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# IDENTIFICATION OF *AQP*S IN CHINESE HAMSTERS AND THEIR EXPRESSION PATTERNS IN TESTES OF DIFFERENT WEIGHTS

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## ABSTRACT

Aquaporins (AQPs) are a class of proteins encoded by *MIP* gene family, which play a critical role in maintaining cell morphology and small molecule transmembrane transport. In recent years, the role of AQPs in reproduction has gradually been revealed. They have been proved to be widely expressed in many species. The testicles of Chinese hamsters have the characteristics of large size and long spermatogenic cycle. This seems contradictory in evolution and has not been fully studied. At present, the whole genome analysis of AQPs in Chinese hamsters and the expression patterns in testes have not been reported. In this study, 13 AQPs were identified and characterized in the genome of Chinese hamster for the first time. Protein sequence analysis of AQPs showed that its structure and function were unified. The concentrations of testosterone are higher in larger testes. The expression patterns of AQPs in testes were different. *AQP5*, *AQP7* and *AQP11* were positively correlated with testicular weight. Sperm count showed that larger testes could produce more sperm and store it in epididymis. It is speculated that under the regulation of testosterone, AQPs affect the excretion of excess substances at the end of spermatogenesis, and adapt to the reproductive competition of Chinese hamsters by regulating the rate of spermatogenesis. The results provide basic resources for further studying the role of AQPs in spermatogenesis of Chinese hamster.

**Keywords**: Chinese hamster; Testis; Reproduction competition; *AQP*s



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## INTRODUCTION

Hamsters often have a fixed reproductive rhythm influenced by the body and environmental signals. For example, Syria hamsters are well known as model animals for seasonal reproduction (Martínez-Hernández, Seco-Rovira et al. 2020). It is believed that the Siberian hamster breeds only during the spring and summer months (Cooke, Jordan et al. 2007). These rhythms are thought to be regulated by the hypothalamus-pituitary-gonadal axis (Wang, Xu et al. 2020). In addition, wild rodents tend to have shorter lifespans and larger populations. As a result, hamsters need to complete breeding activities in a short time and face great intraspecific competition. Individuals with stronger reproductive traits and sperm competitiveness will gain certain advantages.

Testis is the core organ of male mammalian reproductive system. Its size is often used to measure the reproductive ability and reproductive competition intensity of mammals (Parker and Ball 2005, Fisher, Hook et al. 2018). A larger testicular size is associated with increased intrasexual competition in males (Soulsbury 2010). Larger testes can produce more sperm at the same time, thus gaining a competitive advantage. Another important indicator of spermatogenesis is the cycle of the seminiferous epithelium (CSE) (Leblond and Clermont 1952). The duration of the CSE, DCSE, can measure the rate of spermatogenesis. Males with a shorter DCSE have greater access to sperm cells in the same amount of time, allowing them to fertilize as many females as possible (Ramm and Stockley 2010). From an evolutionary standpoint, testicular size and DCSE should complement each other and jointly help reproductive competition.

The Chinese hamster, *Cricetulus griseus*, is a small rodent that inhabits farmland and grassland. Research shows that Chinese hamsters have remarkable large testis and obvious seasonal reproductive characteristics (Zhao, Wang et al. 2022). Another notable feature of Chinese hamster is that it also has a significantly longer DCSE than other rodents. For instance, the DCSE of Chinese hamster is 17.0 days (Oud and de Rooij 1977), while in mouse and Djungarian hamster, it is 8.7 days (Kluin, Kramer et al. 1982) and 7.9 days (van Haaster and De Rooij 1993). Although researchers have tried to explain the extreme traits of reproductive organs and long DCSE of Chinese hamsters

from an evolutionary perspective, there are few studies on whether they interact.

Aquaporins (AQPs) are a class of transmembrane channel proteins belong to major intrinsic protein (*MIP*) supper family. Since Agret won the Nobel Prize in Physiology or Medicine in 2003 for his work on the structure and function of AQPs (Agre 2005), thirteen distinct isoforms of AQP0-12 have been identified from humans and mammals. The main function of AQPs is to transport water and some small uncharged molecules across the membrane, such as glycerol, urea, hydrogen peroxide and small metals (Carrageta, Bernardino et al. 2020). In addition, the function of AQPs in reproduction has also received attention from researchers. AQP3 is considered to be necessary for its physiology and function because it participates in the osmoadaptation and movement of sperm in the female reproductive tract (Chen, Peng et al. 2011). AQP7 has been proposed to play a role in the development of the testis as well as in spermiogenesis, based on its pattern of expression (Calamita, Mazzone et al. 2001). *AQP11* mRNA was identified in testicular tissue and its expression was correlated with higher sperm quality (Laforenza, Pellavio et al. 2017). Because knockout *AQP11* mouse models do not function due to polycystic kidney disease and subsequent early death (Matsuzaki, Yaguchi et al. 2017), researchers usually study the function of *AQP11* through its expression pattern.

