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Research Article

It is all in the Blood: Erythrocyte Characterization of Triploid and Diploid African Catfish, *Clarias gariepinus*

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Abstract

Objective: A study was done to characterize the erythrocyte of triploid African catfish. **Methodology:** Triploid African catfish *Clarias gariepinus* fingerlings were produced via cold shock method and their erythrocyte profiles were compared with normal diploid controls. **Results:** Approximately 99% of triploid erythrocytes were ellipsoidal whereas 97.5% of the diploid erythrocytes were round in shape. All of the erythrocyte parameters showed significant higher values in triploids and exhibited size range exclusive to triploids except erythrocyte cellular volume. The nuclear volume and nuclear area were among the two erythrocyte parameters that showed the largest increment in triploids (76.6 and 55.3%, respectively) with their respective exclusive triploid range at 74.3-150.2 and 22.8-35.2 μm . Both erythrocyte and nuclear major axis increased at a much higher percentage with respect to erythrocyte and nuclear minor axis in triploid specimens, resulting in the ellipsoidal shape of triploid's erythrocytes. The erythrocyte major axis also has the highest percentage (76.0%) of erythrocytes that fall in the exclusive triploid range (11.9-14.9 μm) compared to the other erythrocyte parameters. **Conclusion:** Therefore, it is suggested that determination of triploid African catfish can be performed solely based on the measurement of erythrocyte major axis.

Key words: African catfish, aquaculture, characterization *Clarias gariepinus*, erythrocyte, triploid

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INTRODUCTION

Triploidy is the genomic state of having three complete sets of chromosomes and it is relatively easy to be induced in fish. Triploid animals are produced by inhibiting the release of the first or second polar body through the application of a chemical or environmental stress soon after fertilization¹. Triploid fish do rarely occur in nature and they can be induced as well. Most of triploid inductions were done using cold shock treatments²⁻⁵ and heat shock treatments^{6,7}. Triploids have been successfully produced for many commercially important fish species⁸⁻¹¹ and shellfish¹²⁻¹⁵.

Methods such as erythrocyte measurement, karyotyping chromosome, flow cytometry, microfluorometry and Nucleolar Origin Region (NOR) have been used to determine the triploidy status in organisms. Karyotyping chromosome method is a time-consuming and tedious method that estimates triploid chromosomes in an organism, requires the use of cytotoxic chemical and colchicines to interrupt the mitotic division to give readable chromosome spreads¹⁶. Meanwhile, flow cytometry and microfluorometry are laser-based and fluorescent-based methods capable of estimating the number of triploid cell nuclei, but involve costly and specialized equipments¹⁷⁻¹⁹. From all the methods, erythrocyte measurement is the easiest, quickest and cost-effective method to determine triploidy in an organism without sacrificing the fish and is suitable for commercialization of triploid fishes²⁰.

Several studies have shown that the size of erythrocytes in triploid organisms are generally larger compared to diploid's²¹⁻²³. However, previous study has reported that the distribution size of erythrocyte can overlap between triploid and diploid specimens²⁴. This has risen as an issue as determination of triploidy in an organism based on erythrocyte measurement can be inaccurate. Due to this, detailed characterization of triploid erythrocyte is crucial in order to understand the actual erythrocyte profile of triploid organisms. The characterization of erythrocyte profiles based on species would provide accurate information on triploid organisms for both researchers and farmers. In addition, it can also assist in managing triploid organisms during commercialization as African catfish is an important local fish species cultured in Malaysia. Currently triploid African catfish has been successfully produced by several researchers^{25,26,23} via cold shock treatments. Thus, this study was done to compare the erythrocyte characteristics of diploid and triploid African catfish, *Clarias gariepinus* and to characterize the erythrocyte parameters of triploid *C. gariepinus* for identification of triploidy in this species.

