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Comparative studies on histological and ultra-structure of the pituitary of different ploidy level fishes

LONG Yu, LIU Shaojun, HUANG Weiren, ZHANG Jian, SUN Yuandong, ZHANG Chun, CHEN Song, LIU Jinghui & LIU Yun

College of Life Sciences, Hunan Normal University, Changsha 410081, China
Correspondence should be addressed to Liu Shaojun (email: lsj@hunnu.edu.cn)

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Abstract The histological and ultra-structure of the pituitary in diploid red crucian carp (*Carassius auratus red* var.), triploid crucian carp and allotetraploid hybrids within and after the breeding season were comparatively studied. The result showed that there were six endocrine cell types in the pituitary of these three kinds of fishes, and there was an obvious difference in cell size among different ploidy level fishes. As for the same type of pituitary cells, the cell size was increased gradually with the increasing ploidy level. In the breeding season, the allotetraploid hybrids had higher proportion of gonadotropin cells (GTH) than triploids, and the triploids had higher proportion of GTH than diploids. The results were related to the earlier sexual maturity of allotetraploid hybrids and sterility of triploid crucian carp. On the other hand, among the three kinds of fishes, the proportion of somatotropin (STH) cells in triploids crucian carp was the highest, whereas that in allotetraploid hybrids was the lowest. The results might be connected with the faster growth rate of triploids and slower growth rate of allotetraploid hybrids. In addition, in GTH cells of meso-adenohypophysis after the breeding season, there were many endocrine particles in triploids, while those endocrine particles were released from the cells in allotetraploids and diploids. This result showed that the sterility of triploid crucian carp might be related to the hormone which was not released from the GTH cells. In a word, the present study indicated that the differences in the structure of pituitary among different ploidy level fishes contributed to their difference in the growth rate and gonadal development.

Keywords: diploid, triploid, allotetraploid, pituitary, histological structure, ultra-structure.

Both fertile diploid males and females were found in the F_1 diploid hybrids of the red crucian carp (*Carassius auratus red* var.) (♀) × male common carp (*Cyprinus carpio*) (♂)[1]. F_2 hybrids were obtained by the crossing of F_1. Interestingly, some males and females of F_2 hybrids were able to produce diploid sperm and diploid eggs, respectively, and they fertilized with each other to form the allotetraploid hybrids in F_3. Until now, F_{15} hybrids were generated, and F_3—F_{15} hybrids formed a allotetraploid population, in which both males and females were fertile, and the tetraploidy was maintained. It is the first case in the world to artificially create the fertile successive tetraploid population without any experimental treatment[2,3]. The formation of the allotetraploid hybrids provided an important biological base for the comparative studies of different ploidy level fishes. In production, the main practical interest in allotetraploids is for producing triploid fish, for example, the triploid crucian carp was produced by crossing the males of allotetraploids with the females of Japanese crucian carp (*Carassius auratus cuvieri*), which have shown several enhanced performances such as sterility, faster growth rate, good flesh quality and higher anti-disease ability. The large scale triploid fishes have been cultured in 28 provinces of China. The sterile triploids were also valuable in protecting natural fish resources and creating transgenic triploid fish because of their sterility. Previously, we made a series of reports on the number of chromosomes, gonadal development, DNA content, mtDNA, and other biological characteristics in different ploidy level fishes[4−8]. However, there was no report on the comparative studies of pituitary's structures in the different ploidy level fishes. The pituitary is an important endocrine organ, which regulates the growth and development. Until now, most reports on the studies of pituitary focused on diploids[9−14], but not on triploids and tetraploids. In this paper, by comparing the similarities and differences at the level of histological and ultra-structure in different ploidy level fishes, we indicated that the sterility of triploid hybrids and fertility of allotetraploid hybrids were related to their pituitary's structures.

1 Materials and methods

The red crucian carp (2*n*), triploids (3*n*) and allotetraploids (4*n*) all came from the National Tetraploid Fish Protection Station located at Hunan Normal University, Changsha, China.

