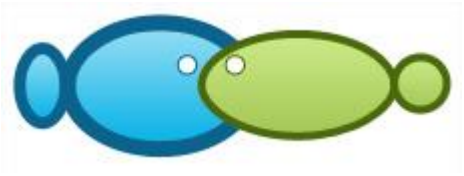

Measurement of zygote DNA content to determine the initial shock time in the striped catfish (*Pangasianodon hypophthalmus*) tetraploid induction

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Abstract. This study aims to determine the influence of initial shock treatment based on measuring zygote DNA content in striped catfish (*Pangasianodon hypophthalmus*) tetraploid induction. The study applied a completely randomized design. The shock was administered at the zygote age of 28, 28.5, 29, 29.5, 30 and 30.5 minutes after the fertilization process, at the temperature of 42°C, for 2.5 minutes. Measurements of the DNA concentrations of each of the above age categories was determined during the incubation period. This is in contrast to earlier tetraploid researches, which determined the age of zygotes by only observing the first mitotic phase, using a microscopic method. The number of samples for each age category was 70 and there was a number of three repetitions. Non-fertilized eggs were used as DNA concentration controls. DNA concentration measurements showed that there was an almost double increase in concentration at the beginning of the zygote stage, in the 27th and 28th minutes compared with the DNA control concentrations. The tetraploid induction treatments resulted in a success rate of 77.66% for zygotes at the age of 29 minutes ($P<0.05$), followed by zygotes with the age of 29.5 minutes with a 72.53% success rate. For 30 minutes, the success rate was 67.58%; for 30.5 minutes, the success of tetraploid induction was 62.64%. For 28.5 minutes, it was 55.13% and the lowest tetraploid induction success rate at the age of 28 minutes, 40.11%. The success of the tetraploid induction treatments was determined by nucleolus analysis (maximum 4 nucleoli) and by the total number of chromosomes (54 pairs).
Key Words: catfish, chromosome, DNA, nucleolus, polyploid.

Introduction. The success of aquaculture activities cannot be separated from the availability of high quality broodstock and population material. One of the efforts to improve broodstock and seed quality for aquaculture is producing tetraploid broodstock (Hershberger & Hostuttler 2005). This is because tetraploid broodstock crossed with diploid broodstocks are able to produce triploid seeds, as it has been reported on rainbow trout (Chourrout et al 1986; Horstgen-Schwark 1993). These triploid descendants will then be distributed in cultivation ponds, presenting faster growth and sterility (Thorgaard 1983; Pandian & Koteeswaran 1998). These have a positive effect on the feed conversion ratio (Wolters et al 1982) and are also able to adapt to various water conditions (Schultz 1980). The most basic determinant of the success of tetraploid induction is knowing exactly when the first mitotic division occurs in the zygote (Dunham 2004). The common method used for observing the first mitosis is the microscopic analysis of the zygote at a given (known) temperature. One of the popular methods today is FCI (First Cleavage Interval) (Hershberger & Hostuttler 2005; Weber et al 2015). However, tetraploid induction in striped catfish based on knowing the time of the first mitotic division by microscopic observations results in a low percentage of tetraploid fish.

Some studies show that tetraploid striped catfish production has a success rate of only 27.67% (Buulolo 2016) and 28.83% (Hartono et al 2016). Both studies used the

first mitotic division for determining the initial time of the shock treatment. To improve the success of tetraploid induction, it is necessary to make some modifications to the treatments, not only in the intensity, but also in the duration and the initial time of induction (Hershberger & Hostuttler 2005). The aim of this study is to determine the best initial time for thermal shock for increasing the success of tetraploid induction in the case of striped catfish (*Pangasianodon hypophthalmus*). The best initial time for shock treatment is determined by measuring changes in the DNA concentration of the zygote. The DNA concentration measurement is performed because the chromosomes in the zygote will duplicate to enter the tetrad phase of meiosis before the first mitosis. To investigate the chromosomal duplication of the zygote reaching the tetrad phase, DNA content analysis is needed.

