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Effectiveness of Heat Shock (40°C) With Different Duration for Tetraploid Formed in Mutiara Catfish (*Clarias* sp.) Juvenile

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Abstract. This study seeks to determine the best method for preventing separating one cell into two cells in early embryogenesis as a means to induce tetraploid in the Mutiara catfish strain (*Clarias* sp.). Heat shocks were applied to newly fertilized eggs. Heat shock that was administered at 40 °C, for 4 min at 30 min post-spawning, was the best method for tetraploid induction, as evidenced by the highest percentage of forming four pronuclei in syngamy (84.12%). Therefore, heat shock induction was employed to further explore the advantage of tetraploid over diploid catfish. After hatching of the heat-shocked eggs, the larvae were allowed to reach juvenile levels of development. At day 30 in culture, the number and amount of chromosomes were determined in juvenile stages, using Silver Nitrate Staining methods, by which the catfish were divided into diploid and tetraploid groups. The average specific growth rate of the tetraploid (22.55%/gr/day) was significantly higher (Pb 0.05) than that of their diploid counterparts (15.42%/gr/day). These results reveal the advantages of growing tetraploid over diploid Mutiara catfish strain and its feasibility for future commercial production.

1. Introduction
Sustainable aquaculture has made a significant contribution to world food security [1,2,3,4,5]. Since the 1980s, there has been an increase in aquaculture production from aquaculture [1]. In theory, polyplody is expected to grow fast and reach a size larger than normal diploid descent due to the enlarged cell size [6] indicating that artificial polyplody can have a real effect on aquaculture.

The results of manipulating chromosome sets in rainbow trout *Oncorhynchus mykiss* by interfering with the first mitotic division in embryonic development to produce individual tetraploids, gynogenesis, and androgenesis are very diverse [7]. Some research has been conducted on tetraploid production as part of a program to develop better broodstock for the aquaculture industry. Tetraploid development is recognized as a potential approach that allows for the direct production of triploid by diploid mounting [8,9].

One assumption is that the induction of tetraploids (equally good in gynogenesis and androgenesis) is based on the time consistency of the first mitotic division. Heat stress or shock with sufficient intensity is applied at the time after fertilization and the duration which effectively gives a disruption in the first division in embryonic development [10]. Polyplody induction, especially the commercial production of triploids and the creation of tetraploid broodstock populations to support this, is an important and successful technique in the field of aquaculture of Oyster *Crassostrea virginica*. In Virginia, about 90% of commercial oysters are triploids [11].
With a few exceptions, a number of commercial triploid oysters are currently produced by crossing diploid females with tetraploid males, becoming possible after a method for producing tetraploid oysters was developed [12]. Compared with polar body II inhibition, the diploid and tetraploid crossing is more appropriate for aquaculture because it cuts the need to make artificial treatments and produce the highest efficiency triploids [13]. The main obstacles to the formation of tetraploid populations are low life pass in a tetraploid generation, decreased the fertility of tetraploids, and undesirable levels of ploidy in offspring in tetraploid spawning [14].

2. Materials and methods
2.1. Broodstock collection, maintenance, and spawning
Sexually mature brood fish of clarias were obtained from ponds maintained by fish breeding research center at the Sukamandi, Subang Regency, Indonesia. They were acclimatized for one month in 6,000 L (4 × 2 × 0.75m) concrete tanks located at the Faculty of Fisheries and Marine Science of Brawijaya University, in Indonesia. During this period, the water quality was maintained at optimum (Temperature = 28.0 ± 2 °C; pH = 7.00 ± 0.5) while the fish were fed commercial diet ad libitum (35% CP) twice daily.

Hormonal dosage and administration, as well as spawning methods used in the study, were as dictated [15]. In brief, both the male and females were injected below the dorsal fin with HCG at 1 ml/kg body weight. Each mating pairs were separated in a different aquarium and allowed to swim for a latency period range between four and eight hours. Three mating pairs were used for each of the three trials reported in this study. The fish behavior during the latency period was closely monitored for pre-spawning signs. As soon as this is observed, the females are removed and stripped into a clean dry petri dish. Simultaneously, the male counterpart is also dissected to take the gonad and squashed to get sperm. Fertilization was then done by mixing the eggs and sperm as well as activation with water. Sperm and eggs are mixed in one container and flattened using chicken feathers. Eggs are spread on the tea strainer as a treatment container. Each treatment container was filled with 200 eggs/tea strainer.

