genetic and phenotypic variations among populations (Hancock et al., 2008; Rodriguez et al., 2017). Therefore, in the face of this environmental fluctuation, *E. miletus* distributed in Hengduan Mountains may have different local adaptation patterns.

In the present study, we selected the adaptive changes in genes of *E. miletus* in Ailao Mountains (ALS, low altitude) and Deqin (DQ, high altitude), and these differences may involve changes in many traits of *E. miletus*. Previous studies have shown that the energy metabolism and skull geometry of *E. miletus* from different parts of the Hengduan Mountains have diverged markedly, and that the phenotypic and genetic changes show adaptations to diverse environments, which are related to environmental factors such as latitude, altitude, and mean annual air temperature (Zhang et al., 2019; Ren et al., 2023). Therefore, on the basis of previous studies, this study speculated that there should be regional differences in the reproductive adaptation strategies of *E. miletus* at different altitudes. Through simplified genome sequencing of *E. miletus* in ALS and DQ, analysis and reproduction related differential genes and differential gene expression measurement, and then reveal the local adaptation of *E. miletus* to environmental differences in reproduction.

2. Materials and Methods

2.1. Samples

66 male *E. miletus* were collected in DQ (n=33) and ALS (n=33) in winter of 2019. The geographical location, climate characteristics, and sample size of each collection location were shown in (Table 1). All young individuals were excluded in the present study. All animal procedures were compliance with the Animal Care and Use Committee of School of Life Science, Yunnan Normal University. This study was approved by the Committee (13-0901-011).

Table 1. Sample sampling point information

Region	Region	Sample number	Site	Altitude/m	Annual average temperature (℃)	Precipitation (mm)	Vegetation types
Deqin	DQ	33	99°03′15″E, 28°35′14″N	3459	4.7	633.7	Alpine meadow
Ailao Mountains	ALS	33	100°42′49″E,24°90′30″N	2217	19.7	597.0	Savanna Shrub and Grass

.2. Sample preparation

After being captured, the animal was immediately executed and its liver was frozen in liquid nitrogen. Total DNA was extracted from animal tissue samples using the phenol/chloroform method (Gautam A., 2022). For each individual, 1-3μg of DNA was cleaved into 200-500 bp Covaris system fragments using (Gene Company, Ltd, Hong Kong, China). According to the Genome size and GC-content of voles, we selected the genome of Microtus ochrogaster as the reference gene, and used the restriction prediction software independently developed by Baimike to perform restriction prediction on the reference genome and select an appropriate restriction scheme. The assembled Genome size is 2.29G in size and 42.25% in GC-content.

2.3. Whole genome sequencing of short read sequences

There are six main steps in constructing a whole genome sequencing library: genomic DNA interruption, adding adapters, connecting Dual index sequencing connectors, PCR amplification and library purification, and gel cutting to select target fragments. Firstly, the extracted DNA was cut into 200-500 bp fragments. Next, the DNA fragments were connected to the A-tail adapter and the selected reads were double ended spliced. Then, PCR amplification was performed and the library was purified. A target fragment of 464-494 bp was selected, and after the library passed the quality inspection, IlluminaHiSeqTM2500 was used for sequencing. In order to evaluate the accuracy of digestion, *Oryza sativa ssp. Japonica*, a rice with a Genome size of 374.3Mb, was selected as the digestion control for sequencing. The download address is: http://rapdb.dna.affrc.go.jp/.

2.4. Data analysis

2.4.1. Data analysis process

The original data obtained from sequencing is identified in the Dual index to obtain the reads of each sample. After filtering the sequencing reads connector, the sequencing quality and data volume are evaluated. Evaluate the accuracy and effectiveness of the experimental process by evaluating the efficiency of RsaI enzyme digestion using Control data. According to bioinformatics analysis, developing genome-wide SNP markers within a population and utilizing representative high-quality SNPs within the population for population polymorphism analysis.

2.4.2. Evaluation of sequencing quality and data volume

This project adopts a read length of 126bp×2 as a follow-up data evaluation and analysis data. The sequencing quality value (Q) is an indicator for evaluating the error rate of single base in high-throughput sequencing, and the higher the value, the lower the error rate of base sequencing. The dual indexes is used to identify the original data obtained from sequencing, remove the reads containing connectors, low-quality reads and reads containing more than 10% of N base, and obtain clean reads of each sample. The sequencing data of each sample is counted, including the number of reads, Q30 and GC-content. At the same time, it is necessary to conduct statistics on the sequencing data of the control sample to ensure the accuracy of the sequencing sample data.

