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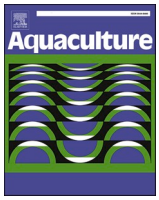

DNA damage may cause the reproductive differences between the male tetraploid fish and diploid red crucian carp

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ABSTRACT

Tetraploid fish are valuable diploid sperm donors and vertebrate models, and their reproductive characteristics have been widely attracted. In previous study, we obtained a new tetraploid fish (4nNT, 4n = 200, AAAB) derived from a cross between female autotetraploid fish (4nAU, 4n = 200, AAAA) and male allotetraploid fish (4nAL, 4n = 200, AABB). Compared with the original parent red crucian carp (RCC, 2n = 100, AA), a high proportion of abnormal sperm was observed in the semen of 4nNT. This study was conducted to gain further insight into the reproductive potential and fertility mechanisms of male 4nNT. During meiosis prophase, 100 pairs of bivalents were observed in germ cells of 4nNT, implying a doubling of crossover events in individual spermatocytes. The sperm concentration, motility (MOT), average path velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL) of RCC were significantly higher than 4nNT, and the lifetime was significantly shorter than 4nNT. In the artificial insemination test, higher fertilization and hatching rates were observed in groups using RCC as sperm donors, and higher abnormality rate were observed in groups using 4nNT as sperm donors. Regarding sperm DNA integrity, the higher comet rate was observed in 4nNT sperm, which may contribute to their lower hatching rate and higher abnormality rate. Transcriptome data indicated that the upregulated genes identified in the testis of 4nNT were significantly enriched in 14 signaling pathways, including the homologous recombination, fanconi anemia pathway, p53 signaling pathway and apoptosis, which are link to DNA damage and repair. This indicates the 4nNT spermatocytes may be unable to fully withstand the DNA damage repair pressure caused by the doubling of crossover events. In conclusion, this study suggests that the reproductive differences between 4nNT and RCC may be caused by DNA damage, our findings provide novel insights into the fertility mechanism of male 4nNT, which is of great significance for improving its fertility, population reproduction, and efficient production of triploids.

1. Introduction

The first allotetraploid and autotetraploid fish lineages that were fertile in both males and females were obtained through distant hybridization, a procedure that is vital for the efficient production of triploid fish (Liu et al., 2001; Qin et al., 2014). Generally, fertile male tetraploid fish are considered to be more commercially advantageous for the production of triploids, given that male gametes can be more readily and sustainably harvested, and are numerically dominant during breeding. In aquaculture, the triploid production technique, which involves crosses between female diploids and male tetraploids, has been employed in the cultivation of aquatic seedlings, including cyprinid fish and triploid pacific oysters (*Crassostrea gigas*) (Chen et al., 2009; Guo et al., 1996; Hu et al., 2019; Luo et al., 2011; Wang et al., 2020a). However, the key hybrid diploid or tetraploid that have successfully broken through the reproductive barrier exhibited a varying degree of reproductive abnormalities, including a reduced number of fertile males, thin semen, and short or absent sperm flagella (Liu et al., 2007,

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2001; Wang et al., 2020b; Wang et al., 2020c). Furthermore, even some fertile tetraploid fish lineages that have been stably inherited for many generations are not as well fertilized as their original diploid parents (Wang et al., 2019). These phenomena present a challenge to the expansion of tetraploid stocks as well as to the production of triploid fish.

Reproductive issues are partially caused by DNA damage during spermatogenesis. Programmed double-stranded DNA breaks (DSB) are formed in the pre-meiotic phase, which is essential for chromosome recombination and segregation in spermatocytes. The programmed DSB should be repaired by the homologous recombination (HR) mechanism. However, improper and delayed repairs trigger apoptosis of the spermatogonia, leading to sterility (Hochwagen and Amon, 2006; Oster and Aqeilan, 2020; Qu et al., 2021). DNA damage to mature sperm is also detrimental to reproduction. In mammals, sperm DNA damage results in loss of genetic material integrity, which can lead to embryonic arrest, growth defects in the offspring, and chromosomal abnormalities (Fernandez-Gonzalez et al., 2008; Gawecka et al., 2013; Marchetti et al., 2015). Fish studies have also demonstrated that sperm DNA damage has adverse effects on fertilized egg development and survival, resulting in abnormality in the offspring (Devaux et al., 2015; Gosalvez et al., 2014; Jeuthe et al., 2022; Perez-Cerezales et al., 2010).