2.2. Experiment design for heat-shock treatments
Six treatment affecting production of tetraploid larvae heat-shock duration were studied: 0 minute (as Control); 1 minute; 2 minute; 3 minute; 4 minute; 5 minute. Optimal heat-shock temperature and duration with respect to production efficiency (%) of tetraploid larvae (hatching rates × tetraploidization rates) were determined. Heat-shocks commenced at 30 min post-fertilization (mpf). Each treatment group consisted of approximately 200 fertilized eggs. Non-treated control groups consisted of gamete samples obtained from the same catfish parental specimens. Each heat-shock treatment was repeated for three times using different batches of fertilized eggs. Eggs that have been treated are kept in aquarium maintenance.

2.3. Rearing of heat-shock treated eggs
Approximately 3,600 heat-shock treated fertilized eggs were transferred to a 100-l incubate tank with gentle aeration and incubated at 27 ± 1 °C. Larvae at early stages after egg yolk absorption (5 DPH) were fed with tubifex sp. and the artificial diets were started feeding on 15 DPH. Total water volume changed daily over the course of larvae rearing, in close circulation and filtered.

Water quality was monitored throughout the rearing period in order to ensure the healthiest environment for the growth of the larvae (pH 7.7–8.2; dissolved oxygen >4 ppm). At days 10, 20, 30 in culture, 30–35 larvae were randomly sampled by net casting, and body weights (BW) individually determined. No attempt was made to separate the sex at these stages. The chromosome number of each animal in the sample was determined as described below. After obtaining the ploidy level results, the BW of the tetraploid and diploid (heat-shocked diploid + no-treatment diploid) were averaged and statistically compared.
2.4. Tetraploid induction and ploidy evaluation

After in vitro fertilization, eggs were collected and randomly distributed in five groups of approximately the same number of individuals. Twelve egg fertilized groups were used to produce tetraploid fish by the immersion of eggs in the water at 40°C for the period of 1 minute; 2 minutes; 3 minutes; 4 minutes; 5 minutes following 30 min post fertilization [16,17]. They constituted the groups of tetraploids obtained by Heat (HT) shocks. The least group was constituted by diploid fish (untreated) and thus considered the control group (CG). Eggs from each group were placed in separate incubate tanks of 75 L of capacity and incubated at 28 ± 2 °C for approximately 1 month until tetraploid test was held. The efficiency of tetraploid induction methods was assessed by means of AgNOR's technique in ten randomly selected fish from each group [18,19].

2.5. Silver nitrate staining methods

The samples were treated by adding a 0.0075 M KCL solution for 30 min at 30°C and were fixed by repeating the process 3 times for 30 min in a 40°C acetic alcohol (methanol: acetic acid = 3:1) solution. Each fixed sample was moved to a petri dish, after which the yolk sac of the individual larvae was eliminated. After chopping each piece into individual samples, removed the extraction buffer (20 µL of cysteine) using the 2-Step Kit and eliminated the nuclear membrane in the cell by pipetting for 30 min. A total of 1 mL of staining buffer was added to the cell that had its nuclear membrane removed. Next, the nucleolus organizer region (NOR) staining was added for 60 min with pipetting. Then added 1 drop of 50% acetic acid to the smear, chopped using a pincette [20]. Staining of nuclear preparation is carried out by silver nitrate (AgNO3) in cell preparations, with 2 drops of A solution (10 gr AGNO3 + 20 ml Aquadest) and 1 drop B solution (2 gr of gelatin + 50 ml of warm equates + 50 ml of glycerin). The two solutions are mixed and flattened. Then put into the staining box with a temperature 45-50°C for 20-25 min [21]. The chromosome metaphase and cells were observed using an optical microscope. The metaphase spread and the round shape of the undamaged cells were examined using a located of the NOR, whereas the nucleolus number was calculated based on the nuclear organization region.

2.6. Statistical analysis

All data were presented as means ± SEM. Significant differences between three or more group means were determined using a one-way ANOVA followed by Tukey's multiple comparisons test (p < 0.05), while significant differences between control and treatment groups were determined using a one-way ANOVA followed by Dunnett's multiple comparisons test (p < 0.05).

3. Result and discussion

3.1 Percentage of tetraploid mutiara catfish

Nucleoli can be calculated by counting on each cell by the silver nitrate staining method. The nucleoli will appear black, while all cells will appear yellow [22]. Based on the observation of nucleoli conducted according to this experiment, it is found that the percentage of positive Mutiara catfish has a diploid nucleus of 3 nucleoli and a maximum of 6 nucleoli. The documentation of nucleoli in Mutiara catfish is presented in Figure 1.
Figure 1. Number of nucleoli Tetraploid Mutiara catfish (a) 4 Nu, (b) 5 Nu, (c) 6 Nu, Nu: Nucleoli.