2.4.3. Evaluation of experimental database construction

By comparing the Control sequencing reads with their reference genome using SOAP software, the efficiency of the double ended alignment in this experiment was 95.68%, indicating normal alignment efficiency. Calculate the actual length of SLAF tags by calculating the proportion of residual enzyme cleavage sites in the inserted fragments of sequencing reads and the position of control sequencing pair end mapped reads in the genome.

2.4.4. SLAF tag development

BWA software locates SLAF tags onto the reference genome and counts SLAF tags and polymorphic SLAF tags on different chromosomes. After obtaining whole genome sequencing readings on the IlluminaHiSeqTM2500 platform, SOAP filters low quality readings and performs filtering steps to obtain high-quality SNP datasets. The development of SNP markers uses the genome of the *Microtus ochrogaster* as the reference genome, aligns sequencing reads to the reference genome using BWA, and develops SNP using GATK and samtools. The intersection of the SNP markers obtained by the two methods serves as the final reliable SNP marker dataset.

2.4.5. Selective sweep

FST and $\theta\pi$ average ratio is widely used for detecting selection. In order to detect selection signals related to local selection, we used the PopGen module of Bioperl software (Version 1.5.2_100) to calculate the FST values for pairwise comparisons using a whole genome sliding window (length 100 kb, step size 10 kb) $\theta\pi$ ratio, z-transform the Fst value, $\theta\pi$ ratio is transformed using log2. There will be both the top 5% Z (FST) and log2 ($\theta\pi$ ratio) value is regarded as the candidate outlier under strong selection. Assign all abnormal windows to the corresponding SNPs and genes to obtain candidate genes.

2.4.6. Gene function annotation

Gene ontology (GO) classifications were extracted from Inter-Pro for selected genes located in specific regions using web resources using DAVID (Version 6.8). We also used DAVID (Version 6.8) to map the *E. miletus* gene to the KEGG (Kyoto Gene and Genome Encyclopedia) database, and used BLAST106 v.2.8.1 to search the public protein database COG (homologous cluster), KOG (cluster of Eukaryote protein neighbors), NT and NR (non redundant protein sequence in NCBI), SwissProt and TrEMBL. Utilize network resources to associate the structural domains and motifs of proteins mapped to genes with publicly available databases Pfam.

2.5. Elisa

The testicular tissues of 6 adult *E. miletus* from DQ and ALS were randomly selected to conduct enzyme-linked immunoassay on the expression content of three genes SOX30, PLA2G4A and GPX5 related to reproduction obtained through selective sweep. SOX30 (SOX30) ELISA kit (No JM-11314R2), cytoplasmic Phospholipase A2 (cPLA2) ELISA kit (No JM-11320R2) and glutathione peroxidase 5 (GPX5) ELISA kit (No JM-11325R2) were analyzed with the IBM SPSS Statistics 26.0, and the regional difference was analyzed with independent sample t-test. The results are expressed as mean ± standard error, with $P<0.05$ indicating significant differences.

3. Results

3.1. Genome quality control and polymorphism analysis

After quality control, 363.16Mb reads were generated, the average Q30 of sequencing was 92.23%, and the average GC-content was 42.09%. A total of 847420 SLAF tags were obtained from 66 individuals, with an average sequencing depth of 9.19x. There were 473646 polymorphic SLAF tags, resulting in a total of 2221486 SNP tags.

3.2. Gene function annotation

The selected genes in DQ and ALS played important biological functions in biological processes, cellular components, and molecular functions. DQ and ALS were selected as Venn map, which shows that there are 359 repetitive functions in biological process (Fig. 1A), 138 repetitive functions in cell components (Fig. 1B), and 163 repetitive functions in molecular functions (Fig. 1C). This study further annotated the selected genes of DQ and ALS into the KEGG pathway, with Fst and $\theta\pi$ ratio, the DQ selected region is

annotated to 223 pathways, while the ALS selected region is annotated to 198 pathways. Among the annotated pathways in these two regions, there are 169 repetitive pathways (Fig. 1D), which were mainly concentrated in 6 aspects: cellular processes, genetic information processing, organic systems, human diseases, metabolism, and environmental information processing. In the annotation of KEGG pathway in DQ and ALS, we found that there were fewer pathways related to glucose metabolism and more pathways related to lipid metabolism in *E. miletus* in DQ area, which confirmed from the side that *E. miletus* had such a change that the activity of biological lipid metabolism was increased when migrating to high altitude areas.

