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mtDNA copy number contributes to growth diversity in allopolyploid fish

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ABSTRACT

Phenotypic differences between diploid parents and their allotriploid offspring are common in aquaculture breeding. Some allotriploid populations exhibit rapid growth rates and increased body weight, which are significant for supporting fisheries development. Understanding the genetic mechanisms underlying these traits is crucial for implementing diverse breeding strategies to achieve high production in fish farming. Here, we collected the following fish species for our study: red crucian carp (*Carassius auratus* red var., 2nRR), common carp (*Cyprinus carpio* L., 2nCC), and two allotriploids ($3nR_2C$ and 3 nRC_2). These allotriploids were obtained through backcrossing an allotetraploid of *C. auratus* red var. × *C. carpio* L. ($4nR_2C_2$, ♂) with *C. auratus* red var. and *C. carpio* L. (♀), respectively. These allotriploids demonstrated faster growth rates compared to their diploid inbred parents, contributing to the Chinese fisheries industry for several decades. We conducted a systematic comparison of mitochondrial DNA (mtDNA) copy numbers in the liver and muscle tissues of 2nRR, 2nCC, $3nR_2C$, and 3 nRC_2 under different seasons. When entering winter (low water temperature: 13 °C), the triploid fish ($3nR_2C$ and 3 nRC_2) exhibited lower mtDNA copy numbers in the muscle, indicating a reduction in individual activity and energy expenditure to facilitate weight maintenance when food availability is limited. Furthermore, we analysed the expression levels of three nuclear-regulated mitochondrial genes (*tfam*, *tfb1m*, and *tfb2m*) and observed an imbalance of allelic expression in *tfam* and *tfb1m* in the two triploid fish. These findings enhance our understanding of the molecular regulatory mechanisms underlying growth trait differences among fish with different ploidy levels.

1. Introduction

Hybridization and polyploidization could rapidly shape various genotypes and phenotypes, providing us with abundant material for studying the contribution of genetic regulation to phenotypes. Interspecific hybridization in some plants, including *Triticum aestivum* × *Secale cereal* [1] and *Brassica nigra* × *B. rapa* [2] is always used to obtain varieties with excellent economic traits. In fish breeding, interspecific hybridization involving different genera or subfamilies has been detected in carps [3,4], salmonid fish [5], and cichlid fish [6]. Red crucian carp (*Carassius auratus* red var.) and common carp (*Cyprinus carpio* L.) shared common hybridization and whole genome duplication (WGD) events, and then diverged at 10.0 million years ago [7]. A nascent allopolyploid lineage ($4nR_2C_2$, F_3–F_{30}) was successfully established by the intergeneric hybridization of them [4,8]. Triploid fish have the characteristic of underdeveloped gonads, allowing for rapid growth [9]. After the interploid hybridization of $4nR_2C_2$ with its inbred parents (common carp and red crucian carp), two allotriploids ($3nR_2C$ and 3 nRC_2) were obtained and exhibit superior growth traits in comparison to their diploid inbred parents [10].

The heterosis of growth traits is a classical quantitative or complex trait [11,12]. The continuous variations led by hybridization and/or polyploidization are described as heterosis and used to improve agricultural production over many years [13–16]. The genetic basis of growth has been addressed in hybrid plants and animals for many years. Three classic quantitative genetic hypotheses (dominance,

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overdominance, and epistasis) were proposed, and their underlying molecular regulatory mechanisms involved genomic variations [17], epigenetic changes [18] and transcriptomic changes [10]. Meanwhile, diverse patterns and interactions of allelic expression in hybrids have always been considered key to growth diversity.