Each 10 samples from three kinds of fishes were selected at random. The pituitaries were removed and divided into two halves. One half was fixed in Bouin's solution for preparation of tissue sections, the other half was fixed in the 3% glutaraldehyde solution for the observation of ultra-structure. Parafin-embedded sections were cut and stained with Wilson-Ezrin pas-MB (PMB, 1954) and R.F phifer AB-pas-OG (APG, 1971). Then, the results were observed and photographed by an Olympus light microscope. Each 20 GTH and STH cells in the meso-adenohypophysis were chosen at random to measure their cell size and the nucleolus size. The experimental data were calculated by statistical software in EXCEL, and all probabilities were calculated by the *t* test.

To the samples fixed in the 3% glutaraldehyde solution, they were moved to 1% osmic acid solution, and then embedded in Epon812. Finally, they were cut and stained with uranyl acetate and lead citrate. The JEM-1230 (made in Japan) electron microscope was used to observe the ultra-structure of the samples.

At the same time, the gonads of all samples examined were also removed and fixed in Bouin's solution. Paraffin-embedded sections were cut and stained with hematoxylin and eosin, then observed and photographed by an Olympus microscope.

2 Results

2.1 Histological structure of the pituitary

The pituitaries of the different ploidy level fishes were similar. In appearance, they looked round or in chicken heart-shaped, and white in color. They located at the ventral of interbrain, connecting with hypothalamus by pituitary stem. and consisted of two parts: neurohypophysis and adenohypophysis. The neurohypohysis was composed of nerve fiber, microvein and neuroglia cells, in which the nerve fibers were distributed and extended to all areas of adenohypophysis. While the adenohypophysis could be divided into three obvious regions: pro-adenohypophysis, meso-adenohypophysis and meta-adenohypophysis (Fig. 1(a), (c), (e)).

2.2 Histochemical structure of pituitary

Based on the different chemical components in different endocrine cells, the pituitary could be divided into acidophile cells and basophil cells[15]. The acidophile cells, such as GTH cell, had carbohydrate protein and were stained to amaranth in PAS reaction. In some of these cells, the oxidized inclusion had acid radical which could be stained to blue in AB reaction. While the basophil cells, such as STH cell, were stained to orange with Orange G, because there was no carbohydrate protein or acid radical in their inclusion. According to the results of PMB staining, six endocrine cell types in the pituitaries of the three kinds of fishes were distinguished. In the pro-adenohypophysis, there were two kinds of cells, Prolactin cells (PROL) were stained to light yellow, which were the main