There are several theories describing the formation mechanism of DNA damage in mature sperm of mammalian; one suggests the production of unrepaired DSB through histone hyperacetylation and topo II mediated chromatin reorganization during spermiogenesis, resulting in DNA damage later on. Additionally, another theory points to oxidative stress, which creates DNA damage in sperm cells when high levels of reactive oxygen species exceed the antioxidant capacity threshold of seminal plasma. Finally, according to the apoptotic abortion theory, certain sperm cells carrying DNA damage escape apoptosis during spermatogenesis, and as a result, these cells end up carrying DNA damage even after differentiation into mature sperm (Muratori et al., 2006; Sakkas et al., 2003; Sakkas et al., 1999; Schulte et al., 2010). In brief, DNA damage has negative impacts on reproduction, and repairing the damage is crucial. Fish have several methods of repairing DNA damage (Kienzler et al., 2013), such as excision repair (ER), photoenzymatic repair (PER), homologous recombination (HR) and non-homologous end joining (NHEJ). Thus, less accumulation of DNA damage and a robust DNA damage repair system are important for maintaining reproduction. However, there is evidence indicating defective DNA damage repair systems in hybrid species (David et al., 2004), which remains to be investigated in tetraploids fish. Meanwhile, it is currently unclear whether the tetraploid fish reproductive phenotype is linked to DNA integrity of sperm.

In recent years, single cell gel electrophoresis or comet assay has been used to detect DNA damage in fish sperm, such as rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) and giant grouper (*Epinephelus lanceolatus*) (Perez-Cerezales et al., 2010; Reinardy et al., 2013; Zidni et al., 2022). This method is widely used in fields such as sperm cryopreservation and genetic toxicology research due to its high sensitivity and ease operation. Computer-assisted sperm analysis (CASA) is a frequently used tool for evaluating sperm motility parameters, which enhances the objectivity of the measurements due to its strong statistical power and reproducibility (Figueroa et al., 2016; Kime et al., 2001). Transcriptome sequencing has been employed to investigate RNA expression in diverse tissues of different fish species during specific periods, which can reveal the molecular mechanisms behind phenotypic variation (Qian et al., 2014; Sudhagar et al., 2018).

In our previous study, a new type of tetraploid fish (4nNT) was acquired through crosses between female autotetraploid fish and male allotetraploid fish. It has been observed that male 4nNT produce semen earlier than red crucian carp (*Carassius auratus* var. *red*, RCC). However, the mature sperm secreted by male 4nNT contained roughly 20 % of anomalous sperm without tails (Hu et al., 2020). Based on the importance of tetraploid fish in aquaculture and its scarcity as a polyploid vertebrate model, the present study aimed to elucidate the fertility mechanism of male 4nNT. We investigated the chromosome pairing during meiosis, sperm concentration, sperm motility parameters, sperm DNA integrity and fecundity. Furthermore, the testis transcriptomes of 4nNT and RCC were comparatively analyzed. This study provides novel insights into the fertility mechanism of male 4nNT, which is of great significance for improving its fertility, population reproduction, and efficient production of triploids.

2. Materials and methods

2.1. Animals

All of the RCC and 4nNT in this study were cultured within the State Key Laboratory of Development of Freshwater Fish at Hunan Normal University.

2.2. Observation of chromosome pairing in the testis

The male 4nNT (n = 3) and male RCC (n = 3), aged between 6 and 7 months, were randomly selected for the preparation of germ cell chromosomes. After dissecting the experimental fish, testes were removed to a clean glass dish. The testes were homogenized using rapid clipping, and the resulting testis homogenate was transferred into a 15 mL centrifuge tube. The appropriate volume of physiological saline (0.8 % NaCl) was added, and the germ cells were dispersed using a 5 mL pipette. The sample should then be allowed to stand at room temperature for a period of 10 min, during which the precipitated tissue blocks should be removed. Subsequently, centrifugation at 1500 rpm for 10 min was performed to collect the germ cells. The germ cells were expanded using a 0.075 mol/L potassium chloride solution and incubated for 2–3 h. Following centrifugation and fixation (methanol: glacial acetic acid = 3: 1) at room temperature. The cells were then distributed on clean slides and stained. To investigate the chromosome behavior during meiosis in germ cells, bivalents were observed using referenced methods (Zhang et al., 2015, 2022).

2.3. Reproductive traits

One-year-old RCC and 4nNT reaching sexual maturity were randomly selected. The sperm concentration was quantified using a hemocytometer. Before measurement, the semen of RCC (n = 6) and 4nNT (n = 6) was diluted with Hanks' balanced salt solution (Solarbio, Beijing, China) in a ratio of 1:100, and then diluted again to achieve a total dilution factor of 10^5. The measurement was conducted three times for each sample (Duan et al., 2016; Sotnikov et al., 2023). The sperm motility parameters were analyzed using the CASA (HT CASA II, Hamilton Thorn, USA), with the following parameters being considered: the percentage of motile sperm (MOT, %), curvilinear velocity (VCL, $\mu m \cdot s^{-1}$), straight-line velocity (VSL, $\mu m \cdot s^{-1}$), average path velocity (VAP, $\mu m \cdot s^{-1}$), amplitude of lateral head displacement (ALH, μm), beat cross-frequency (BCF, Hz), and wobble (WOB, %). The sperm lifetime was defined as the time of the period from the initial activation of spermatozoa to the point at which less than 5 % of them remain motile. Finally, spawning was induced artificially for female 4nNT and female RCC using a combination of human chorionic gonadotropin (HCG, 1000 IU kg^{-1}), luteinizing hormone-releasing hormone A2 (LHRH-A2, 12 μg kg^{-1}) and domperidone (DOM, 2 mg kg^{-1}) (Ningbo No. 2 hormone factory, China). Eggs were collected by squeezing the abdomen of female, and RCC and 4nNT eggs were equally divided into two portions and inseminated with sperm from male RCC and male 4nNT, respectively. The fertilized eggs were placed in glass dishes for hatching, with regular water changes and the temperature of the water maintained at approximately 22 °C. The above procedure generated a total of four artificial insemination combinations, and the rates of fertilization, hatching and abnormality were calculated respectively.