Material and Method

Experimental design. The tetrad phase determination in the striped catfish zygote is determined by measuring the concentration of DNA. The DNA is modified during the cell cycle, the highest level of DNA packaging being achieved in mitosis (Lebedeva et al 2011). The working principle of tetraploid induction is to shock the zygote when the DNA is finished duplicating to form the tetrad (4n) before the first mitotic division. The reason for measuring the DNA content is to determine the exact age of the zygote that has entered the tetrad phase (4n) after fertilization. The DNA content was measured based on a modified procedure (Teare et al 1997). The measurement of zygote DNA concentration was conducted in two stages. The measurements in the first stage were carried out within 5 minutes for each zygote. The second stage was carried out after the first stage, when the DNA concentrations began to increase, in 1-minute intervals for each zygote.

Phase I of the DNA content measurement. 10 g of eggs were collected at 10, 15, 20, 25, 30 and 35 minutes after fertilization (maf), while non-fertilized eggs (eggs containing 2n or diploid) were used as control. Each sample was placed into a 96% ethanol solution to halt zygote development. Furthermore, each sample was comprised of 70 zygotes (0.045 g) for the process of cell destruction, RNA elimination, protein precipitation, DNA precipitation and measurement of its content using GeneQuant. The modification to the method proposed by Teare et al (1997) consists in increasing the volume of the cell lysis solution, protein precipitation solution and a double centrifugation to eliminate the fat in the sample. DNA concentration measurements were repeated 3 times.

Phase II of the DNA content measurement. Once the first stage was completed, for determining a more precise time when the DNA concentration reaches a higher level, samples were collected in 1-minute intervals for each zygote age, starting at 26, 27, 28, 29, 31, 32, 33, 34 and 36 maf. This can be used as a basis to draw conclusions regarding the time frame in which the tetrad phase occurs after fertilization in the striped catfish zygote. The DNA extraction step for each sample is the same as for phase I.

Application of heat shocks based on DNA content. Based on the results of the DNA content analysis, the zygote age for heat shock treatment for inducing tetraploidy is as follows:

P1: 28 maf at 42°C for 2.5 minutes;
P2: 28.5 maf at 42°C for 2.5 minutes;
P3: 29 maf at 42°C for 2.5 minutes;
P4: 29.5 maf at 42°C for 2.5 minutes;
P5: 30 maf at 42°C for 2.5 minutes;
P6: 30.5 maf at 42°C for 2.5 minutes;
P7: Control (without heat shock).

The shock temperature and the duration of shock time refer to modification of Hartono et al (2016); Buulolo (2016) and Ibrahim et al (2017). Each treatment had 3 replications. After the heat shock was completed, the treated zygotes were dispersed in aquariums

with a normal temperature of 28-30°C, about 6800 zygotes per aquarium. The eggs hatched approximately 20-24 hours after fertilization. The swimming larvae were kept in a 40 L plastic container and fed with *Artemia* spp. nauplii for three days, continuing with tubificidae until the age of 7 days.

Spawning. Three pairs of mature males and females were selected from brood ponds to ensure the quantity and quality of sperm and eggs. The adults were selected based on the health status and the maturity stage. To obtain eggs and sperm, the spawning was induced in females by using a combination of hCG and ovaprim (sGnRH+antidopamine). The females were injected with 500 IU of hCG/kg of body weight and after 24 hours were injected with 0.6 mL of ovaprim/kg of body weight. The males were injected with ovaprim in a dose of 0.3 mL/kg of body weight, at the same time with the second injection for the females. After 12 hours from the treatment, both females and males were stripped to get eggs and sperm.