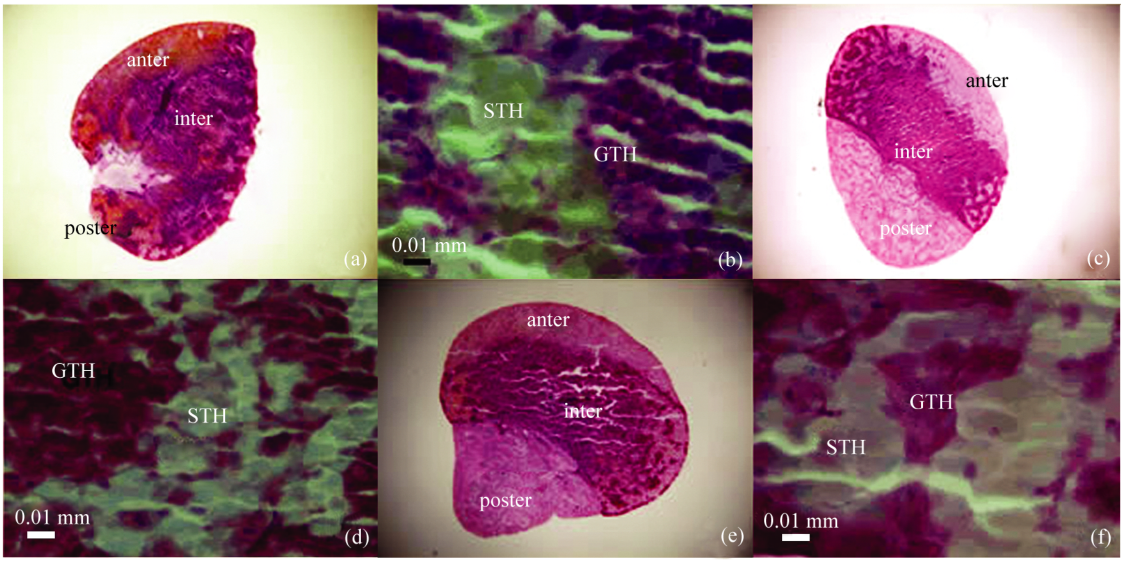

Fig. 1. The microstructure of pituitary and GTH, STH cells in meso-adenohypophysis in different ploidy level fishes. (a) The pituitary of diploid fishes, × 6 (anter, pro-adenohypophysis; inter, meso-adenohypophysis; poster, meta-adenohypophysis); (b) GTH and STH cells in meso-adenohypophysis of diploids. The color of STH cells was orange, and that of GTH cells was amaranth, × 120; (c) the pituitary of triploids, × 6; (d) GTH and STH cells in meso-adenohypophysis of triploids. The color of STH cells was light yellow, and some of them were inconspicuously stained, while the color of GTH cells was deep amaranth, × 120; (e) the pituitary of allotetraploids, × 6; (f) GTH and STH cells in meso-adenohypophysis of allotetraploids. The color of STH cells was yellow, and that of GTH cells was indigo or light amaranth, ×120.

components of pro-adenohypophysis, and adrenocorticotropic cells (ACTH) near the nerve fibers were stained to light amaranth. In the meso-adenohypophysis, there were somatotropin cells, gonadotropin cells and thypotropin hormone cells (TSH), and they were stained to orange or light yellow, amaranth or indigo and light amaranth, respectively. In the meta-adenohypophysis, the major cells were melanotropic stimulating cells (MSH), which were stained to deep purple. Among the six endocrine cells, PROL cell, STH cell and MSH cell were acidophile cells, while ACTH cell, GTH cell and TSH cell were basophil cells.

In the present study, the focus was STH cell and GTH cell in the meso-adenohypophysis of different ploidy level fishes. In general, most STH cells were distributed in both middle and rostral parts of meso-adenohypophysis, but some STH cells conglobately located around the nerve fibers. All of STH cells were elliptic or round, which were arranged out-of-order. GTH cells in elliptic or round shape were mainly distributed at the ventral of meso-adenohypophysis, and they were closely arranged. The similar results of STH and GTH cells were also described by Mattheij[16] and Lu[9,10]. Unlike STH cells of diploid fish which were stained to orange by OG (Fig. 1(b)), triploid fish's STH cells were stained to light yellow (Fig. 1(d)), and tetraploid fish's STH cells were stained to yellow (Fig. 1(f)). Furthermore, GTH cells of diploid fish were stained to amaranth by PAS (Fig. 1(b)), while those of triploids and tetraploids were stained to deep amaranth, indigo or light amaranth, respectively (Fig. 1(d), (f)). The differences of staining results among these three kinds of fishes were not obvious.

The cell size of pituitary increased with the increasing of the ploidy levels in different ploidy level fishes. As for the same type cells, the size of allotetraploids was larger than that of triploids, the latter was also larger than that of diploids. For diploids, triploids and allotetraploids, the average diameter of STH cells was 8.2, 13.7 and 17.0 μm, respectively, and their nucleolus diameters were 3.4, 5.6 and 7.1 μm, respectively. The GTH cells' diameters of the three fishes were 10.1, 14.6 and 18.9 μm, respectively, and their nucleolus diameters were 4.1, 6.1 and 8.4 μm, respectively. It was shown that GTH and STH cells' diameters increased with the ploidy level increasing among the three kinds of fishes. The detailed data about the sizes of the STH and GTH cells in different ploidy level fishes are presented in Table 1. Besides STH and GTH cells, the similar results also existed in the other four kinds of endocrine cells in pituitary.