2.4. Comet assay of sperm

The grade of sperm DNA damage in RCC and 4nNT was assessed using the Comet assay kit (KeyGEN, Jiangsu, China). Clean slides were pre-warmed and coated with 120 μL of 1 % normal melting point agarose gel, then covered with coverslips and cured at 4 °C for 10 min. Semen stripped from the RCC and 4nNT were diluted with Hank's solution, 30 μL of the sperm suspension (approximately 10^4 spermatozoa) was encapsulated in 90 μL of 0.8 % low-melting-point agarose gel and spread on the first layer. The gel-layer slides were allowed to set at 4 °C for 10 min before being lysed in lysing solution at 4 °C for an hour and a half. Subsequently, the slides were rinsed twice with phosphate-buffered saline (Solarbio, Beijing, China) and then immersed in alkaline electrophoresis solution for 20–30 min. The samples were then electrophoresed under an electrical field of 25 v, 300 mA for 15 min, washed thrice with phosphate-buffered saline for 5 min each and stained with propidium iodine for 10 min before being viewed using a fluorescence microscope. Random photographs were taken, and quantitative analysis was performed using Open Comet plugin of Image J (Version 1.50i, US National Institutes of Health, Bethesda, Maryland, USA). In accordance with previous studies (Anderson et al., 1994; Collins, 2004; Liu et al., 2019), sperm were graded into 5 classes corresponding to the following percentages of DNA in the tail: no damage (G0), < 5 %; damage grade I (GI), 5–20 %; damage grade II (GII), 20–40 %; damage grade III (GIII), 40–95 %; damage grade IV (GIV), > 95 %. The comet rate is defined as the sum of damage grades exceeding G0.

2.5. Total RNA extraction and sequencing

During the non-breeding season, male RCC (158.01 ± 25.19 g, n = 9) and male 4nNT (145.09 ± 17.44 g, n = 9) were randomly captured. Following dissection, the testes were expeditiously transferred to a sterile 1.5 mL enzyme-free centrifuge tube. The testes of every three fish were mixed into a sample pool and submerged in liquid nitrogen. RNA extraction was performed from the testes using the standard Trizol method. The purity and integrity of RNA were analyzed using Nanodrop and Agilent 2100. The OD value of high-quality RNA ranged from 2.0 to 2.2 and the RIN value was no lower than 7. Afterwards, Qubit was used to precisely quantify the concentration of RNA. The certified RNA underwent fragmentation, reverse transcription, repair of the ends, and incorporation of sequencing adapters prior to PCR amplification in order to produce the ultimate cDNA library. Qualified cDNA libraries were sequenced using the Illumina HiSeq 2500 platform. All analyses were conducted on clean reads obtained by removing raw reads that contained adapters, reads that contained over 10 % unknown bases, and low-quality reads from the raw data.

2.6. De novo assembly and gene annotation

Due to the limited availability of reference genomes, a De novo assembly approach was adopted to reconstruct the sequencing data. The clean reads were concatenated using Trinity (version 2.4.0) and then clustered using Corset (version 1.05). Sequence annotation was carried out by referencing established databases.

2.7. Screening and pathway enrichment analysis of differentially expressed genes

To quantify gene expression levels, we utilized RSEM (version 1.2.15) and conducted FPKM conversion on Trinity-spliced transcripts. DESeq 1.10.1 was utilized to conduct statistical analysis of differentially expressed genes (DEGs) in each group. The criteria for screening DEGs included P value adjusted (padj) < 0.001 and $|\log_2{}^{(\text{fold change})}| \geq 1$, following prior research on the transcriptome of fish (Gong et al., 2024; Hu et al., 2017; Li et al., 2019; Xu et al., 2015). We carried out pathway enrichment analysis on the DEGs using KOBAS (version 2.0.12) and consulted the Kyoto Encyclopedia of Genes and Genomes (KEGG) to understand the underlying biological processes. The sequence alignment of DEGs was performed using NCBI BLAST 2.2.28+, and the gene co-expression network was visualized using Cytoscape (version 3.9.1) with reference to STRING.