Catfish are known to have a maximum of 3 nucleoli per cell found in the count of 20-50 cells in each individual diploid control. Fish with heat shock has a maximum number of 5 nucleoli per cell and tetraploid individuals have 3-5 nucleoli per cell [23]. In this study, it is known that the number of nucleoli in fish with a shock treatment temperature of 40°C that appears is 1, 2, 3, 4, 5, and a maximum of 6 nucleoli. The variation in the number of nucleoli is caused by NOR (nucleolar organizer regions) which does not form nucleus when cells are not actively synthesizing so that AgNO₃ coloring does not color all nucleoli. NOR is a collection of genes in charge of translating genes into rRNA, then translating into ribosomal proteins that play a role in protein synthesis [24]. Percentage data of tetraploid Mutiara catfish are presented in Table 1.

Table 1. Percentage data of tetraploid Mutiara catfish

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (without heat-shock)</td>
<td>0±0</td>
</tr>
<tr>
<td>A (heat-shock 1 minute)</td>
<td>18.89±1.92</td>
</tr>
<tr>
<td>B (heat-shock 2 minute)</td>
<td>62.22±3.85</td>
</tr>
<tr>
<td>C (heat-shock 3 minute)</td>
<td>64.45±3.85</td>
</tr>
<tr>
<td>D (heat-shock 4 minute)</td>
<td>84.12±1.37</td>
</tr>
<tr>
<td>E (heat-shock 5 minute)</td>
<td>76.67±2.89</td>
</tr>
</tbody>
</table>

Based on the above calculation results, it can be seen that the highest average value is in the treatments D, E, C, B, and A. Then to find out the form of the treatment relationship with the parameters tested then proceed to orthogonal polynomial calculations. Chart of the regression results for the percentage tetraploid of Mutiara catfish is presented in Figure 2.
Figure 2. Chart regression results of tetraploid Mutiara catfish

Based on Figure 2 it can be seen that the administration of temperature shock of 40°C with different shock duration has an influence on the success of tetraploidization of Mutiara catfish, generated and shows a linear pattern with the equation \( y = 20.034 + 13.745x \) with a coefficient of \( R^2 \) is 0.98. The treatment of 1 minute, 2 minutes, 3 minutes, 4 minutes, and 5 minutes has increased value and the highest value at 4 minutes treatment. Heat-shock with duration 4 min gets the average result of tetraploid Mutiara catfish is 84.12%. Tetraploidy can be induced by suppression of early cell division in the zygote. Tetraploid induction has been accomplished by pressure, temperature, or chemical shocking of zygotes in many species including Clarias. The highest percent of tetraploid induction was observed at 72.5% induced by hydrostatic pressure [25].

3.2. Hatching rate of Mutiara catfish eggs

Based on the study the effect of giving a temperature shock of 40°C with different temperature shocks obtained the average results of the percentage hatching rate of Mutiara catfish (Clarias sp.) which can be seen in Table 2.

Table 2. Percentage hatching rate of Mutiara catfish

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average (%) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (without heat-shock)</td>
<td>67.33±1.15</td>
</tr>
<tr>
<td>A (heat-shock 1 minute)</td>
<td>45.17±0.58</td>
</tr>
<tr>
<td>B (heat-shock 2 minute)</td>
<td>29.17±1.04</td>
</tr>
<tr>
<td>C (heat-shock 3 minute)</td>
<td>17.83±1.26</td>
</tr>
<tr>
<td>D (heat-shock 4 minute)</td>
<td>22.00±1.32</td>
</tr>
<tr>
<td>E (heat-shock 5 minute)</td>
<td>7.00±0.87</td>
</tr>
</tbody>
</table>

Based on Table 2, the results of the treatment by giving a temperature shock of 40°C with different temperature shocks have the highest average hatching value in the treatment A than B, D, C, and E. Then to find out the form of the treatment relationship with the parameters tested then proceed to the orthogonal polynomial calculation. Chart of the regression hatching rate results for the percentage of tetraploid Mutiara catfish is presented in Figure 3.

Figure 3. Chart regression result of hatching rate Mutiara catfish
In Figure 3, it can be seen that the hatchability of Mutiara catfish in the treatment of 1 minute, 2 minutes, 3 minutes, 4 minutes, and 5 minutes has a decreased value and the lowest value in the treatment of 5 minutes and the highest value in the treatment of 1 minute. The linear regression equation obtained is $y = 50.683 - 9.05x$ with a coefficient of determination (R2) of 0.99 which can be interpreted that giving a temperature of 40°C at each treatment gives 99% effect on the hatchability of Mutiara catfish (Clarias sp.). The highest average hatching rate after being given a hot temperature shock of 40°C is 45.17% at 1-minute treatment. Hatching rate of individual tetraploid ranged from an average of 10% to 30%. Differences may be inherent in the approaches or may be due to differences in size or shape of the micropyle canal or sperm size [26].