The proportions of acidophile cells and basophil cells in meso-adenohypophysis varied in the three kinds of fishes. In the breeding season, the proportion of acidophile cells and basophil cells in meso-adeno-hypophysis of diploid fishes accounted for about 25% and 75% respectively. In triploids, those cells were about 38% and 62% respectively, and in tetraploids, they were about 20% and 80% respectively. The detailed information is shown in Table 2.

2.3 The ultra-structure of pituitary

Based on the shape and arrangement of rough endoplasmic reticulum (RER), the acidophile cells could be distinguished from the basophil cells under the electron microscope. The RER of acidophile cells was generally arranged around the nucleus regularly, in a ring form[15], such as that of STH cell, while RER of basophil cells like GTH cells was bubble-shaped or pool-shaped. They dispersed in cytoplasm irregularly. In the present study, the focus was the observation of STH and GTH cell. In different ploidy level fishes, the endocrine particles in STH cells were more than those in GTH cells, but the diameter of endocrine particles was smaller than those in GTH cells. With regard to the morphology of cells, STH cells in diploid fishes were nearly round, and those of triploids were also nearly round or irregular shape. The STH cells of the allotetraploids were round or nearly round. On the other hand, all the GTH cells in the three kinds of fishes contained a large number of endocrine particles, which were divided into two types with different sizes. The small one shaped as granules, and the larger one looked like globules. The GTH cells could be distinguished from other five endocrine cells with this special characteristic. The GTH cells in diploids were elliptic or long strip, and those in triploids and allotetraploids were both elliptic, so the shapes of GTH and STH cells in the three kinds of fishes were similar, and differences among them were very small.

After the breeding season, there were lots of cavities in GTH cells of allotetraploids (Fig. 2(b)), which happened similarly in diploid fishes (Fig. 2(a)). It was because lots of endocrine particles were released from the GTH cells after the breeding season. On the contrary, the quantity of the cavities in the GTH cells was few, and many endocrine particles still stayed in the cells (Fig. 2(c), (d)).

In the breeding season, there were lots of endocrine particles arranged closely in GTH cells of diploid (Fig. 2(e)) and allotetraploid fish (Fig. 2(f)). While triploids had an obvious difference from the former two, the number of endocrine particles was smaller, and they dispersed in the endoplasmic reticulum (Fig. 2(g), (h)).

2.4 The gonadal observation of different ploidy fishes in the breeding season

The histological sections indicated that both diploid (Fig. 3(a)) and allotetraploid fishes (Fig. 3(b)) had normal gonads (ovaries and testes) in the breeding season. In the lobules of testes, numerous spermatocytes and mature sperms were found in the ovaries, and many mature ova were observed. But the ovarian structures of triploids were different from those of the diploids obviously (Fig. 3(c)). The ovaries of triploids contained many small oogonium-like cells and only a few large primary oocytes, no matured ova being found, and the testes of diploids consisted of many lobules in which there were many spermatids, no

Table 1 Comparison of STH and GTH cells in meso-adenohypophysis of different ploidy level fishes

Cell type	Fish type	Cell diameter (μm)	Difference of cell diameter (*P* value)	Nucleous diameter (μm)	Difference of nucleous diameter (*P* value)
STH cell	diploid	8.2 ± 0.70		3.4± 0.67	
	triploid	13.7 ± 0.81	3*n* vs 2*n* (<0.001) a)	5.6 ± 0.82	3*n* vs 2*n* (< 0.001) a)
	allotetraploid	17.0 ± 0.69	4*n* vs 2*n* (<0.001) a)	7.1 ± 0.55	4*n* vs 2*n* (< 0.001) a)
GTH cell	diploid	10.1 ± 0.85		4.1 ± 0.72	
	triploid	14.6 ± 0.60	3*n* vs 2*n* (<0.001) a)	6.1 ± 0.89	3*n* vs 2*n* (< 0.001) a)
	allotetraploid	18.9 ± 0.75	4*n* vs 2*n* (<0.001) a)	8.4 ±0.75	4*n* vs 2*n* (< 0.001) a)

a) It means the extremely significant difference between triploid and diploid or between allotetraploid and diploid.