2.8. Quantitative real-time PCR analysis

To validate the reliability of DEGs that have been identified via transcriptome sequencing, we opted to investigate the expression profiles of nine genes, including *bax, top3, casp3, eme1, blm, rfa2, casp8, amh,* and *p53*. The majority of these genes are markers for DNA damage repair and apoptosis. *β-actin* was employed as an internal control, and the qRT-PCR results were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were listed in Table S1.

2.9. Statistics analysis

The data was analyzed using an independent sample *t*-test, with a *P*-value of less than 0.05 indicating a significant difference between the data. The aforementioned procedure was conducted using SPSS Statistics 18.0.

3. Results

3.1. Chromosome pairing

The germ cell chromosomes of 4nNT and RCC were prepared by the air-drying method in order to observe the chromosome behavior at meiosis prophase, and 150 chromosome spreads were observed in each type of experimental fish. The results demonstrated that 50 pairs of bivalents (accounting for 88 %) were present in the germ cells of RCC, while 100 pairs of bivalents (accounting for 82 %) were observed in the germ cells of 4nNT (Fig. 1), with no unpaired chromosomes observed. This suggests that male 4nNT has a cytological basis for meiosis as well as reproductive potential. Furthermore, the paired chromosomes are accompanied by crossover event, indicating that the total number of crossovers of bivalents occurring in an individual germ cell of 4nNT at meiosis prophase was approximately twice that of RCC.

3.2. Sperm concentration and motility parameters

The results of the evaluation of sperm concentration and motility parameters for RCC and 4nNT are shown in Table 1. The average sperm concentration of RCC was $(1.87 \pm 0.33) \times 10^{10}$ cell mL^{-1}, which was significantly higher than that of 4nNT ($(0.70 \pm 0.21) \times 10^{10}$) ($P < 0.05$). With regard to sperm motility parameters, the average MOT of RCC (89.72 ± 3.38) was significantly higher ($P < 0.05$) than that of 4nNT (80.88 ± 6.13), while the average sperm lifetime of RCC (61.00 ± 3.79) was significantly shorter ($P < 0.05$) than that of 4nNT (120.33 ± 9.29). Furthermore, the average values of VAP, VSL and VCL were found to be significantly higher ($P < 0.05$) in RCC than in 4nNT, whereas no significant difference ($P > 0.05$) was observed in ALH, BCF and WOB between the two groups.

3.3. DNA integrity of sperm

The genetic material integrity of RCC and 4nNT sperm was detected through the comet assay, and the results are shown in Fig. 2. The average comet rate (DNA damage rate) of RCC sperm was significantly lower ($P < 0.05$) than that of 4nNT, while the average rate of G0 (DNA undamaged) was significantly higher ($P < 0.05$) than that of 4nNT. In sperm with DNA damage, the predominant type is GI, and there is no significant difference ($P > 0.05$) in the rate of RCC and 4nNT GII sperm. No sperm cells exhibiting grade III or IV damage were observed.

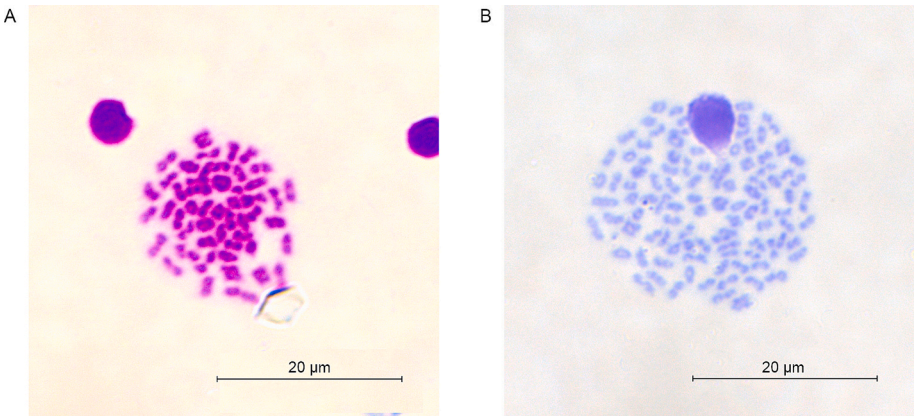

Fig. 1. Observation of bivalents in germ cells of RCC and 4nNT. (A) 50 pairs of bivalents in germ cells of RCC; (B) 100 pairs of bivalents in germ cells of 4nNT. Bar = 20 μm.

Table 1
Sperm concentration and motility parameters of RCC and 4nNT.