3.3. The survival rate of Mutiara catfish larvae
The survival rate of Mutiara catfish is obtained from the calculation of the comparison between fish that live on the 30th day with larvae that hatch at the beginning of the study presented in the form of a percentage. Based on the research the effect of giving a temperature shock of 40°C with different temperature shocks obtained the average percentage of survival of Mutiara catfish (Clarias sp.) which can be seen in Table 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average (%) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>K ( without heat-shock )</td>
<td>7.92±0.30</td>
</tr>
<tr>
<td>A ( heat-shock 1 minute )</td>
<td>5.90±0.60</td>
</tr>
<tr>
<td>B ( heat-shock 2 minute )</td>
<td>8.13±0.91</td>
</tr>
<tr>
<td>C ( heat-shock 3 minute )</td>
<td>15.45±1.42</td>
</tr>
<tr>
<td>D ( heat-shock 4 minute )</td>
<td>14.37±0.48</td>
</tr>
<tr>
<td>E ( heat-shock 5 minute )</td>
<td>31.11±3.85</td>
</tr>
</tbody>
</table>

Table 3. The percentage survival rate of Mutiara catfish

Based on the above calculation results it is known that the highest treatment is in treatment E then followed by treatments C, D, B, and A. Then to find out the form of the treatment relationship with the parameters tested then proceed to orthogonal polynomial calculations. The graph of regression results for the survival rate of Mutiara catfish is presented in Figure 4.
Figure 4. Chart regression result of survival rate Mutiara catfish

In Figure 4 it can be seen that the value of the linear regression equation obtained is $y = -6.9637 + 7.961x$ with a coefficient of determination ($R^2$) of 0.96 which can be interpreted that giving 40°C temperature in each treatment gives 96% influence on the survival of Mutiara catfish ($Clarias$ sp.). The survival rate of Mutiara catfish in 1 minute, 2 minutes, 3 minutes, 4 minutes, and 5 minutes treatment has an upward graph and the lowest value in 1-minute treatment and the highest value in 5 minutes treatment is 45.17%. Owing to their easier mass production from natural polyploid species than from common diploid species, and due to their better growth, survival and flesh quality than their original counterparts [27].

3.4. Specific growth rate Mutiara catfish larvae

Based on research the effect of giving a temperature shock of 40°C with different temperature shocks obtained the average results of the percentage specific growth rate in Mutiara catfish ($Clarias$ sp.) which can be seen in Table 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average (% gr/day) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (without heat-shock)</td>
<td>15.42±0.02</td>
</tr>
<tr>
<td>A (heat-shock 1 minute)</td>
<td>17.08±0.02</td>
</tr>
<tr>
<td>B (heat-shock 2 minute)</td>
<td>18.50±0.09</td>
</tr>
<tr>
<td>C (heat-shock 3 minute)</td>
<td>18.01±0.07</td>
</tr>
<tr>
<td>D (heat-shock 4 minute)</td>
<td>22.55±0.04</td>
</tr>
<tr>
<td>E (heat-shock 5 minute)</td>
<td>20.49±0.03</td>
</tr>
</tbody>
</table>

Based on the above calculation results it is known that the highest treatment is in treatment D followed by treatments E, B, C, and A. Then to find out the form of the treatment relationship with the parameters tested then proceed to orthogonal polynomial calculations. The graph of specific growth rate regression results of Mutiara catfish is presented in Figure 5.
Figure 5. Chart of regression results for the specific growth rate of Mutiara catfish

In Figure 5 it can be seen that the value of the linear regression equation obtained is $y = 16,065 + 1.0867x$ with a coefficient of determination ($R^2$) of 0.999 which can be interpreted that giving 40°C temperature in each treatment gives an effect of 99.9% on the specific growth rate of catfish Mutiara (*Clarias* sp.). The graph of the rate of growth of Mutiara catfish in the treatment of 1 minute, 2 minutes, 3 minutes, 4 minutes, and 5 minutes has an upward graph. In the study conducted by the author, it was found that SGR values given shock treatment of 40°C with the highest average in 4 minutes treatment were 22.55%. The long-term viability and growth performance of induced individual tetraploid. Erythrocyte cellular and nuclear dimensions of individual tetraploid were significantly higher than those of diploids, as expected. Chromosomal and flow cytometric analyses revealed the genomic structure of tetraploids as originating from the doubling of 2n chromosomes, as evidenced by their modal chromosome number and average cellular DNA content, which were double the diploid values [20].

4. Conclusion
Based on the research that has been done, the conclusion is that the treatment of a temperature shock of 40°C with a different temperature shock has a significant effect on the success of tetraploidization in Mutiara catfish (*Clarias* sp.). The temperature shock treatment of 40°C with a temperature shock of 4 minutes is an effective treatment with the highest average value with a success value of tetraploidization of 84.12%.

5. References
[27] Li Z, and Jianfang G 2017 *Aquaculture and Fisheries* 2(3):103-111