Mitochondria are cellular organelles that primarily participate in energy production. They contain an independent genome called mtDNA, which is maternally inherited through the egg. Several studies have suggested that specific variations in mtDNA can influence an individual's growth phenotype [19]. For instance, certain mtDNA mutations have been associated with phenotypes such as inherited short stature or growth retardation [20]. These mutations can lead to mitochondrial dysfunction, affecting energy metabolism and cellular function and consequently impacting the growth process. Additionally, variations in mtDNA have been implicated in other growth-related diseases and phenotypic traits. Research indicates that certain mtDNA mutations are linked to an increased risk of obesity, diabetes, and cardiovascular diseases. The mtDNA copy number significantly decreases with age in various tissues such as skeletal muscle, brain, liver, skin, and dorsal fin [21]. In old mice (24 months), the ratio of mtDNA to nDNA decreases by 75 % compared to young mice (2 months). This change may reflect either a depletion of mtDNA or a decrease in mitochondrial number/content [22]. These findings indicate a close relationship between changes in mitochondrial copy number and growth. However, it is important to acknowledge that the influence of mtDNA on growth phenotypes is a complex interplay between genetic and environmental factors, and there may be considerable variability in research findings. However, the influence of mitochondrial DNA on growth phenotypes remains elusive due to the complexity of this issue, which is influenced by multiple genetic and environmental factors.

The copy number and transcription efficiency of mtDNA in individual animals are closely related not only to the genomic characteristics of mtDNA but also to the expression of specific nuclear genes. Among these nuclear genes, *tfam, tfb1m*, and *tfb2m* play pivotal roles in regulating mitochondrial function. *Tfam*, encoded by nuclear genes, participates in the activation of mtDNA transcription and the regulation of mtDNA copy number [23]. On the other hand, *tfb1m* and *tfb2m* are indispensable for mtDNA transcription, and proteins interacting with POLRMT can modulate mtDNA gene expression to fulfill the physiological requirements of animal metabolism [24].

Here, we obtained two allotriploid fish populations from interploid hybridization of $4nR_2C_2$ with red crucian carp and common carp, in which the type of mtDNA was different. We investigated and compared the mitochondrial DNA copy number between the triploid individuals and their diploid parents, with the aim of addressing the questions: "Does the hybridization and polyploidization process influence mitochondrial DNA copy numbers? Is there an association between changes in mitochondrial copy number and variations in growth phenotypes?" Then, we focused on nuclear-encoded mitochondrial genes, including *tfam*, *tfb1m*, and *tfb2m*, to investigate potential mtDNA -nuclear gene interactions in two triploids with different growth traits. This analysis will help us understand if changes in nuclear gene copy numbers affect mitochondrial DNA copy numbers and whether this is correlated with the growth differences observed in two distinct triploid individuals. This result will help us understand the genetic basis of heterosis from a new perspective.

2. Material and methods

2.1. Samples

The fish used in this study were red crucian carp (*Carassius auratus* red var., 2nRR), common carp (*Cyprinus carpio* L., 2nCC), two allotriploids ($3nR_2C$ and 3 nRC_2), which were obtained from backcrossing an allotetraploid of *C. auratus* red var. × *C. carpio* L. ($4nR_2C_2$, ♂) with *C. auratus* red var. and *C. carpio* L. (♀), respectively. These fishes were fed in different pools (0.067 ha) under identical and appropriate environmental conditions, including water temperatures (4–29 °C), PH (7.0–8.5), dissolved oxygen content (5.0–8.0 mg/L), ample forage, suitable breeding density, etc. These pools were located in the drainage area of Dongting Lake, Hunan, China. Twelve healthy individuals (12 months after hatching) and twelve healthy individuals (19 months after hatching) were collected for this study. Some growth traits, including body length (BL), body height (BH), height of back muscle (HBM) and body weight (BW), were detected for each individual. These fish were deeply anesthetized with 300 mg/L Tricaine Methanesulfonate (Sigma-Aldrich, St Louis, MO, USA) for 10 min (25 °C) in a separate tank. After confirming their death, they were collected for dissection.

2.2. Measurement of DNA content

Peripheral blood samples of 2nRR, 2nCC, $3nR_2C$, and 3 nRC_2 were collected for DNA content determination. The average DNA content was measured using a flow cytometer (Cell Counter analyzer, Partec, Germany). All blood samples were processed according to the standard method [25]. Each sample was measured under the same conditions. To test the deviation of the DNA content between hybrid fish and the sum of 2nRR and 2nCC, Pearson's chi-square test was used in our analysis.