Parameters	Fish type	
	RCC	4nNT
Concentration ($\times 10^{10}$ cells mL^{-1})	1.87 ± 0.33^a	0.70 ± 0.21^b
MOT (%)	89.72 ± 3.38^a	80.88 ± 6.13^b
Lifetime (s)	61.00 ± 3.79^a	120.33 ± 9.29^b
VAP ($\mu m \cdot s^{-1}$)	124.30 ± 3.56^a	117.71 ± 6.13^b
VSL ($\mu m \cdot s^{-1}$)	114.41 ± 3.41^a	106.99 ± 5.83^b
VCL ($\mu m \cdot s^{-1}$)	135.06 ± 2.39^a	128.71 ± 5.11^b
ALH (μm)	5.45 ± 0.73^a	5.09 ± 0.59^a
BCF (Hz)	15.22 ± 3.02^a	16.10 ± 1.67^a
WOB (%)	91.23 ± 0.03^a	91.43 ± 0.02^a

The identical superscript letters within the same row indicate no significant difference ($P > 0.05$), whereas different superscript letters within the same row indicate a significant difference ($P < 0.05$).

3.4. Fertilization, hatching and abnormality rates

The results of artificial insemination in different groups are shown in the Table 2. When the RCC were used as egg donors, the fertilization rate and hatching rate of self-crossing (RCC ♀ × RCC ♂) was significantly higher ($P < 0.05$) than that of hybridization (RCC ♀ × 4nNT ♂), and the abnormality rate of self-crossing (5.11 ± 1.36) was significantly lower ($P < 0.05$) than that of hybridization (14.84 ± 3.47). However, in the case of 4nNT acting as egg donors, the fertilization rate and hatching rate of self-crossing (4nNT ♀ × 4nNT ♂) was significantly lower ($P < 0.05$) than that of hybridization (4nNT ♀ × RCC ♂), and the abnormality rate of self-crossing (34.57 ± 7.36) was significantly higher ($P < 0.05$) than that of hybridization (8.92 ± 1.98).

3.5. Screening and validation of DEGs

The detailed assembly annotation findings for RNA sequencing data are presented in Table S2 and Table S3. According to the screening criteria of P value adjusted (padj) < 0.001 and $|\log_2{}^{(\text{fold change})}| \geq 1$, 15,107 upregulated genes and 3172 downregulated genes were identified in the testis of 4nNT. Nine differentially expressed genes were

Table 2
Comparison of artificial insemination in different groups.

Index	Sperm	Eggs	
		RCC (♀)	4nNT (♀)
Fertilization rate (%)	RCC (♂)	88.52 ± 6.61*	84.57 ± 4.27*
	4nNT (♂)	75.80 ± 3.63*	71.16 ± 6.98*
Hatching rate (%)	RCC (♂)	74.49 ± 3.28*	73.13 ± 3.15*
	4nNT (♂)	67.42 ± 3.00*	62.72 ± 7.82*
Abnormality rate (%)	RCC (♂)	5.11 ± 1.36*	8.92 ± 1.98*
	4nNT (♂)	14.84 ± 3.47*	34.57 ± 7.36*

The asterisk represents significant difference ($P < 0.05$) within the same column.

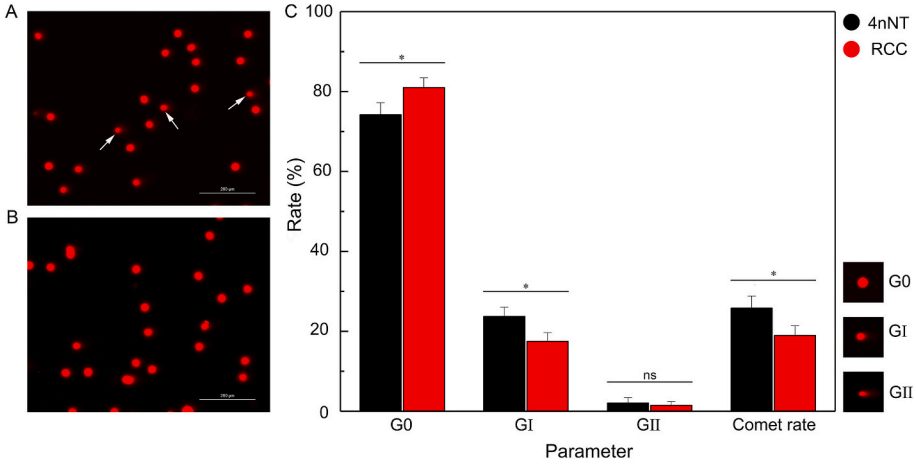

Fig. 2. Comet assay on DNA damage of fresh sperm in RCC and 4nNT. (A) Sperm of 4nNT; (B) Sperm of RCC; (C) Statistics on different grades of DNA damage. The "ns" indicates no significant difference ($P > 0.05$), whereas "*" indicates a significant difference ($P < 0.05$). The arrow indicates sperm with DNA damage, bar = 200 μm.

validated through qRT-PCR, the results demonstrated the reliability of RNA-Seq data (Fig. 3).

