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The Effect of Heat Shock on the Embryonic Development of Tetraploid *Clarias gariepinus*

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Abstract

African catfish *Clarias gariepinus* species have a lot of muscle includes a high concentration of mono- and polyunsaturated fatty acids, as well as a high concentration of linoleic acid, an important fatty acid. This research aims to evaluate the effect of heat shock on the formation of tetraploid African Catfish, *Clarias gariepinus* during embryonic development. Tetraploidization was induced, by a 2 minutes heat shock at 39°C, 40°C, and 41°C approximately 40 minutes after fertilization, each treatment was replicated three times and compared to the control (diploid eggs). The result indicated that the percentage of fertilization and embryonic development during the cleavage, blastula, and gastrula stages was similar in the 39°C, 40°C, and 41°C treatments and control. However, embryonic segmentation was observed earlier during heat shock treatment (39°C, 40°C, and 41°C) compared to the control treatment at 8:30 hours. Furthermore, during the segmentation stages, the 40°C treatment shows more advanced development of the first somite, tail appearance, and early movement embryo compared to other treatments (39°C and 41°C). At 22:30 hours, the 40°C treatment was recorded on an early hatched *C. gariepinus* larva with a fully developed heart, heartbeat, and blood circulation. The findings suggest that heat shock treatment can be used to produce tetraploid food fish with fast growth characteristics that can be applied in mass culture production.

Keyword: *Clarias gariepinus*; embryonic development; heat shock; tetraploid

INTRODUCTION

Certain reasons contribute to the popularity of African catfish *Clarias gariepinus* as a valuable animal protein and widely farmed aquaculture alternative. Excellent nutritional value, high fecundity, early maturity, toughness, resilience to poor water quality, great growth performance, and ease of reproduction in captivity are all characteristics. However, the performance of this fish, like that of others, must be improved in order to meet the ever-increasing need for cheap animal protein induced by the exponential rise in the human population. The production of farmed fish can be increased by producing sterile or triploid seedlings, which increase the growth rate of juveniles, maintain the survival rate, and increase the growth rate of adult fish. The formation of triploid individuals by maintaining polar body II is not always 100% effective and can result in negative side effects and decreased viability; an alternative method of triploid production is by crossing tetraploid (4n) with diploid (2n) (Hartono et al., 2016). Therefore, tetraploid broodstocks are used to produce triploid fry as an interim process for large-scale triploid production (Wu et al., 2019).

Polyploid induction, as a chromosomal alteration strategy, entails the addition of extra chromosome sets to the standard two sets carried by each organism. Many fish species have profited from triploidy induction because of the advantages of decreased reproductive functionality, which improves development and other areas of performance. This is also good for the environment because the fugitives from the aquaculture operation are thought to be unable to mate with wild fish (Zhou & Gui, 2017).

Heat shock is a frequent induction for producing triploidy in fish, however, it is not always effective and can have unfavorable side effects and lower survivability. Several kinds of ploidy, including tetraploidy, have been employed to enhance triploid synthesis. Individuals with triploid features can be created by preserving the polar body II or by mating a tetraploid (4N) with the diploid (2N), which was initially developed for fish tetraploid individuals are created as an intermediate step in the production of triploid fish fingerlings. Hence, a more practical approach for industry is to use tetraploid broodstock for triploid bulk production rather than the more controversial method of transgenic fish production (Rasmussen & Morrissey, 2006).

The majority of triploid fish are sterile, cannot be used as broodstocks, and have a variety of problems, including lower hatching rates and negative side effects when heat shock is applied. Tetraploid induction with temperature shock is one method for mass producing triploid fish. Furthermore, seeds produced by tetraploid matings had higher levels of heterozygozity than seeds produced by shock treatment of diploid parents (Christopher et al., 2019). Therefore, the aim of this study is to assess the efficacy of heat shock treatment in producing tetraploid *C. gariepinus* in order to standardise the method.

METHODOLOGY

Broodstock preparation

Adult male and female *C. gariepinus* were collected from Taman Agroteknologi Pertanian Bestari Jaya in Selangor, Malaysia, with body weights ranging from 1.1 to 2.2 kg. The fish were delivered to the Aquaculture Laboratory, Faculty of Engineering and Life Sciences, Universiti Selangor, Malaysia. When the fish arrived at the laboratory, they were disinfected with a potassium permanganate solution. For one week, fish were kept in two tonnes of fibreglass tanks

with a natural photoperiod for acclimatization. Feeding with commercial pelleted feed (Cargill) was done twice a day, once in the morning (10:00am) and once in the afternoon (5:00pm). In this study, three males and three females were used to perform an induced spawning experiment and to determine the effect of heat shock on early developmental fish.