2.3. Hematoxylin and eosin (H&E) staining

A total 12 individuals of 2nRR, 2nCC, $3nR_2C$, and 3 nRC_2 were collected from 12 months after hatching. A histological staining technique was used to detect the cross-sectional area of a muscle fiber among these fish. After dissecting, the organization block of muscle tissue was fixed in Bouin's solution. After paraffin embedding, staining is performed by first staining the tissue with hematoxylin, a basic dye that stains acidic structures such as the cytoplasm and nuclei blue. Eosin is then used to stain proteins nonspecifically and appears pink. The sections were scanned and imaged using a slide scanner (Pannoramic MIDI), and the results were observed using CaseViewer 2.3 software. Finally, ImageJ v1.8.0 software was used to calculate the average fiber diameter by measuring the total area of fibers in the sections.

2.4. Detection of mtDNA copy numbers

Genomic DNA from the liver and muscle tissues of 2nRR, 2nCC, $3nR_2C$, and 3 nRC_2 was extracted using a universal genomic DNA extraction kit (TaKaRa, Dalian, China). A pair of universal primers (12S rDNA, F: AAATAGAGTGTCCCTTTGAACC, R: TACCGTGTTACGACTTGCCTC) was designed using primer (v. 5.0). A polymerase chain reaction (PCR) was performed to amplify the 12S rDNA from the genomic DNA. The PCR products were separated on a 1.3 % agarose gel using TBE buffer, and single bands were amplified to confirm the efficiency of the primers. The DNA of these samples was then subjected to real-time quantitative PCR (qPCR), and the mtDNA copy number was calculated using $2^{-\Delta\Delta CT}$ (Livak) method.

2.5. Gene expression of tfam, tfb1m, and tfb2m

Total RNA was extracted from tissue samples using the Trizol Reagent (Ambion, Shanghai, China) following the manufacturer's instructions. The concentration and integrity of the total RNA were determined using the 260/280 nm absorbance ratio and agarose gel electrophoresis. The purified total RNA (1000 ng) was used for cDNA synthesis using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Shanghai, China). Primer specificity was verified, and each sample was replicated three times (Table S1 in Supporting Information). A melt curve analysis was performed at the end of qRT-PCR detection to confirm the reliability of each qPCR analysis. The results were obtained using the Applied Biosystems QuantStudio 5 Real-Time PCR system, and the expression values of *tfam*, *tfb1m*, and *tfb2m* were determined using

the $2^{-\Delta\Delta CT}$ (Livak) method.

3. Results

3.1. Growth phenotype among allotriploids and diploids

Using cell flow cytometry, the DNA content in the two triploid fish is about 1.5 times that of their diploid inbred parents (Fig. 1). We detected the weight of four fish, including red crucian carp (*C. auratus* red var., 2nRR) and common carp (*C carpio* L., 2nCC), allotriploids of $3nR_2C$ and 3 nRC_2. There was a significant higher weight in the two allotriploids than the diploids (*t*-test: $p < 0.001$), while 3 nRC_2 exhibited a higher weight than $3nR_2C$ (*t*-test: $p < 0.05$) (Fig. 2a). Analysis of the size of muscle fibers exhibited that the diameter of the two triploids was higher than in their inbred diploid parents (Fig. 2, b-c). Meanwhile, a significant correlation was observed between the values of muscle fiber diameter and body weight (12 months after hatching) in the two triploids (Pearson correlation coefficient: R = 0.96) (Fig. 2d).

3.2. Detection of mitochondrial DNA (mtDNA) copy number

To investigate the relationship between mtDNA copy number and growth rate, we performed mtDNA copy number analyses not just in liver and muscle tissues but also in two development stages (12 and 19 months after hatching). As a key digestive organ, the mtDNA copy number in the liver could reflect food intake and digestion for each individual. As a key motor organ, the mtDNA copy number in muscle could reflect fish activity and energy digestion. For the 12 months after hatching, spring has arrived, and the water temperature has risen to 20 °C. For the 19 months after hatching, it has entered winter, and the water temperature has decreased to 13 °C. Our results at low water temperature (13 °C, 19 months after hatching) exhibited that mtDNA copy number was significantly lower in the triploids ($3nR_2C$ and 3 nRC_2) than in their diploid inbred parents (2nRR and 2nCC) for both the liver and muscle tissues (Fig. 3). Meanwhile, we detected a high correlation value between body weight and the mtDNA copy number of muscle in low water temperature (13 °C, 19 months after hatching) (Pearson correlation coefficient: R = 0.92) (Fig. 3b).