3.6. KEGG pathway analysis and co-expression network of DEGs

In order to gain a comprehensive understanding of the functions of differentially expressed genes, a KEGG pathway analysis was performed on the differentially expressed genes. The results of the enrichment analysis of the upregulated genes are presented in Fig. 4A. Fourteen pathways were identified as significantly enriched, including the p53 signaling pathway, fanconi anemia pathway, homologous recombination, and apoptosis. It is notable that all of these pathways are potentially linked to DNA damage and repair, which may contribute to the reproductive differences between 4nNT and RCC. The specific expression of the upregulated genes was indicated in the pathways (Figs. 4B-E). In order to identify the hub genes, the co-expression networks of the differential genes are presented in Figs. 4F and G.

4. Discussion

Triploids have the characteristics of fast growth, strong stress resistance and sterility. The large-scale breeding of triploids can enhance economic benefits (Wang et al., 2020a). Currently, the number of triploid seedlings obtained by artificial induction is limited, and the economic cost and technical threshold are high, which makes it difficult to be applied on a large scale (Zheng et al., 2023). In contrast, the production of triploids by crossbreeding female diploids with male tetraploids is the efficient way. Hence, reproduction of tetraploids is a pressing issue in polyploid breeding research, with the complex factors involved. In previous study, we obtained 4nNT and found that males have a high proportion of morphologically abnormal sperm, which may affect their reproduction and application (Hu et al., 2020). In this study, we observed the chromosome pairing of male 4nNT during meiosis, and detected the concentration, motility parameters and DNA integrity of the sperm. In addition, a comparative analysis was conducted on the transcriptome data of the testes in RCC and 4nNT. This study provides new insights into the reproductive biology of male 4nNT.

Organisms that engage in sexual reproduction produce gametes through meiosis, a process that is subject to rigorous monitoring (Gao et al., 2024). In the meiosis prophase, the formation of chromosome pairing and the occurrence of chromosome exchange and recombination are crucial events that must be completed (Tian et al., 2024). Normal chromosome synapsis maintains the progression of meiosis. In sterile triploid fish, unpaired chromosomes are observed, suggesting that disrupted chromosome pairing may be the cause of sterility in triploid fish (Zhang et al., 2021; Zhang et al., 2005). In contrast, the chromosome exchange and recombination lead to genetic diversity in gametes and represents a significant source of genetic variation in organisms (John et al., 2016). In this study, we found that 100 pairs of bivalents were formed in male 4nNT spermatocytes at meiosis prophase, with no unpaired chromosomes observed. This suggests that male 4nNT has a cytological basis for meiosis as well as reproductive potential, which is consistent with our previous results (Hu et al., 2020). Meanwhile, compared to the 50 crossover events of 50 pairs of bivalents in RCC, the 100 pairs of bivalents in 4nNT implies that with the doubling of chromosome numbers, individual spermatocytes also underwent doubled chromosome exchange and recombination. It is notable that the process of chromosomal exchange and recombination is accompanied by programmed double-strand breaks (DSBs) in DNA, which are typically repaired through the homologous recombination repair pathway (HR) to ensure the integrity of the genetic material (Longhese et al., 2009; Macaisne et al., 2018; Yadav and Claeys Bouuaert, 2021). This process also determines the cell fate, as only cells that have been successfully repaired can proceed to the next division program, while cells that have not been repaired or that have failed to repair in time will undergo apoptosis.

Sperm concentration and motility parameters are important indicators of the reproductive capacity of a species. In this study, we found that the average sperm concentration in RCC was significantly higher ($P < 0.05$) than that of 4nNT. Sperm motility parameters showed that the MOT of RCC was significantly higher ($P < 0.05$) than that of 4nNT, which may be related to the high proportion of flagellum-free sperm in 4nNT that we previously reported (Hu et al., 2020). However, the lifetime of 4nNT sperm was significantly longer ($P < 0.05$) than that of RCC, which is similar to previous findings and presumably related to the number and ATP content of sperm mitochondria (Duan et al., 2016; Hu et al., 2017). Furthermore, the VAP, VSL and VCL of RCC were found to be significantly higher ($P < 0.05$) than those of 4nNT, whereas no significant differences ($P > 0.05$) were observed in ALH, BCF and WOB. The results of the artificial insemination experiments demonstrated that the fertilization rate and hatching rate of groups using RCC as sperm donors were significantly higher ($P < 0.05$) than those of groups using 4nNT as sperm donors. However, the abnormality rate of groups using RCC as sperm donors was significantly lower ($P < 0.05$) than that of groups using 4nNT as sperm donors, which may be related to the embryonic development in different groups.