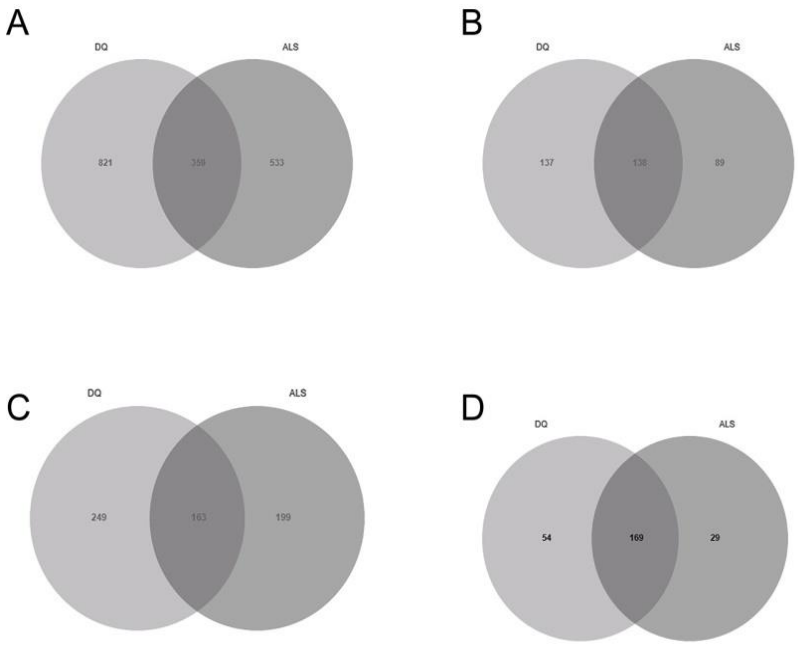

Fig 1. (A) Bioprocess enrichment results in DQ and ALS regions; (B) Results of cell component enrichment in DQ and ALS regions; (C) Molecular functional enrichment results in DQ and ALS regions; (D) KEGG enrichment results in DQ and ALS regions.

3.3. Selective elimination of E. miletus genome

Using Fst and θπ ratio method has identified 504 selected genes in DQ and 384 selected genes were identified in *E. miletus* in ALS (Fig. 2-3). In the screening of selected genes in DQ and ALS regions, we first screened six genes related to energy metabolism: CYP2J1, ELOVL5, ACADSB, ELOVL4 related to fatty acid metabolism; UGP2 is related to the conversion of sugars; ACY1 is related to the metabolism of 2-oxycarboxylic acids. More importantly, we identified three genes related to male reproductive development: PLA2G4A, SOX30 and GPX5.

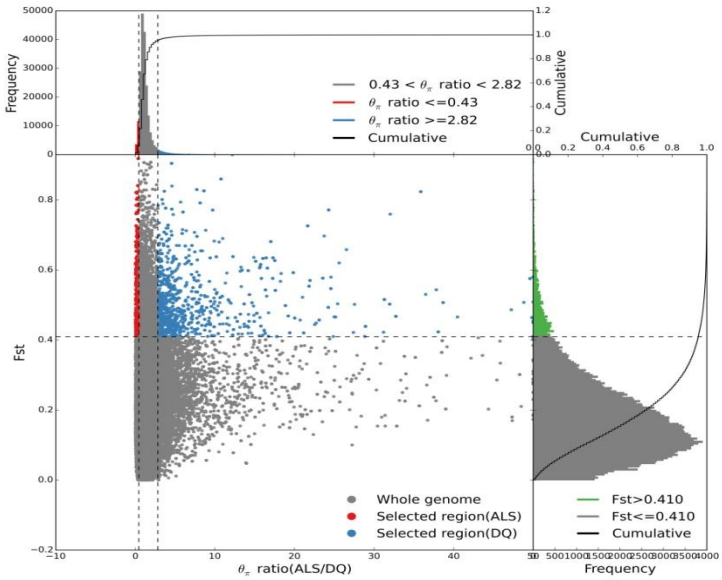

Fig 2. Candidate regions of Yunnan red-backed vole gene in DQ area, represented by abscissa θπ (π ratio), denoted as the Fst value in the vertical axis, corresponds to the frequency distribution map above and the frequency distribution map on the right, respectively. The dot plot in the middle represents the corresponding Fst and θ The ratio of pi. The top red and blue areas are θ The top 5% area selected by π, the green area is the top 5% area selected by Fst, and the blue area in the middle is Fst and θ The intersection of π is the candidate site for DQ.