The present study focuses on the correlation between AQPs and testicular volume of Chinese hamsters. It has been well documented that testicular sizes represent different reproductive competitiveness (Fisher, Hook et al. 2018). We hypothesized that AQPs affect spermatogenesis and maturation in testis of different sizes. In order to test this hypothesis, we examined the weight and testosterone levels of testes. A genome-wide identification and characterization of AQPs in Chinese hamster were performed. The expression of AQPs mRNA was detected to explore the potential effect of AQPs on spermatogenesis and testicular volume regulation. Finally, the spermatogenesis ability of testis was verified by sperm count method

## MATERIALS AND METHODS

**Ethical statement:** All animal protocols were in accordance with Laboratory Animal Guidelines for the Ethical Review of Animal Welfare (GB/T 35892-2018) and were approved by the Biomedical Ethical Committee of Qufu Normal University (No. 2022062). This study was performed in compliance with all ARRIVE guidelines.

**Animals and grouping:** The Chinese hamsters were captured in Qufu in Shandong Province, China and kept separately in the feeding room of our laboratory, with a temperature of 22 ± 2 °C and relative humidity of 55 ± 5%. Water and standard mouse chow (Jinan Pengyue Experimental Animal Breeding Co., Ltd., China) were provided ad libitum. Twenty adult male Chinese hamsters of similar weight were randomly selected, and the right testicles were dissected at 24:00. After weighing the testicles, they were divided into two groups according to the weight: LG (light group) and HG (heavy group). Four testes in each group were randomly selected for ELISA and transcriptome sequencing. The remaining testis tissue was used for RT qPCR. The epididymis linked to the testis were selected for sperm counting.

**Identification of AQPs in Chinese hamster:** To identify AQPs in Chinese hamster, we downloaded reference genome sequence (GCA_000223135.1) from Ensembl database (http://asia.ensembl.org/index.html). Homo sapiens and Mus musculus AQP sequences obtained from UniProt Swiss-Prot datebase (https://www.uniprot.org/) were used for reference. In order to perform HMMER (Hidden Markov Model) search, the MIP binding domain (PF00230) was downloaded from the Pfam database (http://pfam.xfam.org/). The HMMER tool was used to search for MIP domains in Chinese hamster proteomic database. With an E-value of 1e−5. The genome database is first transcribed into cDNA database, and then translated into proteomic database by using TBtools (Chen, Chen et al. 2020).

**Overview of AQPs in Chinese hamster:** Molecular Phylogenetic analysis was constructed by MEGA7 using the amino acid sequences of AQPs. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel 2008) All positions with less than 95% site coverage were eliminated.

In addition, the theoretical molecular weight (MW), isoelectric points (pI), instability index and grand average of hydropathicity (GRAVY) of the AQPs in Chinese hamster were predicted using EXPASY ProtParam tool (https://web.expasy.org/protparam/).The MEME Suite was used to identify the motif. Conservative domains are defined by NCBI batch CD search tool. To gain more insight into the regulatory role of AQPs in biological function in protein–protein interaction, the STRING online database was used to predict the protein interaction networks of AQPs (https://cn.string-db.org/).

**RNA sequencing and transcriptome-based analysis of expression profiling of the AQPs:** Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After mRNA quality inspection, rRNA removal, fragmentation, reverse transcription, agarose gel electrophoresis and PCR amplified, the transcriptome was sequenced using Illumina HiSeqTM 4000 by Gene

Denovo Biotechnology Co. (Guangzhou, China). The detailed method is similar to that of Xia et al (Xia, Wang et al. 2022). FPKM (fragment per kilobase of transcript per million mapped reads) values were calculated to quantify the expression abundance and variations of the *AQP*s using StringTie software.

**Quantitative RT-PCR (qRT-PCR):** Total RNA was routinely extracted from muscles using an RNAiso Plus kit (9108, TaKaRa, Dalian, China) according to the manufacturer's protocols. RNA quality was determined by measuring the OD260/OD280 ratio, with samples showing a ratio of > 1.8 then reverse transcribed into cDNA using a TaKaRa reagent (6110A TaKaRa, Dalian, China) and stored at −80℃ for subsequent reactions. RT-qPCR was performed using a SYBR Premix Ex Taq II kit (CN830A, TaKaRa, Dalian, China). The reference gene was β-actin. The $2^{-\Delta\Delta ct}$ method was used to analyze the relative concentrations of *AQP2, AQP5, AQP7* and *AQP11*. The primers used for RT-qPCR are shown in Table 1.