Artificial fertilization

Both male and female fish (3 males and 3 females) were given ovaprim injections at the same dose (0.5 mL/kg body weight of fish) to induce spermiation and ovulation for induced breading. The treated male and female fish were kept in separate fibreglass tanks. After the fish had been injected for 18-24 hours, ovulation was detected using hand stripping. The abdomen of the vent fish was gently pressed to release the eggs on a bowl, and the fish was left alive. The ovulated eggs were transparent green-brown in color. However, treated males were sacrificed by dissecting and cutting their testes into small pieces in order to provide sperm suspension in clean water and activate the sperm. The milt and eggs were then thoroughly mixed with a bird feather. The experimental design followed to the guidelines established by the Universiti Selangor Research Ethics Evaluation Committee.

Tetraploid induction

Eggs and sperm are mixed for 4-5 minutes by moving the bowl slowly and using bird feathers. The fertilized eggs were thoroughly washed with 0.9% NaCl solution (saline solution) and transferred to a 5 litre container. For heat shock treatment, waterbaths were set up at three different temperatures: 39°C, 40°C, and 40°C. After 40 minutes of fertilization, the eggs were divided into four groups. The three groups were subjected to a two-minutes heat shock at three different temperatures (39°C, 40°C, and 40°C). For other group, fertilized eggs were not subjected to heat shock and were left at room temperature (27°C) naturally as a control. Unfertilized or dead eggs were immediately removed to avoid fungal infection. The 5 litre aquarium tanks were set up for four treatments to study the effect of heat shock on fish embryonic development.

Embryo development observation

For observing early development embryo to larval stage, eggs were sampled every 10 to 30 minutes until hatching in each aquarium tank (39°C, 40°C, 41°C, and control). Ten live eggs were collected from each aquarium tank and transferred to a petri dish for examination under a microscope. The samples were taken in triplicate. The embryonic developmental stages and larval hatching were observed by capturing images under microscopic views of fertilized eggs at various stages of embryonic development for each heat shock treatment and control. Live samples of eggs and hatching larvae were observed, and images were captured with smartphone cameras for use in microscopic imaging. Each developmental stage's appearance time was recorded and analyzed for each treatment.

Data analysis

The mean and standard error of the mean were used to express the data. For mean variation, one-way analysis of variance (ANOVA) was used, followed by Duncan's New Multiple Range Test (DMRT). For the induced spawning experiment, one-way analysis was used to analyse the variation among heat shock treatments, followed by Duncan's New Multiple Range Test to analyse the mean variation. Fertilization rate was calculated as a percentage of the number of fertilized eggs divided by the number of eggs incubated.

RESULTS AND DISCUSSION

Table 1 shows the effects of heat shock on the percentage of live fertilized eggs and egg size

after fertilization in *C. gariepinus*. This indicated that ovaprim and heat shock treatment after fertilization were necessary for successful induce spawning for polyploidy purposes. At temperature of 39°C, 40°C, and 41°C, and two minutes duration, had no significant effect on the fertilized eggs, which ranged from 91 to 95%. When compared to the heat shock treatment of tetraploid catfish *Pangasius hypophthalmus*, the fertilization rate was also not significantly different (ranged 65.63% to 92.31%) However, the hatching rate differed significantly (Hartono et al., 2016). In a similar species but different chromosome manipulation, catfish *C. gariepinus*, the fertilization rate of diploid and triploid eggs using heat shock treatment was 81.10% and 80.09%, respectively (A. Hassan et al., 2018). Other factors that influence fertilization rate include the quality of eggs and sperms, such as matured seed (Roshani et al., 2021), pathogen free, and good genetics produced by high-quality broodstocks. Thus, indicating that temperature shock after fertilization had no effect on fertilization but did reduce hatching rate.

Meanwhile, the post-fertilization egg size was greatest at 40°C heat shock treatment (1.177±0.032), but there was no significant difference between treatments in this study (Table 1). Fertilized eggs in this study were within the size range and mostly the same as reported for a similar species, *C. gariepinus* (A. Hassan et al., 2018; Olaniyi & Omitogun, 2014). Egg size variation was influenced by broodstock quality and size (Fitriliyani et al., 2022), egg yolk contents (Kajiwara et al., 2022), environmental conditions (Jonsson & Greenberg, 2022), and post-fertilization hydration of the eggs (Brandt et al., 2022). More research is needed to determine the impact of these factors on larval survival. According to the findings of this study, egg quality and ovaprim injected *C. gariepinus* produced the best results. Furthermore, heat shock treatment had no significant effects ($p>0.05$) on fertilization rate and egg size after fertilization.