MATERIALS AND METHODS

Broodstocks weighing between 620-920 g were collected from a local dealer and maintained in one-tonne tank at the UMT freshwater hatchery until matured and ready to be used for breeding. Daily feeding using pellet (approximately 3% of b.wt.) was applied. Healthy and matured broodstocks were injected with GnRH (OVAPRIM, Canada) at a dosage of 0.5 mL kg⁻¹ for males and 1.0 mL kg⁻¹ for females²⁷. A minimum acclimatization period of 10 h post-injection was given for each isolated individuals prior eggs and sperm collection. Eggs were collected in a clean bowl by stripping the female softly along her abdomen. Simultaneously, male was dissected and its sperm sac was cut open and squeezed to obtain the stored sperms. The collected eggs and sperms were mixed evenly before subjecting the fertilized eggs to cold shock method. The cold shock method was based on baseline parameters set for this species^{2,28,29}. Triploidy was induced by exposing fertilized eggs at 5°C for 20 min, 3 min after fertilization. Eggs without cold shock treatment were regarded as control (diploid). Three replicates were carried out for each triploid and diploid treatment. Each treatment was maintained separately in 100 L tank with strong aeration until hatching.

After 2 weeks, 20 fingerlings from each treatment were randomly selected for erythrocyte characterization. Blood was obtained by cutting the caudal fin using a pair of surgical scissors without sacrificing the fish. Triploidy was assessed by erythrocyte measurement method and slide was prepared using dry blood smear method^{21,30}. A drop of blood was dripped on a glass slide and gently smeared using a cover slip. The smeared blood was air dried for 2 min before fixing with 95% alcohol and air dried again. The slide was then stained with 10% Giemsa stain for 1 h before washing off the excess Giemsa stain with distilled water at room temperature. The Giemsa-stained slide was air dried, mounted with distyrene plasticizer and xylene (DPX) and sealed with a cover slip. A compound microscope at 40X magnification (Nikon Eclipse 80i, Japan) was used to measure the cell major axis, cell minor axis, nucleus major axis and nucleus minor axis (Fig. 1). The smeared erythrocyte was sectioned into five different blocks (Fig. 2). Ten erythrocytes were measured for each one block. A total of 50 erythrocytes were measured for each individual. Formulae below were used to calculate the cell volume, nucleus volume, cell area, nucleus area and percentage of triploid. Data was statistical analyzed by using Independent t-test:

$$\text{Volume of erythrocyte} = 4/3 \times \pi \times (A/2) \times (B/2)^2$$

$$\text{Area of erythrocyte} = \pi \times A \times B$$

RESULTS

Triploid African catfish were successfully produced using cold shock technique with a success rate of 96-100%. Approximately 99% of triploid erythrocytes were ellipsoidal while 95.7% of diploid erythrocyte had round shape (Fig. 3, 4).

Triploid's erythrocyte major axis ($12.4 \pm 0.7 \mu\text{m}$), erythrocyte minor axis ($8.3 \pm 0.7 \mu\text{m}$), nucleus major axis ($6.0 \pm 0.6 \mu\text{m}$) and nucleus minor axis ($3.3 \pm 0.4 \mu\text{m}$) were significantly larger ($p < 0.05$) compared to that of diploid's (Table 1). The erythrocyte area, nucleus area, erythrocyte volume and nucleus volume were significantly larger in triploid specimens as well with an increment of 26.4, 35.6, 31.7 and 43.4%, respectively. Ratio of diploid:triploid was highest at erythrocyte nuclear volume (1:1.77) followed by nuclei area (1:1.55) and erythrocyte volume (1:1.46) (Table 1).

The increment of one chromosome number in triploid fishes showed a range size which only exist on triploid fish but do not exist in diploid fish yet, there is overlapping between

triploid and diploid erythrocyte size. This showed that not all triploid erythrocyte were bigger in size than diploid. However, even though there were overlapping in erythrocyte size, there were a range of sizes that only occurred in triploid erythrocytes. Triploid's erythrocytes have larger ranges in almost all erythrocyte parameters, except for erythrocyte volume, even though the lower range limit was still very much higher in triploid ($237.0 \mu\text{m}^3$) compared to the erythrocyte volume of diploid ($114.7 \mu\text{m}^3$). Excluding erythrocyte volume, all other erythrocyte parameters showed an upper exclusive triploid range (Table 1). The erythrocyte parameter with the highest percentage of triploid erythrocytes being larger than the normal diploid erythrocyte range was erythrocyte major axis followed by nucleus major axis and nuclear area with a 76.0, 54.0 and 21.0%, respectively (Table 1).