The eggs were placed in separate dry containers, whereas milt was collected in a diluent vial with a physiologic solution (0.9% NaCl) and stored in ice-filled containers until the fertilization step. Furthermore, sperm and eggs were mixed evenly while stirring, using dry feathers for approximately 1 minute. Fresh water was added to activate the sperm in the fertilization process while stirring evenly for approximately 1 minute. To remove the adhesive force, the eggs were washed with soil suspension for 5 minutes and then rinsed with clean water. The fertilized eggs were dispersed in funnel hatching jars at 28-30°C until ready for the thermal treatment.

Nucleolus and chromosome observations. For nucleolus observations, the tissue samples were collected from caudal fin and then fixed in Carnoy solution. The sample was dried using tissues and 50% acetic acid was added to obtain the cell suspension for dry specimen preparation. The dry prepared specimens were stained using silver nitrate and examined under a microscope with 400x enlargement (Howell & Black 1980; Carman et al 1992).

For the preparation of chromosome observation, samples of live larvae were first soaked in a solution of colchicine with a dose of 70 mg/L for 7 hours. The fish were soaked in KCl 0.075M solution for 60-90 minutes. The next step is similar to the previous procedure of preparing the nucleolus with staining, using Giemsa 60% (Kligerman & Bloom 1977; Carman et al 1992; Bencsik et al 2011). 40 test samples were observed in order to determine the success rate of tetraploidization induction.

Statistical analysis. The data analysis of the research results is done statistically, using F Test (ANOVA). If the results of the analysis showed significantly different results among treatments, it was followed by the Tukey test to determine the treatments with the best response at 95% confidence level. The software used is Minitab 16 and Microsoft Excel.

Results and Discussion

DNA concentration. Based on the DNA concentration, Table 1 shows that the increase in DNA concentration level starts from a zygote aged ≥ 10 minutes. This is caused by the fact that some zygotes has begun to do chromosome doubling. The DNA concentration of the zygote increased compared with the initial concentration of the unfertilized eggs (control) as presented in Table 1. The increase in the DNA level starts when the zygote aged ≥27 minutes. It can be concluded that more than 50% of zygotes reach the tetrad phase. This data can be used as a basis for determining the age of zygotes for the start of the tetraploidization optimization. As for the age of zygote exceeding 31 minutes, the DNA concentration has reached more than twice compared with the controlled DNA, meaning that the phase of tetrad has reached mitosis. Statistical analysis also showed that there was a significant change in DNA concentration among different zygote ages ($P<0.05$) starting from the zygote age of 27 minutes after fertilization.

Table 1

DNA concentration measurements in the zygotes of striped catfish

Time After Fertilization (minute)	*DNA (µg/zygote)*	*Purity (%)*
Control	5.12±0.43	86.33±4.04
10′	6.70±1.05	80.67±13.28
15′	7.08±0.41	97.33±4.62
20′	6.40±0.64	88.67±10.02
25′	6.48±0.33	90.33±3.21
26′	5.97±0.30	88.00±2.65
27′	7.62±0.81	79.00±4.00
28′	7.28±0.35	80.67±1.15
29′	8.77±1.21	93.00±5.00
30′	8.87±1.13	89.00±4.36
31′	10.93±0.53	95.00±4.36
32′	10.13±0.95	90.67±6.03
33′	11.01±0.17	86.33±5.69
34′	11.70±0.52	77.00±6.56
35′	11.00±0.20	83.00±2.65
36′	10.74±1.10	88.00±4.58

Successful formation of tetraploids. Heat shocks of 40°C for 2.5 minutes were applied to each aged zygote. Treatment P3 is the best treatment with a percentage of success of 77.66±6.62%, while treatment P1 and P2 have the lowest percentages of tetraploidy induction success, 40.11±5.41% and 55.13±5.44%, respectively. The statistical results show that the thermal shock induction for a 29-minute-old zygote has a significant effect on the formation of the tetraploid individual ($P<0.05$). The overall treatment results are presented in Table 2.