**Table 1 Primer sequences of *AQP*s of Chinese hamster.**

| Primers | Primer sequences (5' -3') | Annealing temperature |
|---|---|---|
| AQP2 | F: TCCTCCGAGCTGCCTTCTATGTG | 60℃ |
| | R: GCGTTGTTGTGGAGAGCATTGAC | |
| AQP5 | F: GCTGCCATCCTCTACTTCTACTTGC | 61℃ |
| | R: GGTGCTCTTCCCAGTCCTCCTC | |
| AQP7 | F: TGAGGCAGAGGTGATAGGCATCC | 60℃ |
| | R: CAGCCAGCAATGAAAGTGAAGAAGC | |
| AQP11 | F: CTGATGCTGTTGGTCGTGCTACTC | 61℃ |
| | R: GGCTAGAAACTCCAAGACGAAGGC | |
| β-actin | F: GAGACCTTCAACACCCCAGC | 61℃ |
| | R: ATGTCACGCACGATTTCCC | |

**Epididymal sperm count:** Caudal region of the epididymis was extracted with 1 mL of physiological saline solution. Cut through the epididymal tails with ophthalmic scissors and shock for 3 min. Incubated at 37 ℃ for 10 min. Then took the supernatant and added the same volume of Eosin dye. 20 μL of the mixed liquid was placed on a pre-warmed counting chambers of the hemocytometer. Using a microscope (Olympus, Japan) at 40×, spermatozoa were counted in at least 10 fields (Najafi, Farokhi et al. 2016).

**Hormonal analysis:** Weigh not less than 50mg testicular tissue sample and add PBS (0.01mol/l, pH = 7.2) at a ratio of 1:9, which is equivalent to adding 9ml PBS to 1g tissue. Homogenization was performed on ice with a tissue homogenizer. The supernatant was centrifuged at 5000 rpm for 15 minutes. Take the supernatant for inspection. Intratesticular testosterone concentration was quantified using hamster ELISA kits (HB013-Hr, Hengyuan, Shanghai China) according to standard protocol instructions and information provided by the manufacturer.

**Statistical analyses:** SPSS version 26.0 was used for all of the statistical analyses. The date was presented as the means + SD. One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was used to analyze the experimental data. The results were deemed statistically significant at $P < 0.05$. The correlation between the data was analyzed by Person correlation.

## RESULTS

**Testicular weights and hormonal levels of Chinese hamsters:** The selected testicles showed that the weight of LG was significantly lower than that of HG (Figure 1A). However, there was no difference in the body weight of the owners of these testicles (Figure 1B). These results suggest that the difference in testicular size is not due to body weight or nutritional status. The levels of testosterone (Figure 1C) in LG was also lower than those in HG. The difference of hormone indicates that the testes of Chinese hamsters may be in different physiological states.

**Identification and overview of AQPs in Chinese hamster:** Thirteen AQPs were identified in Chinese hamster. Their detailed information were summarized in Table 2. After referring to humans and mouse, we divided the AQPs of Chinese hamsters into three subgroups. AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 belong to the orthodox aquaporins. AQP3, AQP7, AQP9, and AQP10 belong to the aquaglyceroporins. AQPs 11 and 12 are a group of unorthodox AQPs, also known as superaquaporins (Figure 2A). Phylogenetic trees of AQPs were derived using maximum likelihood (Figure 2A). The consistency of function and evolution confirmed the correctness of the identification results. In Chinese hamster, 5 putative motifs of AQPs were identified via MEME website (Figure 2B), and motif 2 and motif 3, located in the MIP

domain, are present in all 13 AQPs (Figure 2A). Protein-protein interaction network of AQPs was generated by the STRING database (Figure 2C).

**Expression pattern and correlation of *AQP* genes in testis of different sizes:** The expression patterns of Chinese hamster *AQP* genes in testis were detected using transcriptome sequencing. Nine *AQP* genes were detected in the Chinese hamster testis, as shown in Figure 3A. *AQP2* (Figure 3B), *AQP*5 (Figure 3C), *AQP7* (Figure 3D) and *AQP11* (Figure 3E) showed significant differences between HG and LG via RT-qPCR. Correlation between expression patterns were shown in Figure 4A. The expression of *AQP*5 (Figure 4B), *AQP*7 (Figure 4C), and *AQP*11 (Figure 4D) correlated strongly with testicular weight among individuals.

**Epididymal weight and sperm count:** The sperm count of HG was higher than that of LG (Figure 5A). Statistical analysis after sperm count showed that there was a significant difference between the two groups (Figure 5B). Epididymis in HG is heavier (Figure 5C), which was consistent with the weight of testis.