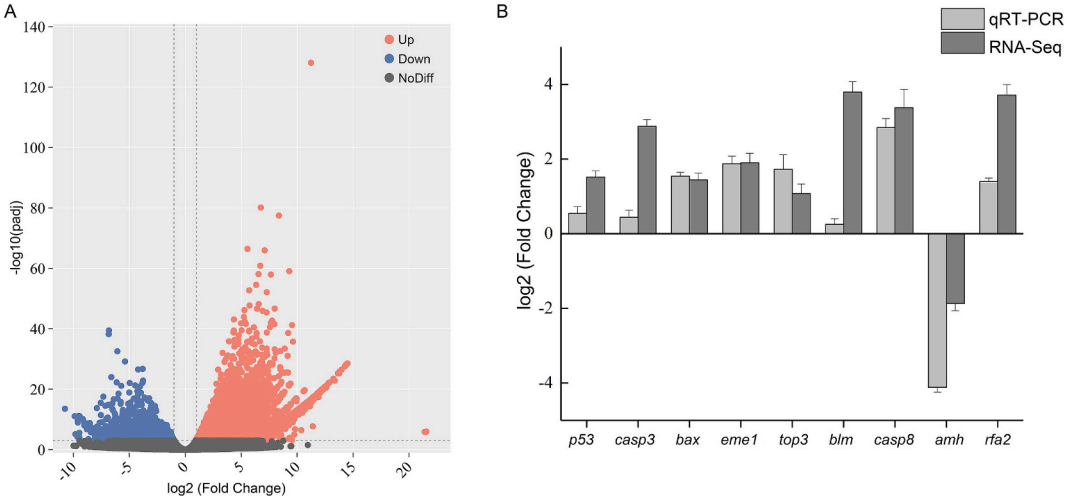

Fig. 3. Identification and verification of DEGs in testis transcriptome. (A) The distribution and expression levels of DEGs. The red dots indicate the upregulated DEGs, while the blue dots represent the down regulated DEGs. (B) The comparison of nine DEGs determined by qRT-PCR and RNA-seq. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