3.3. Imbalance of allelic expression

The copy number and transcription efficiency of animal mtDNA are not only closely associated with the characteristics of the mtDNA genome but also have a strong correlation with the expression of specific nuclear genes. In this study, we selected the *tfam*, *tfb1m*, and *tfb2m* genes to investigate their effects in regulating mtDNA copy number through nuclear gene regulation. By studying the expression levels of these three nuclear genes in liver and muscle tissues during different seasons (November: ~13 °C, April: ~20 °C), we found that the expression levels of *tfam* and *tfb1m* were higher in 2nRR compared to 2nCC (Fig. 4a). Additionally, in the two triploid fish, the expression of allele R (originating from parental 2nRR) was higher than that of allele C (originating from parental 2nCC) for *tfam* and *tfb1m* (Fig. 4b). In *tfb2m*, we observed a similar expression level between alleles R and C in both the two triploid fish (Fig. 4b).

4. Discussion

This study investigated the cross-sectional diameter of skeletal muscle fibers in allotriploid fish with different growth traits. It was found that the differences in individual size resulting from growth rate variations were positively correlated with the diameter of muscle fibers. The size of an individual's body is closely related to the number and size of muscle fibers. During the early stages of individual growth, the number of muscle fibers primarily influences body size. However, after reaching a certain stage of development, the number of muscle fibers is no longer the main factor affecting individual size. Instead, the size of muscle fibers plays a more critical role [26]. This study revealed that body size and muscle fiber size are closely associated in the two allotriploid fishes at the growth stages of 12 months and 19 months after hatching. In summary, the cross-sectional area of skeletal muscle fibers can provide important data for studying growth phenotypes at specific developmental stages.

The copy number of mtDNA in cells is closely related to metabolic activity [27]. After hybridization and polyploidization, the original regulatory mechanisms of the species change with the appearance of heterozygous alleles and different mitochondrial genomes [28,29]. The copy number of mtDNA in allotriploid fish varies with seasonal changes