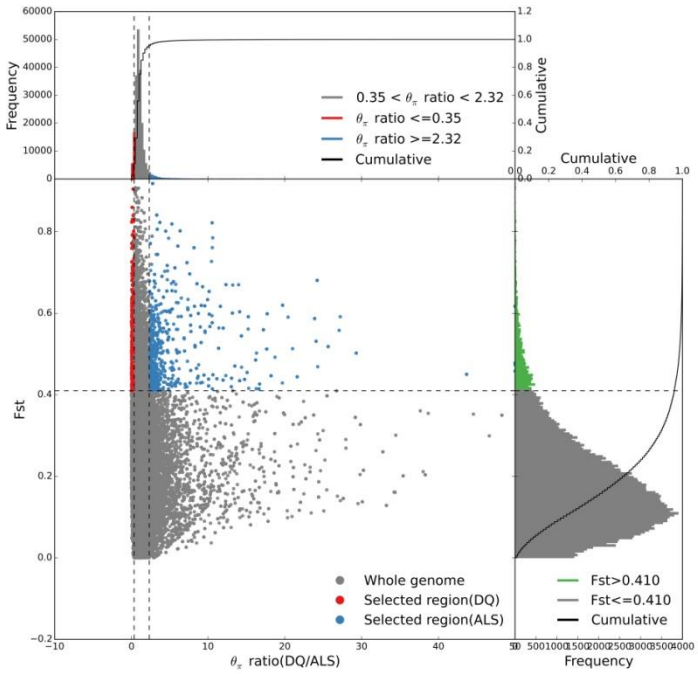

Fig 3. Candidate regions of Yunnan red-backed vole gene in DQ area, represented by abscissa θ The ratio of π (π ratio), denoted as the Fst value in the vertical axis, corresponds to the frequency distribution map above and the frequency distribution map on the right, respectively. The dot plot in the middle represents the corresponding Fst and θ The ratio of pi. The top red and blue areas are θ The top 5% area selected by π, the green area represents the top 5% area selected by Fst, and the middle red area represents Fst and θ The intersection of π is the candidate site for ALS.

3.4. Enzyme linked immunosorbent assay results

The average levels of SOX30, PLA2G4A and GPX5 in DQ were 62.08 ng/L, 10.19 μg/L and 8.88 pmol/ml, respectively, which were significantly higher than those in ALS (62.08 ng/L, 9.07 μg/L and 8.14 pmol/ml). It was found that the levels of these three genes in the DQ were highly significantly different from those of these three genes in the ALS (Fig. 4).

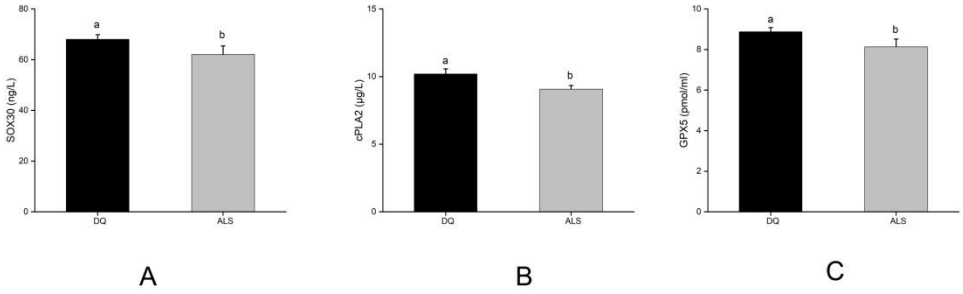

Fig 4. (A) Analysis results of SOX30 content in DQ and ALS regions; (B) Analysis results of cPLA2 content in DQ and ALS regions; (C) Analysis results of GPX5 content in DQ and ALS regions. Different letters denote significant differences between DQ and ALS regions.