Table 2

Success of tetraploid induction (%) using 42°C for 2.5 minutes at various zygote ages

Replicate	*Initial time of heat shock (maf)*					
	28′	28.5′	29′	29.5′	30′	30.5′
1	38.46	53.85	76.92	76.92	61.54	57.14
2	46.15	61.54	84.62	69.23	76.92	69.23
3	35.71	50.00	71.43	71.43	64.29	61.54
Average	40.11±5.41^{c}	55.13±5.44^{bc}	77.66±6.62^{a}	72.53±3.96^{ab}	67.58±8.2^{ab}	62.64±6.12^{ab}

Note: values given under different superscript letters mark a significant difference ($P<0.05$ level).

The basis for obtaining the successful tetraploid induction is presented in Table 3. The age of the embryos was recorded and the maximum number of nucleoli was observed after 7 days since hatching.

Tabel 3

Nucleolus observation from the caudal fins of tested fish for each treatment

Sample	*Initial time of heatshock (maf)*					
	28´	28.5´	29´	29.5´	30´	30.5´
1	×	×	√	×	√	×
2	×	×	×	√	√	√
3	√	√	×	√	×	√
4	×	√	√	×	√	√
5	×	×	√	√	√	×
6	√	√	√	√	√	×
7	√	√	√	√	×	√
8	×	×	√	√	×	√
9	×	√	√	×	×	√
10	×	√	√	√	√	√
11	×	×	×	√	√	√
12	√	×	√	√	×	×
13	√	√	√	√	√	×
14	×	√	√	√	√	√
15	√	√	√	√	√	×
16	×	√	×	×	√	√
17	×	√	√	√	√	√
18	√	√	√	√	√	√
19	√	×	√	√	√	√
20	√	×	√	√	√	√
21	√	×	√	×	×	√
22	×	√	√	×	×	×
23	×	√	√	×	×	×
24	√	×	×	√	√	√
25	×	√	√	√	√	√
26	×	×	√	√	√	√
27	√	√	√	×	√	√
28	×	√	√	×	√	√
29	×	×	×	√	√	×
30	√	×	×	√	√	×
31	√	×	×	√	√	√
32	×	√	√	√	√	√
33	×	×	√	√	×	×
34	√	√	×	√	√	√
35	√	×	√	√	√	√
36	×	√	√	×	×	×
37	×	×	√	×	×	×
38	×	×	√	√	×	×
39	×	√	√	√	×	×
40	×	√	√	√	×	√
Percentage	40.11	55.13	77.66	72.53	67.58	62.64

Note: (√) tetraploid; (×) diploid.

Figure 1A shows that the nucleolus of the diploid striped catfish has a maximum number of 2 nucleoli, while in Figure 1B there is a maximum number of 4 nucleoli. Nevertheless, both diploid and tetraploid individuals presented 1 or 2 nucleoli, while tetraploid individuals also showed 3 nucleoli.