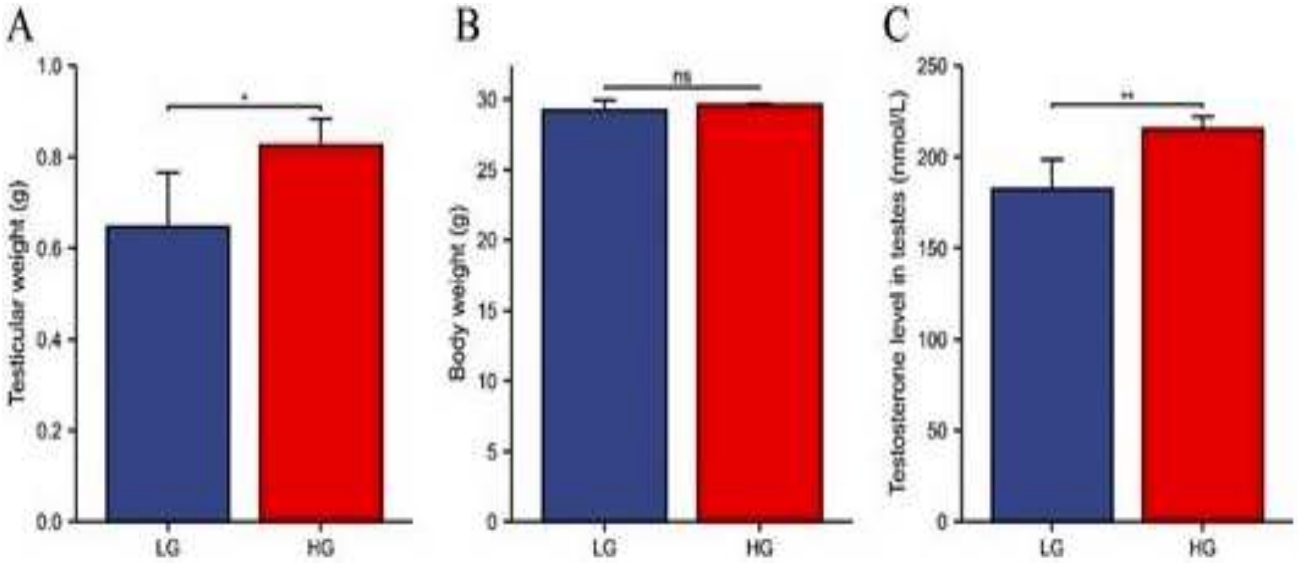


**Figure 1 Body weight and hormonal levels for Chinese hamster with different testicular weight. (A) Comparison of randomly selected testicular weights between LG and HG. (B) Body weight of selected testicular owners in LG and HG. (C) Testicular homogenate testosterone levels of hamsters in LG and HG. Values are expressed as means + standard deviation; LH, luteinizing hormone. *, $P < 0.05$; **, $P < 0.01$; ns, No significant difference.**

**Table 2. Summary of AQPs in Chinese hamster.**

| Gene | Amino acid length (aa) | MW | pI | Instablity index | Aliphatic index | GRAVY |
|---|---|---|---|---|---|---|
| AQP0 | 263 | 28244.87 | 9.46 | 35.42 | 102.05 | 0.554 |
| AQP1 | 269 | 28821.46 | 6.95 | 33.48 | 116.77 | 0.475 |
| AQP2 | 250 | 26545.83 | 5.56 | 41.49 | 119.44 | 0.673 |
| AQP3 | 292 | 31489.84 | 7.07 | 21.18 | 105.86 | 0.551 |
| AQP4 | 299 | 31522.83 | 6.47 | 31.46 | 110.54 | 0.586 |
| AQP5 | 265 | 28230.07 | 8.45 | 36.42 | 114.15 | 0.657 |
| AQP6 | 294 | 31162.51 | 8.75 | 24.95 | 109.80 | 0.673 |
| AQP7 | 299 | 32473.03 | 7.73 | 34.83 | 101.74 | 0.462 |
| AQP8 | 261 | 27362.41 | 6.29 | 34.33 | 119.69 | 0.756 |
| AQP9 | 295 | 31858.43 | 7.65 | 31.13 | 99.46 | 0.668 |
| AQP10 | 223 | 23514.24 | 5.06 | 36.53 | 101.88 | 0.414 |
| AQP11 | 271 | 30422.33 | 8.55 | 36.47 | 114.50 | 0.668 |
| AQP12 | 290 | 31506.96 | 8.84 | 34.01 | 105.66 | 0.519 |

AQP0-12, Aquaporin 0-12; MW, Molecular weight; pI, Isoelectric point; GRAVY, Grand average of hydropathicity.