4. Discussion

When a population is in a new environment, gene expression in the previous environment is no longer under selection; hence, gene expression in the previous environment might gradually deviate from its original value (Chen et al., 2023). Due to geographical differences, *E. miletus* in DQ and ALS showed different genetic diversity, that is, *E. miletus* in these two regions have different degrees of genetic variation, which is crucial for a species to survive in a constantly changing environment (Ren et al., 2023). *E. miletus* is a key link in the food web of Hengduan Mountains. Because of its large biomass, it is very important for the local ecosystem (Hu et al., 2020). The enrichment of highly divergent genes with functions in sperm and reproduction could indicate that these functions are important for adaptation to highlands (Everitt et al., 2023); hence we obtained the differential genes of *E. miletus* in DQ and ALS through selective elimination. Here we mainly describe the impact of *PLA2G4A, SOX30, GPX5* in DQ on male reproductive development.

PLA2G4A gene plays an important role in the fertility of mice, the production of eicosanoid by inflammatory cells, brain injury and allergic reaction, because the expression product cPLA2 of this gene has greater selectivity for lipids containing arachidonic acid compared with other cytoplasmi cPLA2, The expression product of *PLA2G4A* is related to the direct production of Arachidonic acid and the initiation of icosane like cascade reaction (Bonventre et al., 1997; Uozumi et al., 1997; Sapirstein and Bonventre, 2000; Tithof et al., 2007). There were many studies on the function of *PLA2G4A* gene, many of which mainly focus on its impact on animal reproduction. Some studies have found that *PLA2G4A* can express in mouse testes and play an important role in testicular interstitial cells. The cascade of Arachidonic acid triggered by the expression product of this gene is a key medium in the response of mature interstitial cells to LH stimulation, and is necessary for the normal development of the structure and function of testicular interstitial cells and testicular pellets, Moreover, *PLA2G4A* plays an important role in sexual ma-

turity processes such as spermatogenesis and male sexual accessory gland growth (Kurusu et al., 2011).

SOX30 plays a key role in gonadal development, and may play different roles in different stages and different sexes. The epididymis of *SOX30* deficient mice show disorder of testicular morphology, multinucleated spermatogenic cells, few sperm, and no sperm in the seminiferous tubes and testes. The mice with *SOX30* deficiency will uniquely damage testicular development and spermatogenesis, Moreover, with the increase of age, the reduction of testicular weight becomes more serious, but does not affect ovarian development and female reproductive ability. *SOX30* has been proved to be a preferential hypermethylation gene at the promoter. After *SOX30* methylation inactivation, spermatogenesis will be uniquely damaged. However, after the gene is re expressed, spermatogenesis and fertility can be restored, and there is no significant difference between its offspring and those of wild type mice, and can survive for more than a year (Han et al., 2020).

The expression product *GPX5* belongs to the glutathione (GSH) peroxidase family. The body uses GSH as a specific electron donor substrate to catalyze the reduction of various peroxides, including lipid peroxides (Huang et al., 2016). *GPX5* is different from other members of the family. It lacks the classic selenocysteine residue, which has been proved to be crucial to its activity (Marchesi and Feng, 2007). All members of the family express in the epididymis, but other members (GPX1-4) are cytoplasmic enzymes that exist in epididymal cells. GPX5 is secreted in the epididymal cavity. GPX5 is a powerful antioxidant scavenger in the caudal lumen of mouse epididymis. It can protect sperm from oxidative damage, which can damage sperm integrity. Although the deletion of this gene does not have a significant impact on mouse reproduction in young mice, it was found in mice aged one year and over that male mice that knocked out the gene mated with wild-type female mice, resulting in increased mortality and miscarriage rates in young offspring, Further research has found that such miscarriage and death are related to the quality of sperm itself (Chabory et al., 2009). Mammalian sperm plasma membrane is rich in unsaturated fat acids, and unsaturated fat acids are easy to be oxidized, so oxidative damage is a major threat to sperm (Vernet et al., 2004), so GPX5 may play a key role in the process of sperm maturation (Chabury et al., 2009). GPX5 mainly binds to sperm in the epididymis and protects them from peroxide attacks during subsequent maturation (Aitken, 2009). Moreover, GPX5 bound to sperm acrosome may play a role in preventing premature acrosome reaction by binding to lipid peroxide. Otherwise, lipid peroxide may interact with Phospholipase 2 and induce replacement reaction when sperm is stored in the epididymis (Williams et al., 1998).