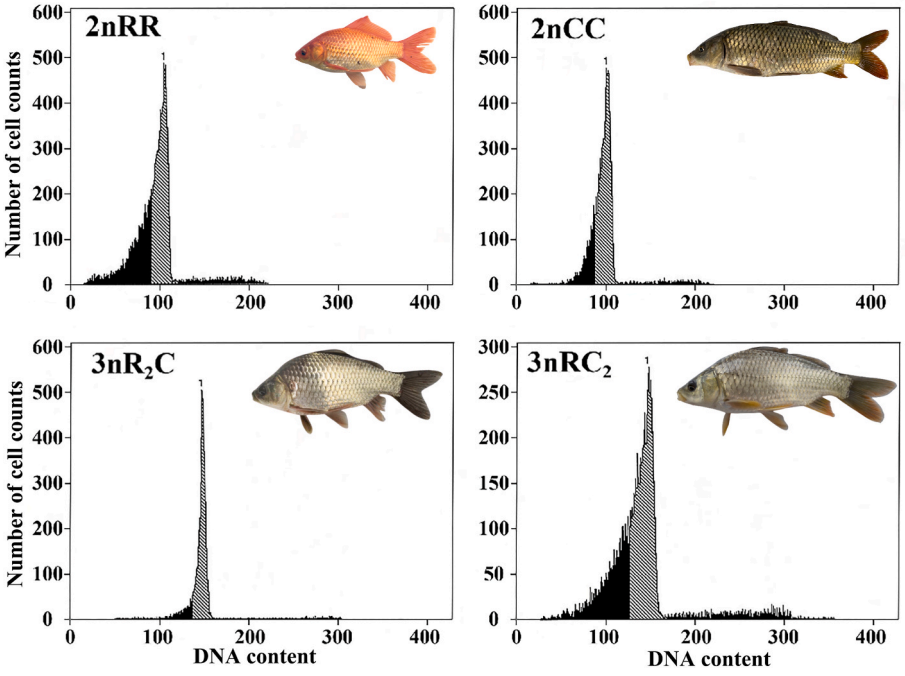

Fig. 1. Appearance and ploidy level among 2nRR, 2nCC, $3nR_2C$, and $3nRC_2$.

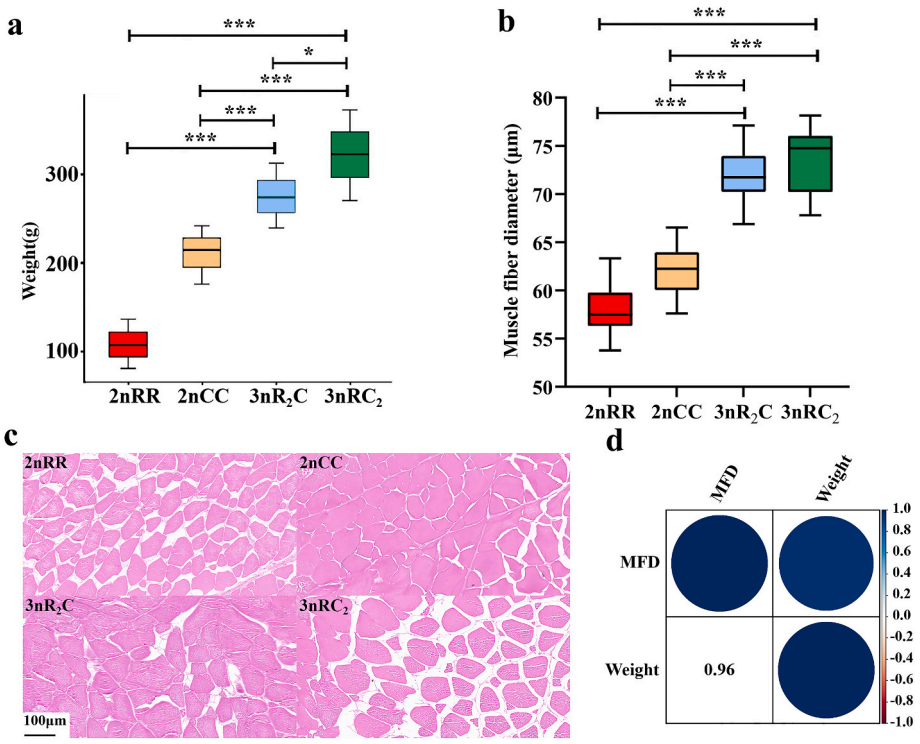

Fig. 2. Growth traits among 2nRR, 2nCC, $3nR_2C$, and $3nRC_2$. a. Body weight in 12 months after hatching. "*" represent "0.01 < *p*-value <0.05" using independent samples *t*-test, while "***" represent "*p*-value <0.001". b. Mean cross-sectional diameter of muscle fibers. c. Cross-section of skeletal muscle (H&E staining) showing the myofibers of the two triploids and their inbred parents (2nRR and 2nCC) (n = 3 biologically independent samples). Scale bar = 100 μm (20X). d. Correlation between body weight and diameter of muscle fibers. MFD: muscle fiber diameter.

