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The rate of embryonic development and hatching in transgenic G_3 mutiara catfish (*Clarias gariepinus*) eggs at room temperature

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Abstract
Mutiara catfish is one of the most important catfish strains developed in Indonesia. However, the growth stability of cultured non-transgenic Mutiara catfish fluctuates. This problem can be solved by developing transgenic Mutiara catfish (containing the African catfish growth hormone gene, *Cg*GH) to improve the stability of fish growth. Controlled handling of eggs is carried out to reproduce this superior fish in each generation. The egg handling in a controlled system was performed to reproduce this superior fish in each generation. The G_3 transgenic Mutiara catfish eggs production was carried out with three different broodstock pairs (pairs A, B and C). The results of observations of the rate of embryogenesis and hatching of the G_3 eggs showed that eggs from pair A were faster than B and C. Transgenic catfish eggs hatch 1-1.5 hours faster than non-transgenic fish eggs at room temperature.

Keywords: Transgenesis, embryogenesis, mutiara catfish, handling eggs

Introduction
The problem of providing fish seeds cannot be separated from the controlled handling of eggs, considering that the mortality of hatching fish eggs in natural conditions almost 70% [1]. Conditions for hatching fish eggs in nature experience many obstacles such as poor water quality, pests and diseases of fish causing the development of egg embryos to be damaged and the percentage of hatching eggs is small. Handling of fish eggs, especially the eggs of transgenic fish as a superior fish species that require a controlled aquarium to obtain a greater number of hatching eggs in the production of generations of transgenic fish [2]. The process of developing transgenic fish egg embryos related to egg hatching rate requires a study of transgenic fish egg embryogenesis. Induction of foreign genes inserted into the genome of related fish is thought to accelerate the hatching rate of transgenic fish eggs, as shown in a study on G_2 transgenic Mutiara catfish eggs [3]. Generally, the average hatching rate of catfish eggs ranges from 18-20 hours depending on the hatching temperature [4, 5, 6]. However, no information has been obtained for the embryogenesis and hatching rate of transgenic catfish eggs. Therefore, this research was conducted for the controlled handling of transgenic fish eggs to increase the production of transgenic fish larvae.

Materials and Methods
Identification of the G_2 transgenic Mutiara catfish broodstock
Screening for identification of three pairs of the G_2 transgenic Mutiara catfish broodstock was carried out by PCR using male and female tail fin samples. RNA extraction of broodstock fin samples was performed using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and RT-PCR using My Taq OneStep RT-PCR (Bioline, London, UK) following the kit instructions. PCR programme settings as follows: 48°C for 20 min; 40 cycles of 95°C for 1 min, 95ºC for 10 s, 60°C for 30 s and 72°C 30 s; and 72°C for 5 min. Transgenic positive broodfish were indicated by the presence of a 600 bp band parallel to the *Cg*GH gene band in the pCMV-*Cg*GH plasmid (positive control) as the primer amplification product of GH-F (5'-ATGGCTCGAGTTTTGGTGCTGCT-3') and GH-R (5'-CTACAGAGTGCAGTTGGAATCCAGGG-3') [7, 8].

Maturation and spawning of G_2 transgenic Mutiara catfish broodstock

Furthermore, three pairs of G_2 transgenic Mutiara catfish were matured for 1.5 months in a fibre tank measuring 1.3 m in diameter and 1 m deep filled with water with a height of 60 cm, installed a water heater with a temperature of 30 ± 1 ºC and given aeration to maintain dissolved oxygen and photoperiods were set as 12 h light and 12 h dark. During rearing, broodfish were given a mixture of commercial feed (33% protein content) and *pindang tongkol* (35.80% protein content) (65:35 ratio) with a frequency of three times a day as much as 5% by weight of biomass. After the maturation process is complete, the next step is to observe the broodstock which has matured gonads and are ready to be spawned. The maturity of broodstock gonads was determined by examining the females and male genital papillae; pink genitals are an indicator of sexual maturity [9]. Three pairs of the G_2 transgenic Mutiara catfish broodstock that were mature and ready to be spawned with three spawning treatments, namely A (female-1 transgenic × male-2 transgenic), B (female-2 transgenic × male-2 transgenic) and C (female-3 transgenic × male-3 transgenic). Each cross of the G_2 broodstock can be seen in Figure 1 and the length and weight of the broodfish is in Table 1.