Table 1 The effect of heat shock treatment on the diameter and number of *C. gariepinus* fertilized eggs.

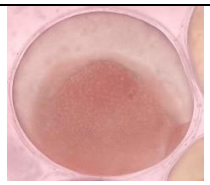
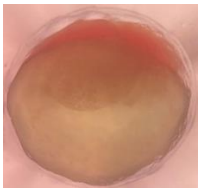
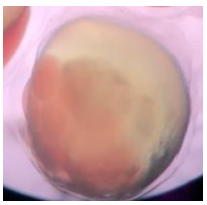
Treatments	Live fertilized eggs (%)	Eggs size post fertilization (mm)
Control	94.667±1.202[a]	1.150±0.023[b]
39°C Heat Shock	93.333±1.202[a]	1.170±0.025[b]
40°C Heat Shock	93.667±0.882[a]	1.177±0.032[b]
41°C Heat Shock	91.333±0.882[a]	1.167±0.019[b]

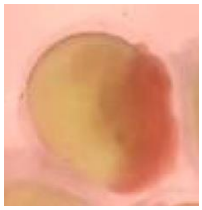
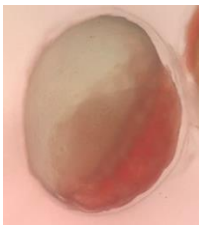
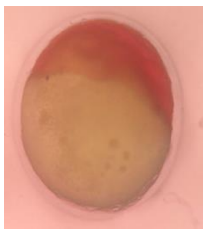
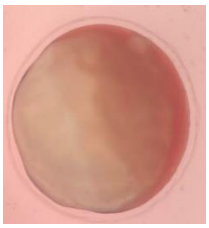
Data are mean ± SEM and based on subsamples of 100 eggs.
Duncan's new multiple range test finds that means with the same superscriptletter within columns are not significantly different ($p>0.05$).

Table 2 summarizes the characteristics of *C. gariepinus* embryonic development in the laboratory, from fertilized eggs to hatching. Embryonic development stages such as zygote, cleavage, morula, blastula, gastrula, somite, and hatching were observed. The ontogenic events observed in *C. gariepinus* were comparable to those observed in other catfishes (Adebiyi et al., 2013;A. Hassan et al., 2018; Rizal et al., 2020). Temperature is one of the factors that influence embryo development. The period of embryo development is slower in low temperature conditions (24°C) than in high temperature conditions (27°C) (Adebiyi et al., 2013; Brandt et al., 2022). In this study, *C. gariepinus* hatched at 23:45±3.38 minutes after fertilization. When compared to fertilization, hatching, and embryogenesis of diploid and triploid eggs of *Anabas testudineus* (A. Hassan et al., 2018), the cell stage does not end at the 64-cell stage, but continues to the 128-cell stage, where the seventh division results in the production of 128 daughter cells of relatively small size. Therefore, each species has a stage variation throughout

the entire embryonic cycle before becoming an adult fish. This information record on *C. gariepinus* embryonic development is useful for observing the effects of heat shock treatments.

Table 2 Characteristics of *C. gariepinus* embryonic development under laboratory condition.

Image	Developmental stages	Developmental period	Characteristics
	Fertilized egg	0:19±0.58 hour	Red spot (blastodisc), yolk mass (vegetal pole).
	One-cell stage	0:30±0.58 hour	The protoplasmic membrane protrudes at the animal pole or at the submicropilar region.
	Two-cell stage	0:38±0.67 hour	As is characteristic of telolecithal eggs, the first mitotic division occurred meridional and meroblastic, leading to the formation of two blastomeres.
	Four-cell stage	0:53±1.15 hour	The creation of the second mitotic specific terms split occurred at a correct angle to the first division, resulting in four blastomeres.
	Eight-cell stage	1:04±0.58 hours	The third metaphase was identical with the first split in that it contained two parallel lines that ended in four paired cells.

	16-cell stage	1:18±1.86 hours	The fourth metaphase resulted in 16 blastomeres and was similar with the first cell breakage. The cells were organized in a four-by-four blocking layer.
	32-cell stage	1:25±0.88 hours	Early morula stages, when continual splits and plunging necklines result in the heterogeneous production of numerous cells that are hard to count.
	64-cell stage	1:46±1.53 hours	The major divisions were indeed latitudinal, resulting in the creation of a new layer of cells.
	Morula stage	3:13±3.51 hours	Several further cell growths resulted in a large number of blastomeres.
	Blastula stage	3:45±3.84 hours	The blastocoele proliferated to create a roof construct that encased a large portion of the egg.
	Early gastrula	4:03±1.67 hours	The blastoderm grew, and the commencement of epiboly started gastrulation with a freely moving transition phase wave.