Table 2 Proportion of acidophile and basophil cells in meso-adenohypophysis of different ploidy level fishes in the breeding season

Fish type	Acidophile cells (%)	Basophil cells (%)
Diploid	25	75
Triploid	38	62
allotetraploid	20	80

mature spermatozoon being observed. In addition, only two strips fat tissue located on the gonad places in some triploids, neither ovary nor testes being observed. There was no germ cell in the fat tissue except for the fat cells (Fig. 3(d)).

3 Discussion

3.1 Comparison of endocrine cells in pituitaries of different ploidy level fishes

The endocrine cells in pituitaries of different ploidy level fishes had obvious difference in cell size. Besides, there were differences in the distribution of

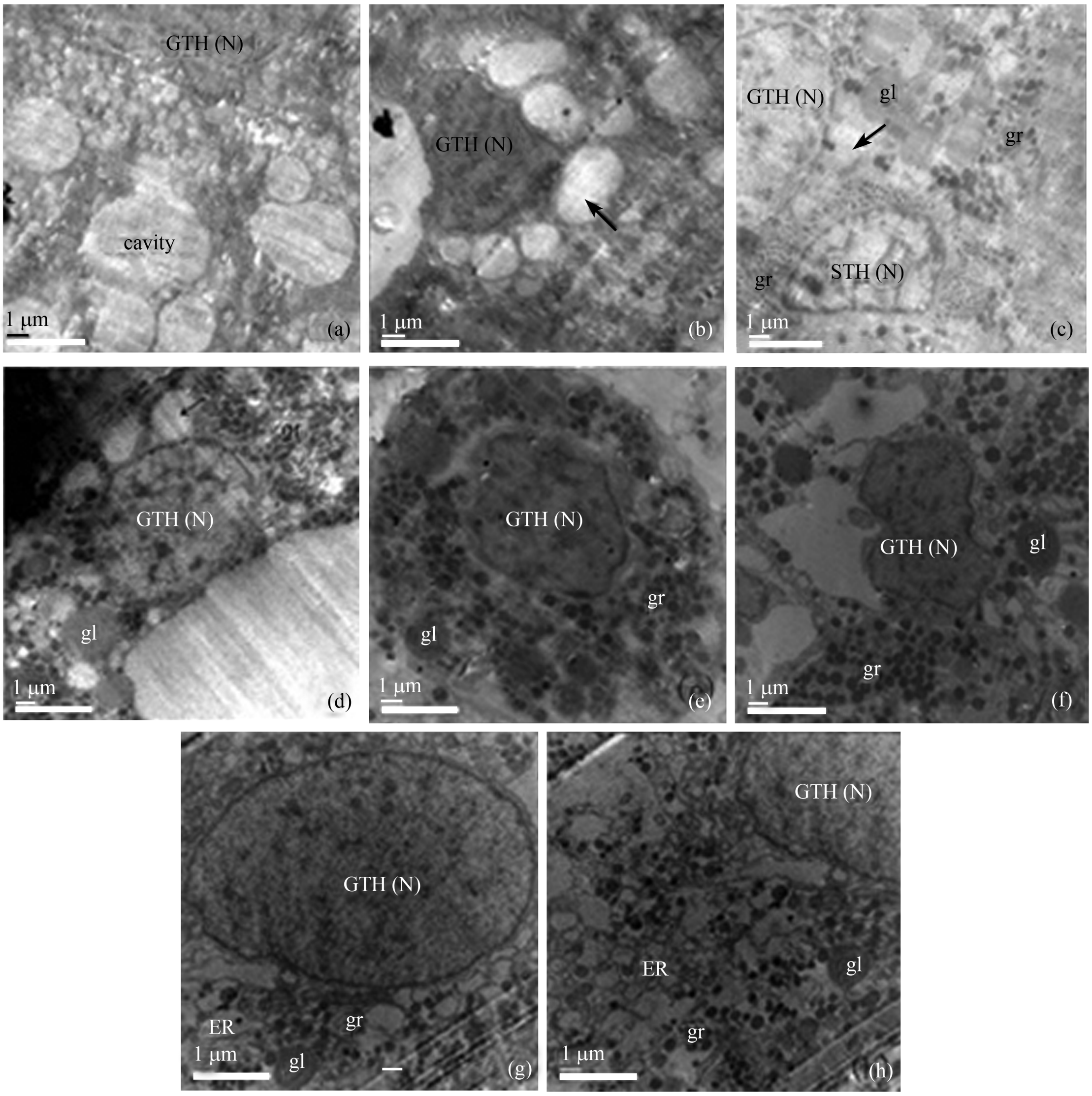

Fig. 2. The ultra-structure of GTH cells in meso-adenohypophysis of different ploidy fishes. (a) GTH cells in meso-adenohypophysis of diploids after the breeding season, ×15000 (N: nucleus); (b) GTH cells in meso-adenohypophysis of allotetraploids after the breeding season, ×15000 (→: cavity); (c) GTH and STH cells in meso-adenohypophysis of triploids after the breeding season, ×15000 (N: nucleus, gl: globules, gr: granules, →:endoplasmic reticulum dispersed in cytoplasm); (d) GTH cells in meso-adenohypophysis of triploids after the breeding season, ×15000 (→: cavity); (e) GTH cells in meso-adenohypophysis of diploids in the breeding season, ×15000; (f) GTH cells in meso-adenohypophysis of allotetraploids in the breeding season, ×15000; (g) and (h) GTH cells in meso-adenohypophysis of triploids in the breeding season, ×15000 (ER: endoplasmic reticulum in bubble-shaped or pool-shaped).