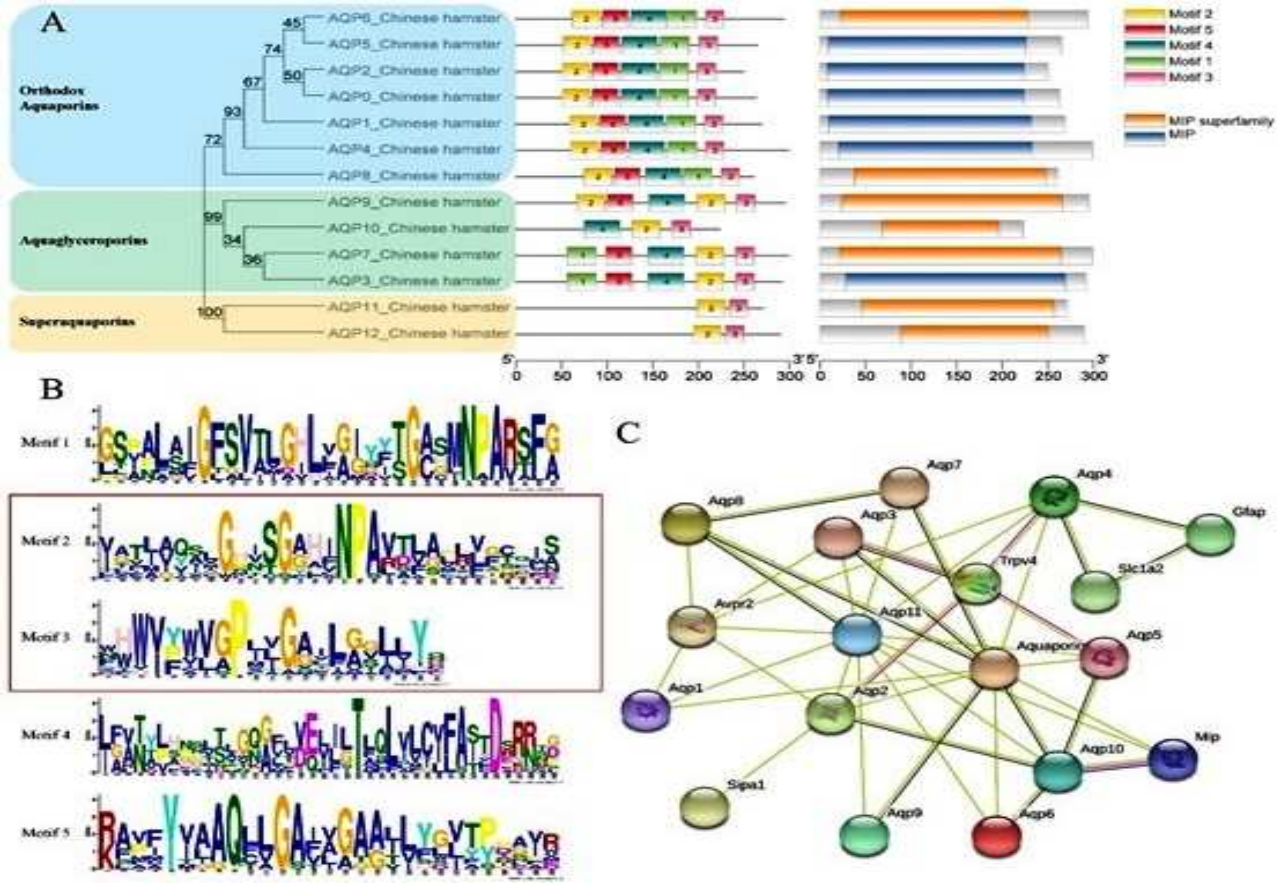


**Figure 2. Structure and function analysis of AQPs in Chinese hamster. (A) Identification, phylogenetic relationship and motif analysis of AQPs. (B) Seqlogo of 5 motifs predicted by MEME. (C) Protein-protein interaction network of AQPs constructed by STRING database.**