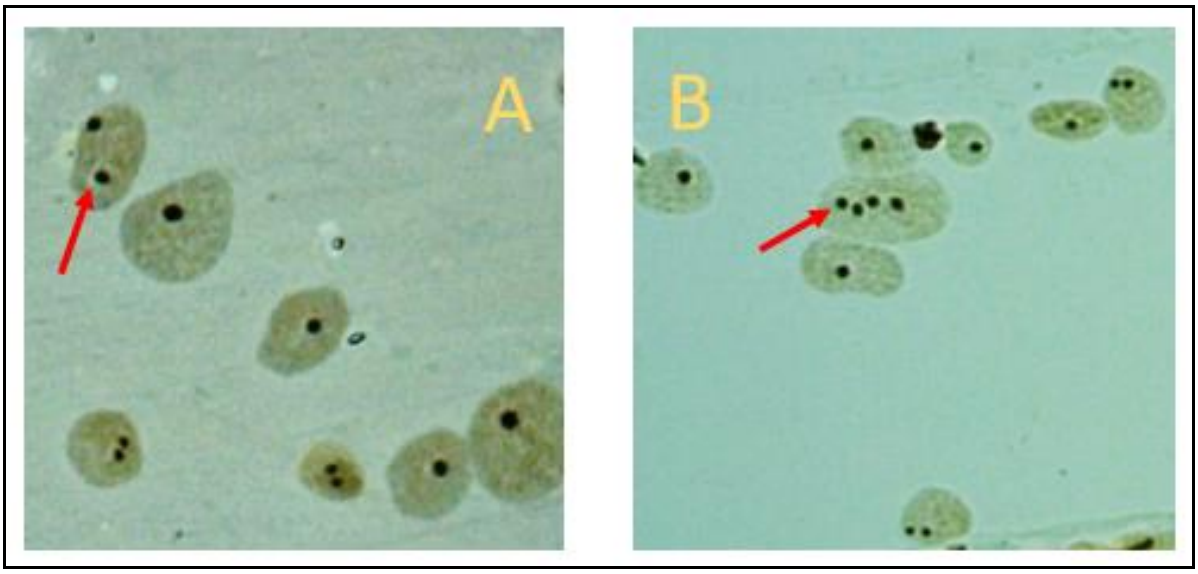

Figure 1. A - Diploid nucleoli; B - tetraploid nucleoli (400x magnification).

Furthermore, observations made for the number of chromosomes revealed that striped catfish diploid individuals had 27 pairs of chromosomes (Figure 2A) and tetraploid individuals had 54 pairs of chromosomes (Figure 2B).