The research on *SOX30* is less than that on the other two genes. The current research shows that *SOX30* is involved in the development of mouse testes and spermatozoa, involving a variety of downstream genes, and its specific pathway mechanism for regulating sperm has not been thoroughly studied, but the mechanism of *PLA2G4A* and *GPX5* for mouse testes development and spermatogenesis is relatively thorough. This article provides a general summary of the relationship between *PLA2G4A* and *GPX5* genes. The concentration of *GPX5* transcripts is androgen dependent (Huang et al., 2016). Androgen, such as testosterone, is secreted by testicular interstitial cells, which are the main source of testosterone. When interstitial cells (not seminiferous tubule) are incubated with cholesterol, testosterone and androstenedione will be produced (Zirkin and Papadopoulos, 2018). Testosterone and androstenedione secreted by leydig cells need not only cholesterol as raw material, but also LH. The generation of LH requires gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH and the G protein-coupled receptor of pituitary gonadotrops combine to start the GnRH signal pathway. This signal pathway can regulate the generation of LH. The generated LH combines with the G protein-coupled receptor on the surface of testicular interstitial cells to activate the PKA-cAMP signal pathway to produce a large amount of cAMP, cAMP stimulates the accelerated transport of cholesterol, the raw material for steroid synthesis, to mitochondria, after that, cholesterol became testosterone and androstenedione through a series of

enzymes (Zirkin and Papadopoulos, 2018). Androgens such as testosterone can stimulate the expression of GPX5 in the epididymal head.

In the present study, it was found that temperature had a significant impact on the testicles. A larger testicular volume was observed in males receiving additional heat, and temperature had a significant impact on male reproductive status. All males receiving additional heat were in a reproductive state (Martin et al., 2020), indicating that temperature is also an important factor affecting male reproductive system development. The average annual temperature in the DQ region is relatively low; therefore, *E. miletus* in DQ maintained the normal development of their male reproductive system by selectively clearing *PLA2G4A, SOX30* and *GPX5*.

The high altitude hypoxia experienced at an altitude of ≥ 2500 meters poses many challenges to human health, survival, and reproduction, which is the result of a decrease in oxygen supply due to a decrease in atmospheric pressure (Bigham, 2016). Compared with the normoxic control group, long-term exposure to low-pressure hypoxia can lead to a decrease in LH and plasma testosterone levels (Farias et al., 2008). The continuous decrease of LH is related to the influence of hypothalamus pituitary activity during hypoxia, and the decrease of testosterone level may be related to spermatogenesis (Farias et al., 2008). In addition, exposure to high-altitude areas is associated with an increase in the production of reactive oxygen species (ROS), which is produced during the reoxidation stage of intermittent continuous low-pressure hypoxia (Farias et al., 2005; Nanduri et al., 2008). The increase of ROS production leads to the state of insufficient sperm production (Turner and Lysiak, 2008). Moreover, due to the impact of oxidative stress, sperm DNA is still sensitive to ROS, and oxidant free radical attacks endanger the integrity of gamete Genetic material. Therefore, the existence of antioxidant mechanisms (especially antioxidant enzymes such as GPX5) during fertilization becomes very important, They can maintain and protect sperm from oxidative stress caused by hypoxia (Zepeda et al., 2014). The results of expression of three genes also confirmed that DQ was significantly higher than ALS, which is crucial for its survival.

5. Conclusion

In conclusion, we screened the differential genes in DQ and ALS by selective elimination method, and three genes, *PLA2G4A, GPX5* and *SOX30*, were screened from the genes in DQ. Through further detection of the content of these three genes, we found that the content of these three genes in DQ was higher than that in ALS. This may be a reproductive strategy derived from the relatively poor environment of *E. miletus* in DQ to maintain the population level. We believe that the three genes *PLA2G4A, SOX30* and *GPX5* of *E. miletus* in DQ area were selected not only to maintain the normal male reproduction, but also to lay a foundation for their offspring to better adapt to the harsh local environment, so as to maintain a certain population level.

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