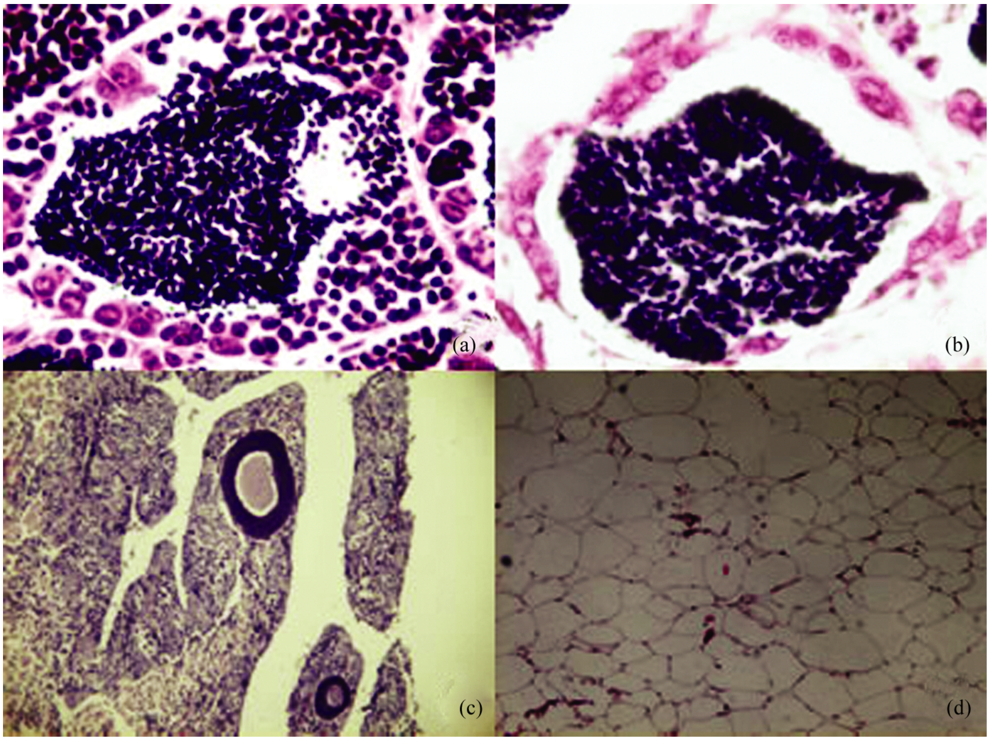

Fig. 3. The gonadal structure of different ploidy fishes in the breeding season. (a) the mature testes of diploid, numerous of mature sperms were found in the lobules of testes, ×120; (b) the mature testes of allotetraploids, numerous of mature sperms were also found in the lobules of testes, ×120; (c) the ovaries of triploids, there were many small oogonium-like cells and only a few large primary oocytes in it. ×20; (d) the fat tissue "gonad" of triploid. The fat cells arranged in reseau, and the fat particles were dissolved by organic impregnant during the process of tissue sections, turned to blurry. No germ cells could be found in it. ×12.

acidophile cells and basophil cells, and the exclusion of granules in GTH cells after the breeding season. Since the GTH and STH cells were related to the growth and gonadal development, they should be paid more attention to in the present study.

As the proportion of STH and GTH cells in pituitary changed regularly in different seasons, the acidophile cells such as STH cells were more than basophil cells such as GTH cells in meso-adenohypophysis of the three kinds of fishes in the breeding season. In the breeding season, the number of GTH cells in pituitary increased, the size of these cells enlarged, while the number of STH cells decreased relatively. The proportion of STH cells in meso-adenohypophysis was 25% in diploids, 38% in triploids and 20% in allotetraploids, respectively. Compared with diploids, the number of STH cells in triploids was 1.5 times of that in diploids. As indicated in our previous study, the triploids had shown several enhanced performances such as higher survival rate, faster growth rate, which was 21.78% and 70.83% faster than Japanese crucian carp (*Carassius auratus cuvieri*) and *Carassius auratus var pengze* respectively[4]. However, allotetraploids grew more slowly than the diploids and triploids. So it indicated that the proportion of STH cells in pituitary might be related to the growth rate. On the other hand, the proportion of GTH cells in tetraploids was the highest among these three kinds of fishes. Our previous study on gonadal development of allotetraploids also showed that the female allotetraploids reached sexual maturity when they were 1 year old, which was earlier than common carp, but the male allotetraploids reached maturity at age of 150 days, which was earlier than both crucian carp and common carp[5]. So it indicated that the male and female allotetraploids reached sexual maturity earlier than both diploids and triploids, which might result from the larger proportion of GTH cells with strong secretive ability in allotetraploids.