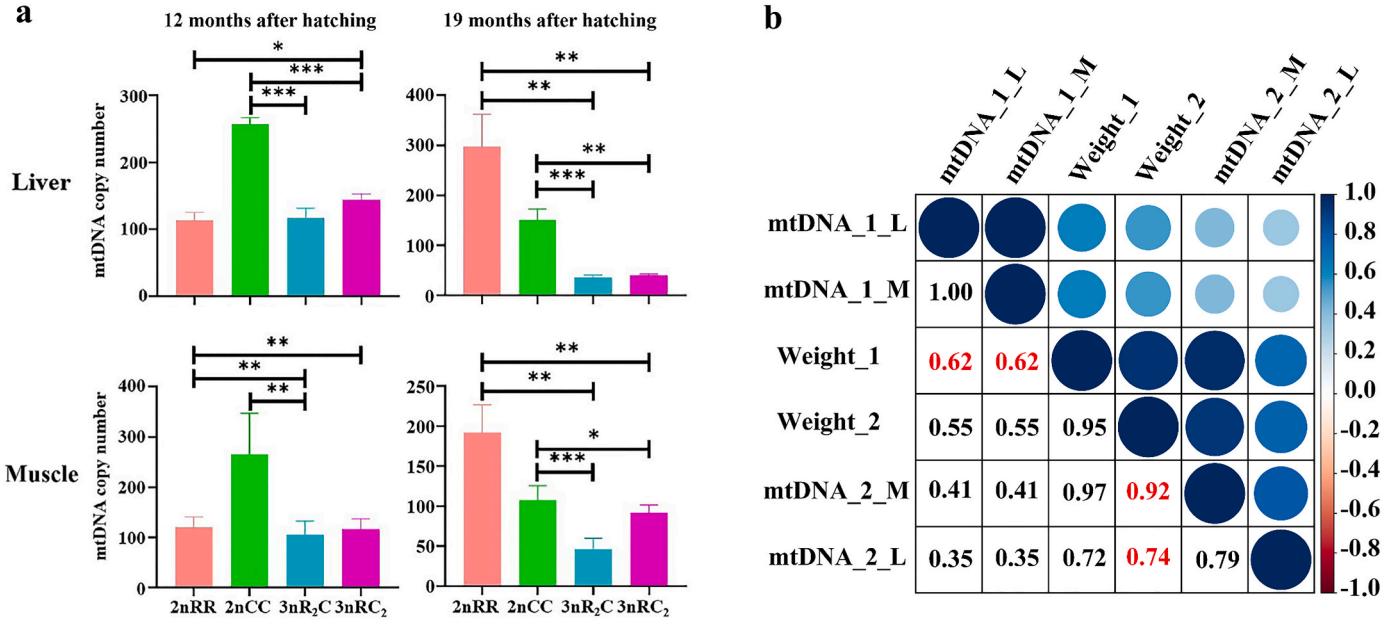

Fig. 3. mtDNA copy number and the relationship with body weight. a. mtDNA copy number in liver and muscle tissues. "*" represent "0.01 < *p*-value <0.05" using independent samples *t*-test, "**" represent "0.001 < *p*-value <0.01", "***" represent "*p*-value <0.001". b. Correlation between body weight and the mtDNA of liver and muscle. "1" represents 12 months after hatching, while "2" represents 19 months after hatching.

caused by factors such as water temperature and food availability. The copy number of mtDNA in muscle is closely related to the individual's movement [30,31]. Although behavioral observations and data are lacking, we speculate that a lower copy number of mtDNA may be associated with reduced movement and energy consumption. This situation is likely to benefit energy conservation during low water temperature conditions such as winter, resulting in a slower decline in body weight [32]. An individual's total energy intake is typically allocated to two primary aspects: growth and development, and daily life activities. For triploid fish, the decrease in mtDNA copy number in muscle, observed after they enter the winter season in November, suggests a reduction in individual activity and energy expenditure. This