DISCUSSION

The erythrocyte shape between triploid and diploid varies because of the increment of one chromosome set in the triploid fish. The change in erythrocyte shape from round to ellipse and obvious increase in size in triploid *C. gariepinus* was observed in triploids of other fish species as well, such as red tilapia *Oreochromis niloticus*³¹, loach *Misgurnus anguillicaudatus*³², wels *Silurus ganis*³³ and shi drum *Umbrina cirrosa*³⁴. The increase in overall erythrocyte size was expected as number of chromosome increases in triploid specimens^{20,35-38}. The change in cell shape and increase in cell size is postulated to be a cytoplasmatic adjustment to the increase in nuclear size-enlargement of the cellular major axis more than the cellular minor axis of the triploid's erythrocytes^{39,40}. This explains the change of erythrocyte shape

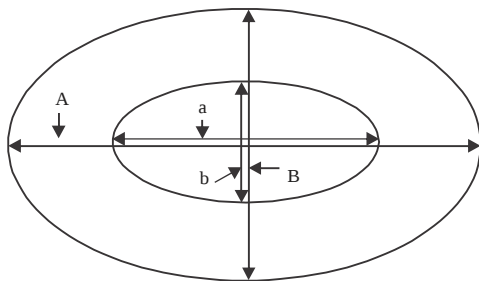


Fig. 1: Sketch of erythrocyte measurement, A: Cell major axis of erythrocyte, B: Cell minor axis of erythrocyte, a: Nucleus major axis of erythrocyte and b: Nucleus minor axis of erythrocyte

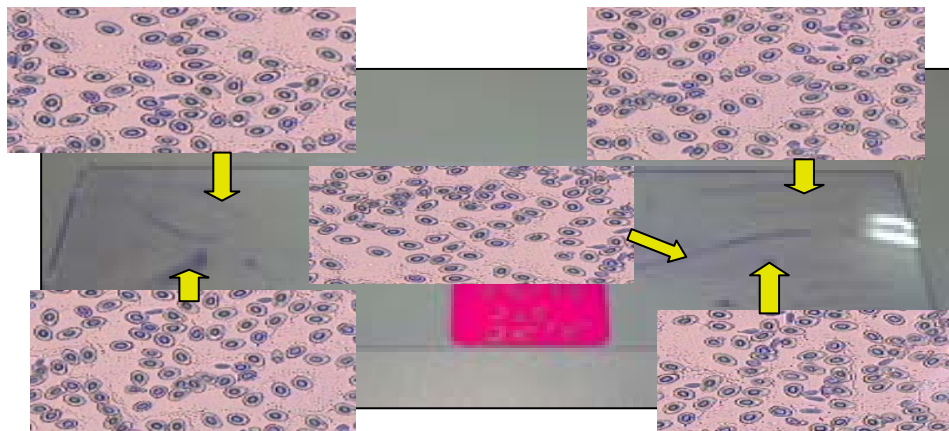


Fig. 2: Randomly sampled block design

from round in diploid *C. gariepinus* to ellipse in triploid specimens as the erythrocyte major axis increased by 25.3% but the erythrocyte minor axis only increased by 7.8% in

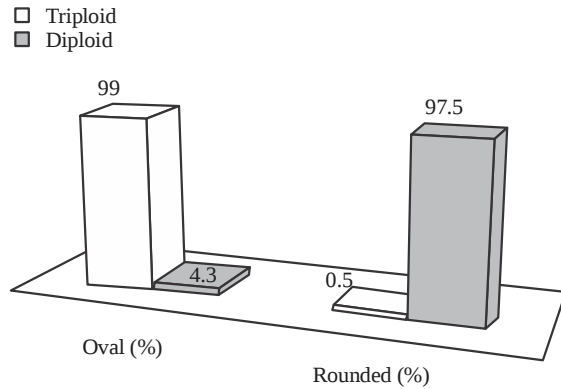


Fig. 3: Percentage of erythrocyte shape (Oval or rounded) between diploid and triploid

triploid individuals. Similar patterns of increment was reported in caspian salmon *Salmo trutta caspius*, where the ellipsoidal shape of triploid's erythrocyte is due to the higher increment of erythrocyte major axis 27% compared to the 22% increment of the erythrocyte minor axis⁴¹.