Similar to the GTH cells in diploids, the GTH cells of allotetraploids were also full of endocrine particles, which were arranged closely. Based on the close relation between GTH cells and gonadal development, the normal structures of the pituitary of the allotetraploids supported the normal development of their gonads. The gonad's structures also indicated that allotetraploids, just like diploids, were able to produce normal eggs and spermatozoa, showing that both the male and female of allotetraploids were fertile. But in the GTH of triploids, the reduction of the endocrine particles in it probably contributed to their abnormal development, even to their sterility. The results indicated that the difference among different ploidy level fishes might result from the degeneration of endocrine particles in GTH cells or an uncertain factor caused the decreasing of particles. Furthermore, the differences also existed in the structures of GTH cells in different ploidy level fishes after breeding season. After breeding season, in the GTH cells of both the diploids and allotetraploids, there were a lot of cavities caused by the releasing of the endocrine particles. The similar results were also reported by other studies[15]. Interestingly, there were fewer cavities in GTH cells of triploids than those of diploids and allotetraploids. The probable reason was that some factors restrained the releasing of endocrine particles in the GTH cells of triploids. Learning from our previous studies, the indexes of ovaries, testes and the fat tissue "gonad" found in triploid crucian carp were respectively lower than those of the ovaries, testes and the average value of the ovaries and testes found in the Japanese crucian carp (*Carassius auratus cuvieri*). The indexes of the ovaries and the testes in the Japanese crucian carp were respectively 2.85 and 1.94 times of the indexes of the ovaries and the testes in the triploid crucian carp. The index of the average value of the ovaries and testes in the Japanese crucian carp was 5.60 times of that of the fat tissue found in the triploid crucian carp[17]. The index of gonad in the red crucian carp was 16%—21.3%[1], which was 5.6 times of the indexes in the triploids as well. Based on the tissue sections, both the ovaries and testes in triploids were not able to produce the mature ova and sperm, suggesting that they were sterile. In addition, after the culture of more than 10 years, we did not find that the triploids could produce the offspring, which further proved their sterility. Because the development of GTH cells was related to the gonadal development, the abnormal gonad of triploids might connect with the repressed releasing of endocrine particles in GTH cells. But what factor causes the repressed releasing needs further study in future.

3.2 The relationship between cell size of pituitary and ploidy levels

Concerning the same type cells in pituitary, the size of cell and nucleus in allotetraploids was larger than that in triploids, and the triploids cells' size was larger than that of diploids. Our study showed that the diploid eggs of allotetraploids with diameter of 0.17 cm were larger than haploid eggs of diploid fishes with the diameter of 0.13 cm. The head of the diploid spermatozoa produced by allotetraploids were 2 times of that of the haploid spermatozoa produced by common carp[2]. Moreover, the studies on blood and blood cells including red blood cell, neutrophil cell and mononuclear cell indicated that the ratio of cell size among allotetraploids, triploids and diploids was 4∶3∶2. In addition, with increasing of ploidy, the ratio between short diameter and long diameter of the red blood cells decreased[18]. Generally speaking, the increasing of nucleolus size was related to the increasing of chromosomes' number, and the cell size also increased with the increasing of nucleolus size in order to maintain the certain ratio of nuclear and cytoplasm. Thus, the sizes of cell and nuclear in the polyploidy fish were generally larger than those of diploids. However, not all the organs and bodies of polyploidy were larger than those of diploids. It was to say that the size of tetraploid body was not surely two times of diploids when they had the same age. It was because with the increasing of cell size, the number of cells decreased, so that the size of organs and bodies would be similar in different ploidy level fishes[19]. The allotetraploids had four sets of chromosomes, whose chromosome number and DNA content were two times of those in their diploid parents (common carp and red crucian carp). With more chromosomes and more genetic material, the pituitary cells and nucleolus of allotetraploids were bigger than those of diploids. The presence of the larger pituitary cells of triploids than those of diploid fishes was due to the similar reason, but it also needs more evidence to prove.

3.3 The comparison of gonad structures among different ploidy level fishes

In the breeding season, both diploid and allotetraploid fishes had normal ovaries and testes, consisting of numerous mature sperms in the lobules of testes and many mature ova in the ovaries, respectively. The mature eggs and white milt could be stripped from the female and male allotetraploids respectively, proving that both male and female fishes of allotetraploids were fertile. But in triploids, there were only spermatids in testes, no mature spermatozoon was observed. The spermatids were also found to be degenerated. In the ovaries of triploids, there were only a few primary oocytes which could not develop into mature ova. In addition, some triploids only had two strips of fat tissue located on the gonad places with no germ cells in it. All of the evidence indicated that the development of triploids' gonad was repressed. The presence of sterile gonads found in triploids was consistent with the results of gonadal structures described by our previous studies[4].

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