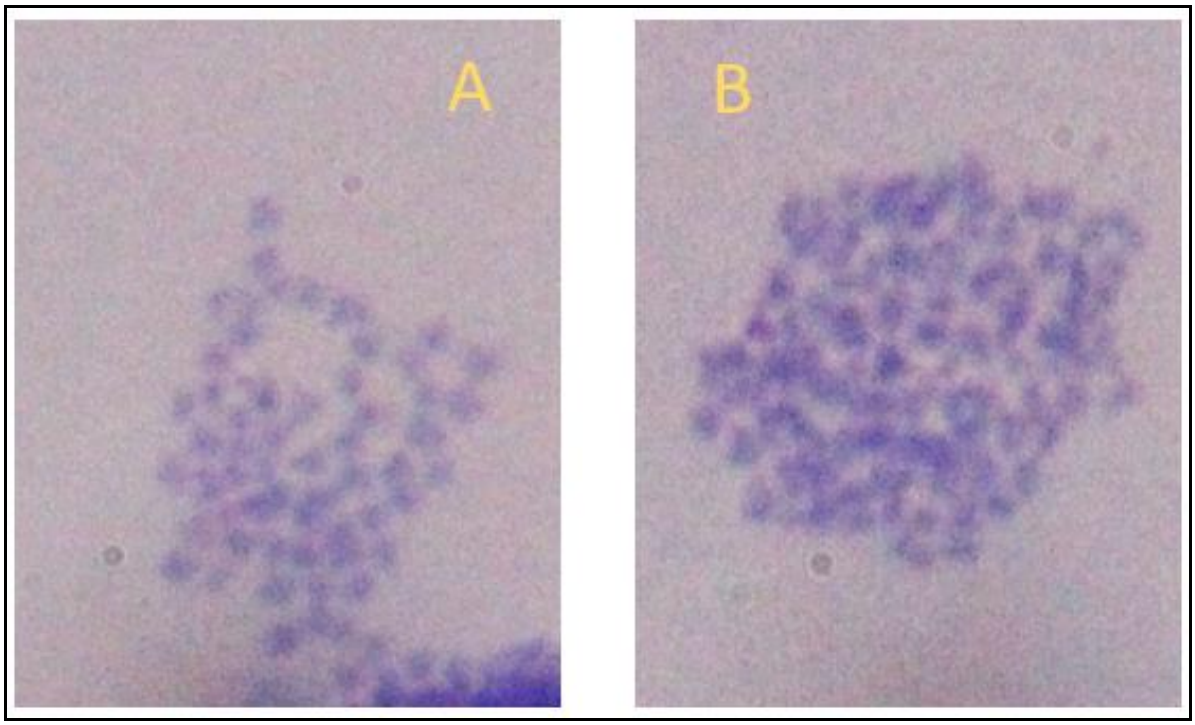

Figure 2. A - Diploid chromosomes; B - Tetraploid chromosomes (1000x magnification).

According to Palomino et al (1995), the results of DNA extraction show an increase in the amount of DNA content of the tetraploid *Leucaena conferiflora* compared to the diploid individuals. Buulolo (2016) mentions that microscopic observations indicate that the first

division of the striped catfish zygote occurs at 32 minutes. This is also in line with the statement of Lebedeva et al (2011) which states that DNA is modified during the cell cycle, the highest level of DNA packaging being achieved in mitosis. Inducing thermal shock for striped catfish at various zygote ages determined by DNA content is a method that has comparable and even better results than other studies in which 26 to 28% induction rates were obtained (Buulolo 2016; Hartono et al 2016). The results from this study are much higher than what Bidwell et al (1985) obtained for channel catfish (*Ictalurus punctatus*), 62%, and what Haniffa et al (2004) obtained for stinging catfish (*Heteropneustes fossilis*), 48%. This result is equivalent to tetraploid induction success rate of 80% for Nile tilapia (*Oreochromis niloticus*) (Gamal et al 1999) and close to tetraploid induction results of 100% for rainbow trout (*Oncorhynchus mykiss*) (Hersberger & Hostuttler 2005).

Based on the results obtained, it can be said that heat shock is very effective to induce tetraploidy in striped catfish. This is in accordance with the discoveries of Refstie et al (1977), Thorgaard et al (1992), Yang & Guo (2006) and Hartono et al (2016), which state that artificially polyploidy can be obtained by applying heat shock in eggs that have been normally fertilized. The temperature shock is suitable for manipulating the chromosome set of species with relatively small eggs (Piferrer et al 2009). In addition, according to Dunham (2004), the success of the treatments depends on the time of shock initiation, shock rate and duration. The best time to start the shock treatment varies among species, but especially for inducing tetraploidy, it is related to the time of the first mitotic division during the embryo development. Related to embryonic age, the measurement of DNA concentration proved to be a method to determine the initial time of the shock.

According to Bencsik et al (2011), nuclear measurements are always consistent. If the volume of the nucleus increases, then the cytoplasmic volume will increase proportionally, so that the cell size will be larger. In theory, the triploid individual will have a larger cell than diploid one and tetraploid cells will be larger than triploid and diploid cells. The increase in dimension may vary across cells and tissues. Nucleolus analysis is also called the NORs method, NORs analysis being one of the easiest and cost effective methods of ploidy measurement. This method can be applied to the larval fish without concerns for the well-being of the fish. Previous studies stated that the maximum number of nucleoli in diploid individuals was 2 nucleoli (Ibrahim et al 2017; Buulolo 2016; Hartono et al 2016) and the number of chromosomes range from 54 to 56 pieces, 27-28 pairs of chromosomes (Ibrahim et al 2017). The observation and counting of the chromosome number of the tetraploid fish is one method to ensure the success rate of polyploidization induction activity (Pradeep et al 2011). To see the apparent differences of two populations (diploid and polyploid), chromosome analysis is the correct method of use (Nishiyama et al 2016).

Conclusions. The conclusion of this study is that the zygote DNA concentration can be used as a method to determine the initial time or age of zygotes for inducing tetraploidy in striped catfish with heat shock treatments. It can also be applied to other fish species of economic interest.

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