The results from this study clearly demonstrated that erythrocyte parameters such as the nuclear and cellular area and volume were significantly higher in triploid *C. gariepinus* than its diploid counterparts. Several studies have shown that triploid samples can be distinguished by observing the nuclear volume which is typically 1.5 times larger than normal⁴²⁻⁴⁴. The triploid erythrocyte nuclear volume of *C. gariepinus* in this study was 1.77 times larger than diploid's erythrocyte nuclear volume, similar to that found in red tilapia *O. niloticus* (1.68x larger in triploid) by Jayaprasad *et al.*³¹ and thai silver barb *Puntius gonionotus* (1.63x larger in triploid) by Koedprang and Na-Nakron⁴⁵. These are still usual compared to the erythrocyte nuclear volume of triploid stinging catfish,

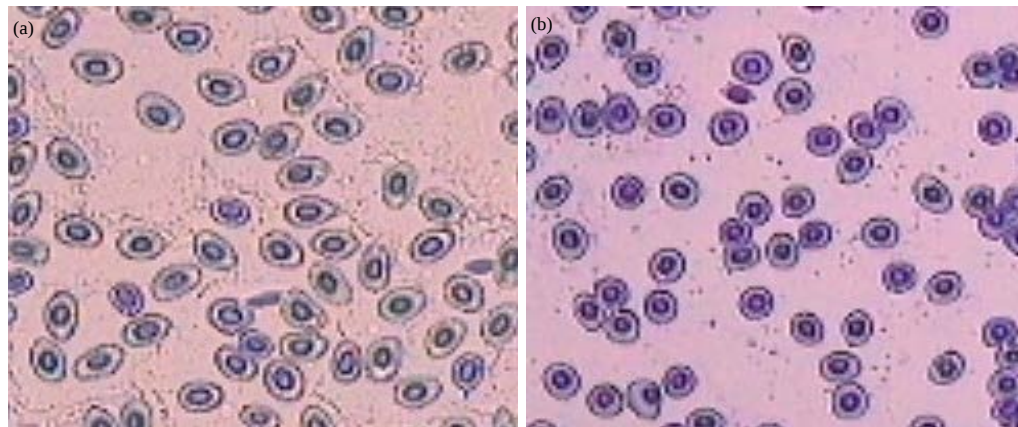


Fig.4(a-b): Erythrocyte of triploid and diploid, (a) Triploid erythrocyte more ellipsoidal and larger than diploid and (b) Diploid erythrocytes more rounded compared to triploid's

Table 1: Comparison of range size, mean and ratio of erythrocyte between triploid and diploid African catfish (*Clarias gariepinus*) sample (n = 100)

	Range (μm)		Mean (μm)		Ratio (Diploid: triploid)	Increment (%)	Exclusive triploid range (μm)	Percentage of triploid erythrocyte in exclusive triploid range	Percentage of triploid erythrocyte in overlapping range
Erythrocyte parameters	Diploid	Triploid	Diploid	Triploid					
Erythrocyte major axis (μm)*	7.8-11.8	10.9-14.9	9.9±0.7	12.4±0.7	1:1.25	25.3	11.9-14.9	76.0	24.0
Erythrocyte minor axis (μm)*	4.8-10.7	6.1-10.6	7.7±0.7	8.3±0.7	1:1.08	7.8	10.3-10.6	0.3	99.7
Erythrocyte cellular area (μm²)*	35.8-92.1	56.2-108.1	59.9±7.5	81.4±8.0	1:1.36	35.9	92.2-108.1	12.0	88.0
Erythrocyte cellular volume (μm³)*	114.7-619.8	237.0-619.3	311.0±65.9	455.2±78.6	1:1.46	46.4	-	0.0	100.0
Nucleus Major Axis (μm)*	3.0-6.2	3.3-6.9	4.4±0.5	6.0 ±0.6	1:1.36	36.4	6.3-6.9	54.0	45.0
Nucleus Minor Axis (μm)*	2.1-4.9	2.1-6.4	3.3±0.4	3.8±0.5	1:1.15	15.2	5.0-6.4	3.0	97.0
Nuclear Area (μm²)	5.5-22.7	7.6-35.2	11.4±2.1	17.7±3.4	1:1.55	55.3	22.8-35.2	21.0	79.0
Nuclear Volume (μm³)*	8.1-74.2	11.6-150.2	25.6±7.4	45.2±14.3	1:1.77	76.6	74.3-150.2	10.0	90.0

*Parameters with significant difference (p<0.05)

Heteropneustes fossilis which was 2.28 times increment in triploids instead from usual 1.5 times for other species²⁴. The diploid:triploid ratio of erythrocyte nuclear in *C. gariepinus* (1:1.55) was consistent with the diploid:triploid ratio found in other fish species such as 1:1.50 in red tilapia *O. niloticus*³¹, 1:1.56 in wels *S. glanis*³³ and 1:1.50 in sea bass *Dicentrarchus labrax*³.