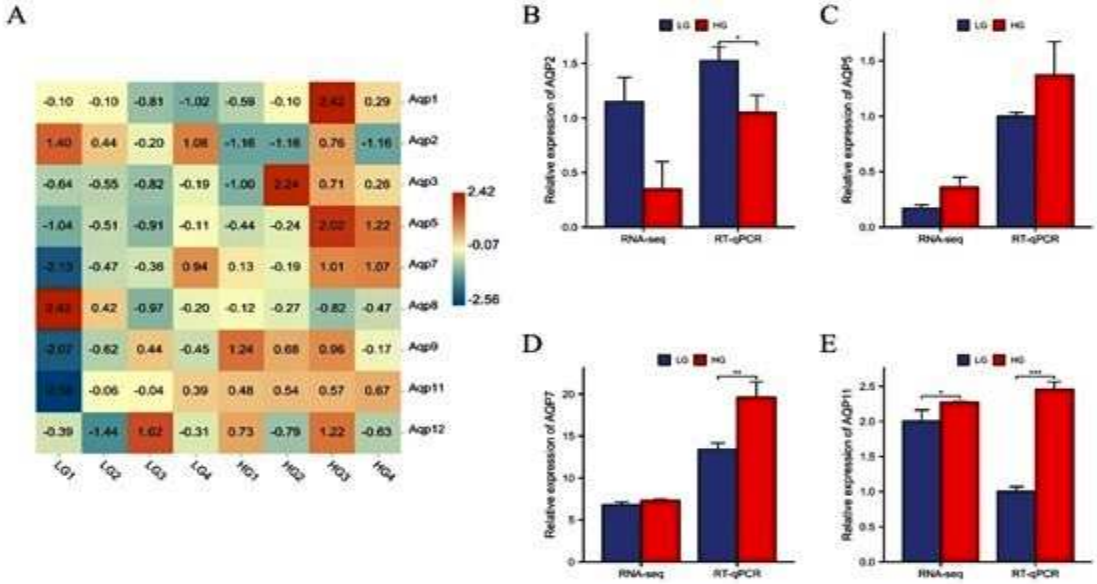


**Figure 3 Expression pattern and correlation of AQP genes in testes of different sizes. (A) Expression patterns of AQPs in testis. (B) Relative expression of AQP2 in LG and HG. (C) Relative expression of AQP5 in LG and HG. (D) elative expression of AQP7 in LG and HG. (E) elative expression of AQP11 in LG and HG. Values are expressed as means + standard deviation. $^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$.**

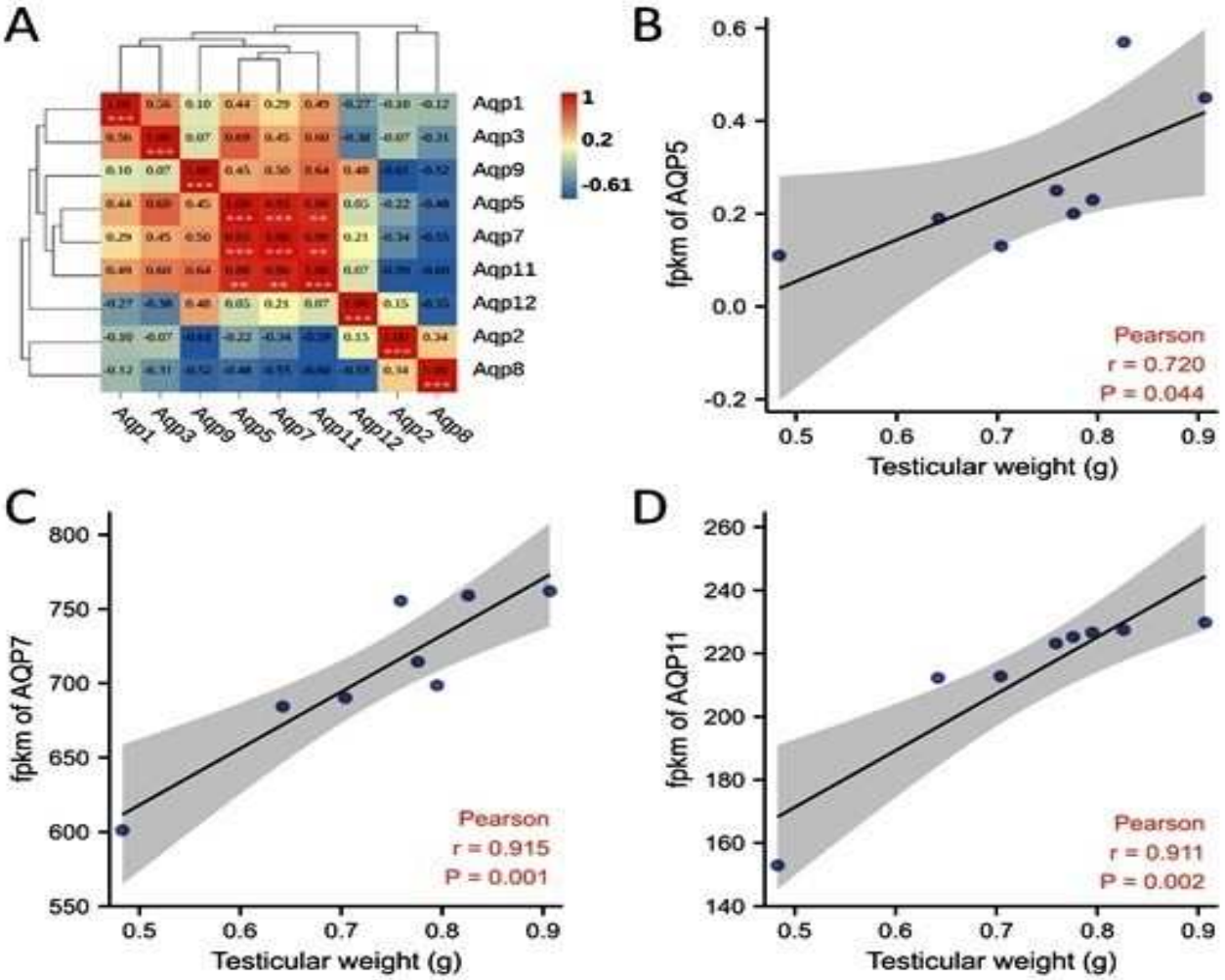


**Figure 4 Correlation of AQPS and testes of different sizes. (A) Correlation analysis of AQPs expression in testes. (B) Correlation analysis between AQP5 and testicular weight. (C) Correlation analysis between AQP7 and testicular weight (D) Correlation analysis between AQP11 and testicular weight.**