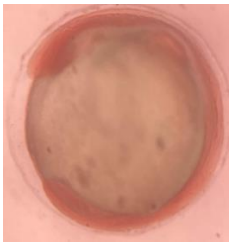
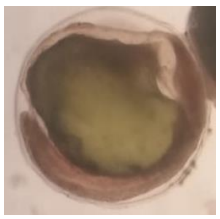
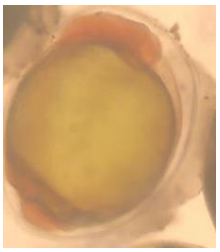
	Late gastrula	7:49±2.03 hours	The closure of the blastopore brought about the end with this motion, as well as morphogenesis, or the formation of developmental axes, started. The polster (cephalic bud) and tail bud are discovered.
	Somite stage	9:04±1.00 hours	The twin groups of cells that formed the vertebral column along the back of an embryo.
	Advance somite stage	15:21±4.98 hours	Somite formation then developed cephalocaudally.
	Hatching	23:45±3.38 hours	The embryo's escape from the chorion or yolk capsule via the tail.

Table 3 depicts embryo development at different heat shock temperatures and at laboratory temperature (27°C). This demonstrates that different heat shock treatments produce each stage at different durations. There was a significant difference in hatching rate between laboratory temperature (27°C: control) and heat shock treatments (39°C, 40°C, 41°C), which ranged from 1350 to 1425. When compared to heat shock treatment of tetraploid catfish *Pangasius hypophthalmus*, hatching rates decreased as shock temperature and duration increased (Hartono et al., 2016). This demonstrates that both the shock temperature and duration treatments had a direct effect on the hatching rate. (Lebeda & Flajshans, 2015) revealed a similar result, that temperature shock reduces the hatching rate of Siberian sturgeon fish eggs (*Acipenser baerii*). In this study, heat shock temperature of 40°C produced the best results on *C. gariepinus*. An early hatched *C. gariepinus* larva with a fully developed heart, heartbeat, and blood circulation was subjected to the 40°C treatment. The findings suggest that heat shock treatment can be used to create tetraploid food fish with fast growth characteristics that can be used in mass culture production.

Table 3 Development of embryos at various heat shock temperatures and time periods. Numbers are means ± standard errors.

Stages	Control	39°C	40°C	41°C
Fertilized egg	19±0.58[a]	20±0.67[a]	20±1.45[a]	20±0.58[a]
One-cell stage	30±0.58[a]	29±0.58[a]	29±0.88[a]	29±0.33[a]
Two-cell stage	38±0.67[a]	36±0.88[a]	36±1.33[a]	36±1.45[a]
Four-cell stage	53±1.15[a]	54±0.58[a]	53±1.33[a]	54±1.00[a]
Eight-cell stage	64±0.58[a]	62±1.00[a]	63±1.2[a]	62±1.33[a]
16-cell stage	78±1.86[a]	77±1.53[a]	77±1.76[a]	76±1.33[a]
32-cell stage	85±0.88[ab]	83±1.00[a]	82±0.33[a]	87±1.45[b]
64-cell stage	106±1.53[a]	104±1.00[a]	103±0.58[a]	104±0.58[a]
Morula stage	193±3.51[b]	186±1.00[a]	184±1.20[a]	184±0.88[a]
Blastula stage	225±3.84[a]	228±4.37[a]	222±3.71[a]	221±4.06[a]
Early gastrula	243±1.67[a]	241±2.00[a]	237±1.53[a]	240±2.40[a]
Late gastrula	469±2.03[a]	464±4.36[a]	462±2.65[a]	464±2.91[a]
Somite stage	544±1.00[b]	507±3.28[a]	504±1.53[a]	509±6.03[a]
Advance somite stage	921±4.98[b]	911±4.93[ab]	904±1.33[a]	914±2.00[ab]
Hatching	1425±3.38[c]	0*	1350±2.31[a]	0*

Data are mean ± SEM and based on subsamples of three eggs.
Duncan's new multiple range test finds that means with the same superscript letter within rows are not significantly different ($p>0.05$).

CONCLUSION

Tetraploid induction after 40 minutes of fertilization is a suitable time for all temperature shock treatments and yields positive results at an early stage of embryo development. However, for embryo development until hatching, shock temperature of 40°C and duration of 2 minutes were found the best parameters comparable to the control or standard method. Furthermore, it appears that different temperature shocks cause significant differences in embryonic development at the advanced somite stage. Therefore, the precise parameters for tetraploid induction require further study. The findings could help with tetraploid induction as an interim method for producing triploids.

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