Several studies have stated that the measurement on major axis is the most accurate and variable value for the differentiation of triploid and diploid individuals as triploidy affected the erythrocyte major axis more than the minor axis^{2,46,21,11}. In this study, erythrocyte major axis has the highest percentage of triploid erythrocyte (76%) in the exclusive triploid range. Thus, in order to reduce determination time, solely based on erythrocyte major axis parameter is sufficiently effective to determine ploidy level in African catfish. Similar observations were also reported by Peruzzi *et al.*⁴⁷ and Gheyas *et al.*²⁴.

The overlapping in size of triploid erythrocyte characters could be due to biological factors. In blood, the shape and size of erythrocytes varies as they progress from immature to mature stages. Studies showed that immature erythrocyte is reported to be rounder while the nucleus is more centrally located as compared to mature erythrocyte⁴⁸. Thus, overlaps in erythrocyte size between triploid and diploid fishes were expected, as seen in this study.

Although, previous researches agree that the erythrocyte size of triploid African catfish fish is larger than the diploid²¹⁻²³, no report has yet been published on the percentage of overlapping between diploid and triploid erythrocyte size and percentage of erythrocyte sizes in triploid that is larger than diploid erythrocyte. This is the first report on the characteristics of triploid African catfish erythrocyte and also on triploid fish. It is suggested to observe the differences through the percentage of overlaps and the range of size present only in triploid individuals. For example, the size range between 11.9 μm until 14.9 μm of erythrocyte major axis only exists in triploid individuals (Table 1). These exclusive triploid ranges in erythrocyte parameters serve as a baseline for future ploidy determination in African catfish, *C. gariepinus*.

Therefore, this study demonstrates the potential of using erythrocyte measurement method to accurately distinguish between triploid and diploid African catfish. There might however, exists slight variation in readings of erythrocyte parameters due to factors such as erythrocyte sample size and specimen's life cycle. A study by Karami *et al.*²³ reported the mean of cell major axis for triploid African catfish to be smaller (11.86 ± 0.1) compared to the result in current study (12.40 ± 0.7). This is because, this study was conducted using

50 erythrocytes for each triploid fish compared to only 10 erythrocytes used by Karami *et al.*²³ for analysis. Besides that, the size of erythrocyte changes in different life cycle. The average erythrocyte size in the fingerling of Atlantic salmon, *Salmo salar* was 101.13 μm^2 and for adult fish, the erythrocytes was slightly larger⁴⁹, 103.12 μm^2 .

In fish, red blood cells have shown differences in shape if taken from different parts of the body and at different ages. Nilsson *et al.*⁵⁰ showed that the erythrocyte became more elongated when squeezed between pillar cells of gills. They observed that in rainbow trout *Oncorhynchus mykiss* and roach *Rutilus rutilus* where the erythrocytes of both fish species were ellipse in the arterioles and arteries. During the erythrocyte passage through the gill lamella, the mean erythrocyte length was increased to $18.4 \pm 2.1 \mu\text{m}$ for trout and to $18.1 \pm 2.7 \mu\text{m}$ for roach. Similar observation was also noted in an earlier histological observation of fish secondary lamella⁵¹. Besides that, the change in erythrocyte size can occur in other part of fish body⁵¹. In this study, all fish blood was taken from the caudal fin and at the same age to ensure uniform conditions.

CONCLUSION

This study successfully highlights the differences between triploid and diploid African catfish based on erythrocyte measurement. The erythrocyte major axis is the most suitable parameter to differentiate between triploid and diploid African catfish as approximately 76% of the triploid erythrocytes were in the exclusive triploid range (minimal overlapping with diploid's range) for this parameter. The usage of erythrocyte measurement method in determining triploid African catfish culture will benefits researchers and aqua-culturists as it is rapid, accurate and does not require sacrificing of fish samples.

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