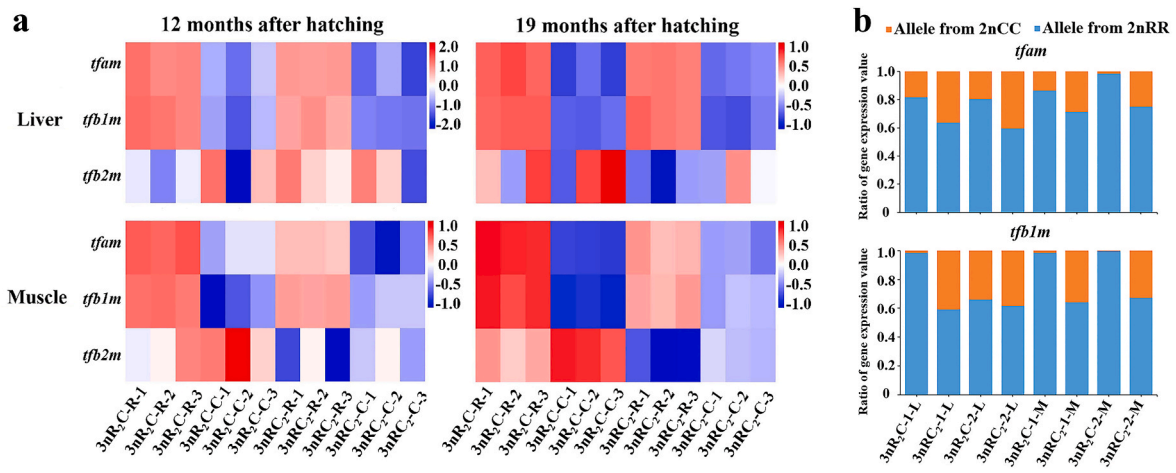

Fig. 4. Allele-specific expression of *tfam* and *tfb1m*. a. Heatmap of allele-specific expression in the two triploids. b. The high ratio of expression in allele R (originating from 2nRR) for *tfam* and *tfb1m*.

adaptation allows them to better maintain their weight when food availability is limited during the winter season. Consequently, this phenomenon appears to reflect a growth strategy that might be associated with the growth advantage of triploid fish compared to their diploid inbred parents. Analyzing the changes in mtDNA copy number in multiple organs of allopolyploid individuals throughout their life activities provides important data not only for exploring the phenotypic variations but also for understanding the biological functions of mtDNA copy numbers in animals.

Regarding the expression levels of three key nuclear-encoded mitochondrial genes (*tfam*, *tfb1m*, and *tfb2m*), we further confirmed that after polyploidization, more genes in individuals were upregulated. Additionally, in hybrid individuals, the expression of *tfam* and *tfb1m* genes consisted primarily of the allele originating from red crucian carp, indicating a partially dominant expression phenomenon of heterozygous alleles, which may have potential effects on individual growth and development. However, further research is needed. Currently, we have not found direct evidence of the regulation of mtDNA copy number variations by these three nuclear-encoded mitochondrial genes. It is speculated that the changes in mtDNA copy number may be jointly regulated by many other factors.

5. Animal ethics declarations

The animal work was approved by the academic committee in Hunan Normal University. We declare that animal handling complied with the relevant guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Engineering Center of Polyploidy Fish Breeding of the National Education Ministry, Hunan Normal University, Hunan, China.

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CRediT authorship contribution statement

Hong Zhang: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mengxue Luo:** Visualization, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yakui Tai:** Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Mengdan Li:** Supervision, Software, Resources, Project administration. **Jialin Cui:** Resources, Methodology, Funding acquisition. **Xin Gao:** Methodology, Investigation, Funding acquisition. **Li Ren:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shaojun Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Shaojun Liu is the editor-in-chief for Reproduction and Breeding and was not involved in the editorial review or the decision to publish this article. All authors declare that there are no competing interests.

Abbreviations:

2nRR	diploid *C. auratus* red var
2nCC	diploid *C. carpio* L.;
$4nR_2C_2$	an allotetraploid of *C. auratus* red var. × *C. carpio* L.;
$3nR_2C$	triploid fish obtained from hybridization between *C. auratus* red var. (♀) and an allotetraploid of *C. auratus* red var. x *C. carpio* L. ($4nR_2C_2$ ♂)
$3nRC_2$	triploid fish obtained from hybridization between *C. carpio* (♀) and $4nR_2C_2$ (♂)
BL	body length
BH	body height
HBM	height of back muscle
BW	body weight
PCR	polymerase chain reaction
H&E staining	Hematoxylin and eosin staining
qPCR	real-time quantitative PCR
MFD	Muscle fiber diameter
mtDNA	mitochondrial DNA

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.repbre.2023.12.008.

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