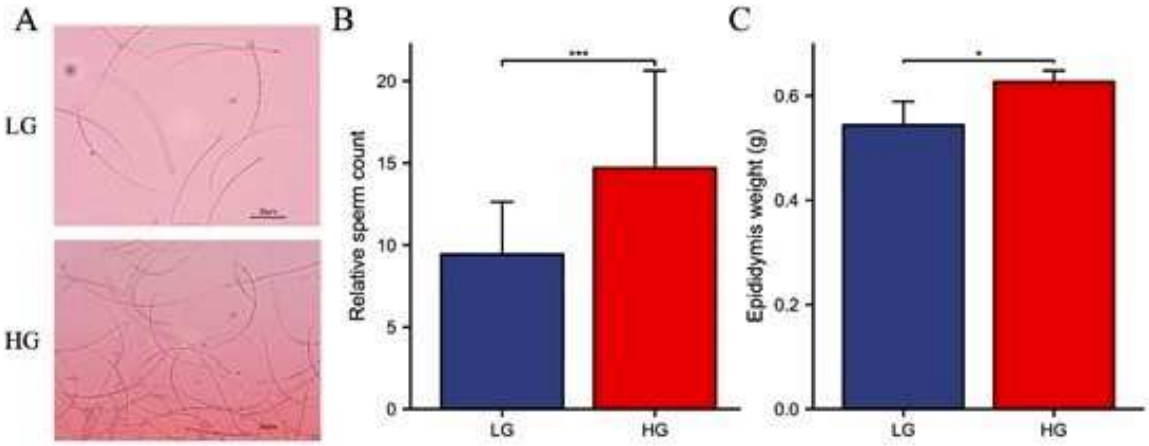


**Figure 5 Epididymal weight and sperm count of Chinese hamster in LG and HG. (A) Observation of sperm smears of Chinese hamsters with microscope. (B) Comparison of sperm counts between LG and HG. (C) Weight of epididymis connected to selected testicles Values are expressed as means + standard deviation. *, $P < 0.05$, ***, $P < 0.001$.**

# DISCUSSION

In this study, the weight of testis of adult Chinese hamsters with similar body size varied greatly among different individuals. Through the study of physiology, biochemistry and molecular biology of testis of different sizes, it is inferred that larger testis can provide some advantages for Chinese hamsters in reproductive competition. One possible way is that *AQP* gene family affects sperm production by regulating the transport of small molecules in sperm cells and Sertoli cells.

ELISA results showed that heavier testes contained higher level of testosterone. Testosterone is of great significance for mammalian reproduction. It is necessary for testicle growth, sperm production, and sperm motility. A study of the testicles of birds proved that increasing testosterone levels allows males to compete more efficiently for females (Garamszegi, Eens et al. 2005). This increase in efficiency comes mainly from testosterone's promotion of sperm competition. As with other vertebrates, sperm competition among rodents is common. Sperm development and maturation is a complex systematic process. One of these steps is the transmembrane transport of water and small molecules in sperm cells, in which AQPs are involved.

One of the important work of this study is the identification and analysis of *AQP* gene family in Chinese hamster genome for the first time. The identification of gene family is a fundamental research, which helps researchers to understand the information in the gene group more comprehensively. Alternatively, phylogenetic studies among family members may reflect important events in the evolution of this species. A total of 13 *AQP* genes were identified in the Chinese hamster genome and divided into 3 subgroups: ①Orthodox aquaporins include AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8. ②Aquaglyceroporins include AQP3, AQP7, AQP9 and AQP10. ③Superaquaporins include AQP11 and AQP 12. Furthermore, phylogenetic, sequence structure, and domain homology analyses supported their annotations. This is similar to researches in humans (Ribeiro, Alves et al. 2021).But in mice, AQP10 is a pseudogene that produces protein with no functional activity (Morinaga, Nakakoshi et al. 2002). By means of bioinformatics, AQP10 cannot be identified in mouse genome through conserved domain.