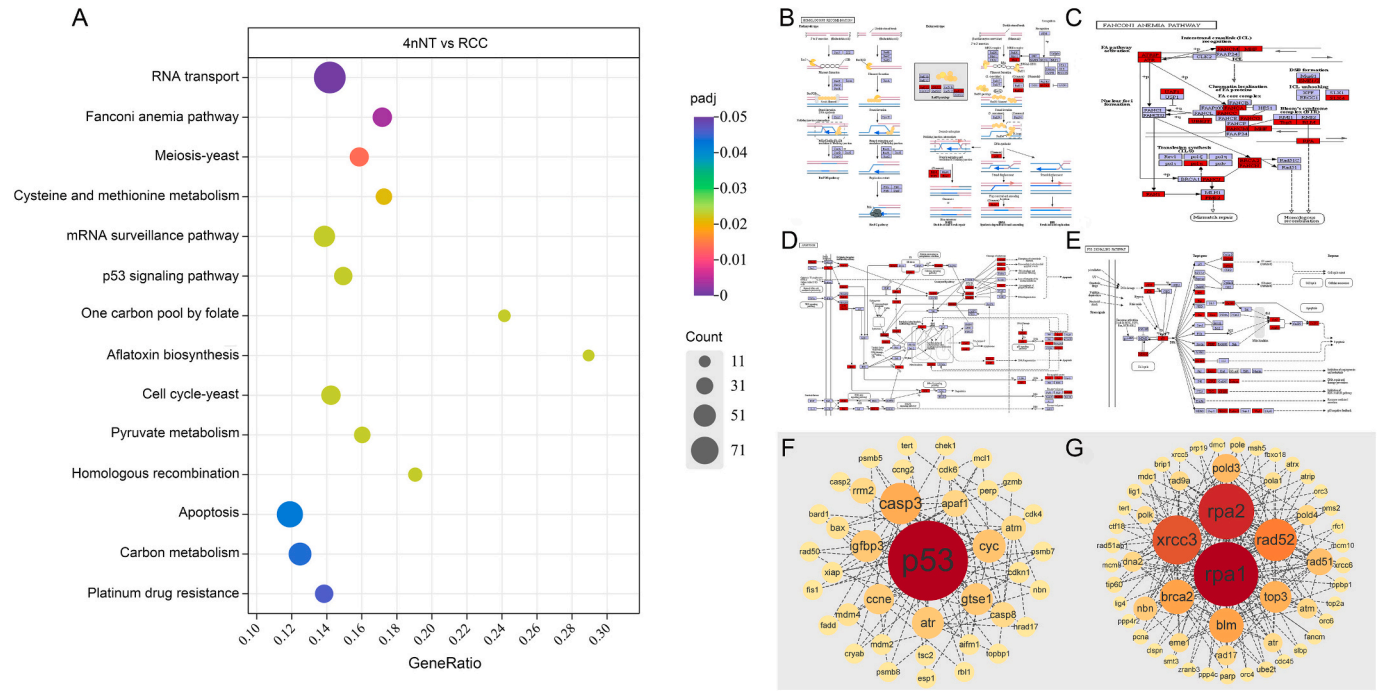

Fig. 4. KEGG pathway analysis and co-expression network of DEGs between 4nNT and RCC. (A) Significantly enriched KEGG pathways for upregulated genes. The colour bar represents the numerical range of padj, and the size of the dots represents the number of DEGs. (B) Homologous recombination. (C) Fanconi anemia pathway. (D) Apoptosis. (E) p53 signaling pathway. Upregulated genes are marked in red. (F) Co-expression network of DEGs involved in p53 signaling pathway and apoptosis. (G) Co-expression network of DEGs involved in homologous recombination and fanconi anemia pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The DNA integrity of sperm is frequently evaluated as it relates to the success rate of reproduction in aquatic animal. In this study, the DNA integrity of 4nNT and RCC sperm was investigated through comet assay. The results demonstrated that the comet rate of 4nNT sperm was significantly higher ($P < 0.05$) than that of RCC sperm. The predominant type of sperm DNA damage was grade I (GI), with a negligible proportion of grade II (GII). Previous studies have indicated that mild DNA damage to sperm does not affect fertilization (Gosálvez et al., 2014). However, there are also studies that have demonstrated that sperm DNA damage can lead to embryonic development arrest or fetal malformation (Devaux et al., 2011; Perez-Cerezales et al., 2010). Interestingly, a study has shown that sperm with damaged genomes can fertilize eggs, but their damaged genomes do not participate in embryonic development, resulting in abnormal haploid fish (Miskolczi et al., 2005). In this study, we observed that the abnormality rate of groups using 4nNT as sperm donors was significantly higher ($P < 0.05$) than that of groups using RCC as sperm donors in artificial insemination experiments. The current findings suggest that this may be affected by sperm DNA damage. Furthermore, further exploration is needed to determine whether there is haploid in the deformed offspring.

In this study, testes transcriptome data showed that upregulated genes were significantly enriched in 14 pathways, including p53 signaling pathway, apoptosis, fanconi anemia pathway, and homologous recombination. During meiosis, homologous recombination (HR) is the mechanism for repairing programmed double-strand breaks (DSBs) during crossover events of bivalent. The enrichment of up-regulated genes of 4nNT in the HR pathway suggests that it may promote damage repair in meiosis, which may be required for the doubling of the crossover events in its individual spermatocytes. However, the up-regulated genes of 4nNT were also significantly enriched in the p53 signaling pathway and apoptosis, implying that spermatocytes of 4nNT may undergo severe apoptosis. One of the triggers of spermatocyte apoptosis is endogenous DNA damage, which includes unrepaired programmed DSBs. According to the results of the sperm concentration and transcriptome data, the HR mechanism of 4nNT spermatocytes may be unable to fully withstand the DNA damage repair pressure caused by the doubling of crossover events. A comparable perspective was proposed in a study examining the reproductive mechanism of the triploid scallop (*Nodipecten subnodosus*) (Galindo-Torres et al., 2022).

5. Conclusions

In conclusion, the present study indicated that 4nNT have a cytological basis for meiosis as well as reproductive potential. However, the higher proportion of DNA damage in sperm and the imbalance between DNA damage repair and apoptosis may impair the reproduction of 4nNT. Therefore, it is proposed that a refined breeding system for specialized fish should be established. On the one hand, strict management of the cultivated water should be carried out to avoid reproductive impact caused by environmental pressure. On the other hand, the addition of functional substances to diets with the purpose of enhancing antioxidant capacity may be beneficial for reproduction. Finally, the development of substances or technology with apoptosis inhibitory effects may be a promising approach. However, this is contingent upon a comprehensive and nuanced understanding of 4nNT spermatocyte apoptosis.

Ethics statement

This study was authorized by the Animal Care Committee of Hunan Normal University, and all experimenters obtained professional training certification. In accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in China, all experimental fish were provided with improved living conditions. Prior to conducting the experiment, the fish were fully anaesthetized and appropriately disposed after experimentation.

CRediT authorship contribution statement

Haitao Zhong: Writing – original draft, Visualization, Investigation, Data curation. **Hong Chen:** Writing – original draft, Validation, Investigation. **Mingli Liu:** Visualization, Validation, Investigation. **Chiye Zhao:** Validation, Investigation. **Chaoying Luo:** Visualization, Data curation. **Zheduo Xiong:** Data curation. **Yan Li:** Visualization. **Yilin Wu:** Investigation, Data curation. **Yuheng Wang:** Validation. **Chun Zhang:** Methodology, Data curation. **Chang Wu:** Investigation, Data curation. **Qizhi Liu:** Software, Data curation. **Yu Sun:** Resources, Investigation. **Shi Wang:** Resources. **Ming Wen:** Resources. **Fangzhou Hu:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Shaojun Liu:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742283.

Data availability

The RNA sequencing data used in this study have been deposited in the NCBI Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra/, accession number: SRR30156031, SRR30156032, SRR30156033, SRR30156034, SRR30156035, SRR30156036).

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