There are five motifs predicted in AQPs, of which motif2 and motif3 are common to all AQPs, so it is speculated that they are the core motif. The orthodox aquaporins have all five motifs. In addition, the motif order and the classification of AQPs show high correlation. The network diagram of protein-protein interaction shows that there are complex interaction and coexpression networks between AQPs.

In testis, AQPs play a critical role in maintaining water homeostasis and the microenvironment (Yang, Song et al. 2005). Spermatogenesis encompasses a number of complex biological processes, in which water homeostasis is indispensable (Zhang, Tan et al. 2012). It is believed that changes in water homeostasis will alter spermatogenesis and sperm motility (Chen, Peng et al. 2011). In the heavier testes of Chinese hamsters, more sperm counts and differential expression of *AQP2*, *AQP5*, *AQP7* and *AQP11* were detected. In addition, *AQP5*, *AQP7* and *AQP11* were highly correlated with testicular weight. Therefore, it is inferred that the moderate expression of *AQP*s in Chinese hamsters will provide them with more viable sperm and increase the chance of fertilization in the fierce reproductive competition. The mechanism is that after testosterone binds to specific receptors on Sertoli cells, it regulates *AQP*s to ensure the appropriate ionic and nutritional environment for spermatogenesis and to ensure the integrity of the blood-testis barrier (Basaria 2014).

One puzzling phenomenon is that Chinese hamsters have both relatively large testicles and longer DCSE than other rodents. From the perspective of evolution, it seems unreasonable. Studies have shown that testicles need to produce more sperm in a competitive environment (Firman, Garcia-Gonzalez et al. 2018). Longer DCSE forces Chinese hamsters to grow seemingly cumbersome giant testicles and epididymis to produce and store sperm (Meyer, Klose et al. 2021). Too large testicles are not conducive to individual survival. But from the perspective of sexual choice, handicap theory may explain this phenomenon(Zahavi 1975, Grafen 1990). Larger testes not only have physiological function, but it may also be one of the visual signals of female sexual selection (Emlen, Warren et al. 2012).

AQPs may mediate the regulation of DCSE by testosterone. Kerr et al confirmed that the late stage of spermatogenesis is sensitive to testosterone (Kerr, Millar et al. 1993). At this time, the sperm cells are deformed obviously and form a hook. It is well known that AQPs play a key role in regulating cell morphology. Therefore, specifically expressed AQPs at the end of spermatogenesis may be regulated by testosterone, which may affect the duration of some stages of the DCSE.

DCSE plasticity has been demonstrated within a species and even within an animal (Giannakara, Schärer et al. 2016). Vivian et al believe that the DCSE of Chinese hamsters in winter is longer than that in other seasons (Meyer, Klose et al. 2021). The reason is that the testis of Chinese hamsters decreases under the influence of short photoperiod. Our previous studies have also proved a series of physiological and functional changes of hamster testis under different photoperiods, including the change of testosterone level.(Zhao, Wang et al. 2022). However, the plasticity mechanism of Chinese hamster DCSE is still unknown. The differential expression of

AQP2, AQP5, AQP7 and AQP11 in testis with different weight may be the reason affecting the plasticity of DCSE. Therefore, AQPs provide a new way to explain the contradiction between reproductive competition and DCSE in Chinese hamsters.

The difference of AQPs and sperm number between LG and HG suggest that the expression of AQPs may affect the probability of fertilization. In humans, AQPs have been shown to play an important role in coping with the osmotic changes from epididymal fluid to cervical mucus (Laforenza, Pellavio et al. 2017). Its involvement in the process of human fertilization is also recognized (Huang, He et al. 2006). Therefore, it is speculated that the expression of AQPs in the population may affect the population growth. But the mechanism of population fluctuation is complex and systematic. It is not enough to evaluate the AQPs of male reproductive system alone. Exploring AQPs and other related genes in the bisexual reproductive system may provide a better theoretical basis for the study of mammalian population fluctuations and may also provide benefits for the treatment of male infertility.

**Conclusion:** In conclusion, 13 AQPs were identified and characterized in the genome of Chinese hamster for the first time. According to the expression pattern of AQPs, it is speculated that AQP5, AQP7 and AQP11 affect the excretion of excess substances at the end of spermatogenesis under the regulation of testosterone, and affect the reproductive competition of Chinese hamsters by regulating the spermatogenesis rate. Therefore, this study provides a basis for the study of AQPs in rodents and a new idea for predicting the population fluctuation of Chinese hamsters.

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**Competing interests:** The authors declare no competing interests.

**Data Availability:** Raw data of transcriptome of this study are available from the corresponding author upon reasonable request.

**Authors' contribution:** Laixiang Xu and Jinhui Xu conceived and designed research. Shuo Wang and Yongzhen Feng performed experiments. Xingchen Wang, Huiliang Xue and Ming Wu analyzed data, interpreted results of experiments, prepared figures. Shuo Wang and Jinhui Xu drafted manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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