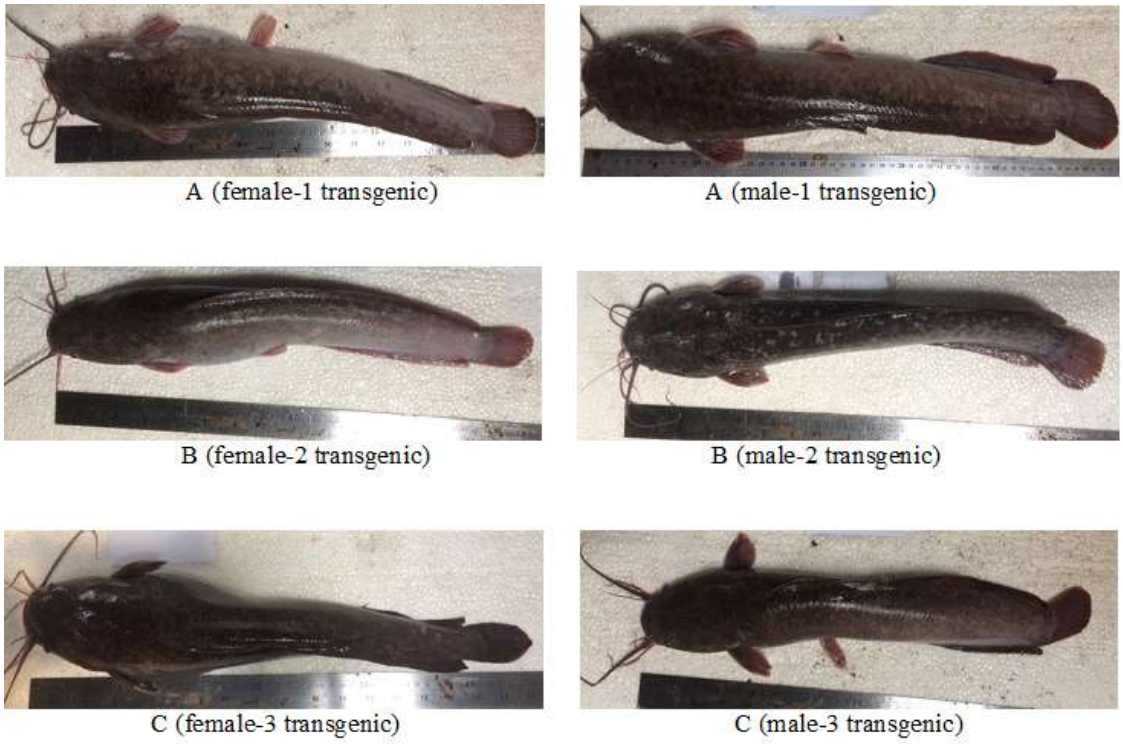


Fig 1: Mating of G_2 broodstock pairs

Table 1: Length and weight of the G_2 broodstock

Crossing of G_2	Transgenic broodstock	Length (cm)	Weight (g)
A	Female-1	46.5	980
	Male-1	54	1180
B	Female-2	35.5	400
	Male-2	44	600
C	Female-3	43.9	500
	Male-3	41.7	360

The spawning of broodstock was used as a semi-artificial method (with hormone induction and the use of kakaban an egg-attachment substrate placed on the bottom of a round fibreglass tank). Dosage of ovaprim hormone injection (Syndel Laboratories Ltd., British Columbia, Canada) was 0.5 mL/kg body weight of female catfish and 0.4 mL/kg body weight of male catfish. Immediately following injection, the broodstock pairs were transferred to their spawning tank (1000 L), which was maintained at controlled water temperature, oxygen level, and water height (35 cm).

Twelve hours after injection, eggs attached to the *kakaban* substrate were examined. Fertilized eggs were then transferred into a glass aquarium (40 × 25 × 25 cm) filled with approximately 30 L of water maintained at 26°C ± 1°C, with homogenous aeration [3].

Embryonic development and hatching of the G_3 transgenic Mutiara catfish eggs

The embryonic development of G_3 transgenic Mutiara catfish eggs was observed in the spawning results of three different pairs of G_2 transgenic Mutiara catfish broodstock and compared with the reference of *C, gariepinus* egg embryo development as an indicator of the rate of development and hatching of transgenic fish eggs. Observations on the development of fish egg embryos (cleavage, morula, blastula, gastrula, organogenesis, hatching larvae) were carried out in each G_2 broodstock pair, by taking four samples of fertilized eggs as replicates under a microscope. The duration of eggs to hatching in each spawning broodstock was calculated starting from the cleavage stage to the larvae hatched (in minutes).

Results and Discussion

Verification of transgenic broodstock

The results of PCR amplification for screening G_2 positive transgenic Mutiara catfish were indicated by the presence of a 600 bp DNA band (the *Cg*GH gene) as insertion of the African catfish growth hormone gene in the transgenic Mutiara catfish genome. Three G_2 females and three G_2 males used for egg production were G_3 positive for transgenic fish with the presence of *Cg*GH fragments in the caudal fin sample genome (Figure 2).

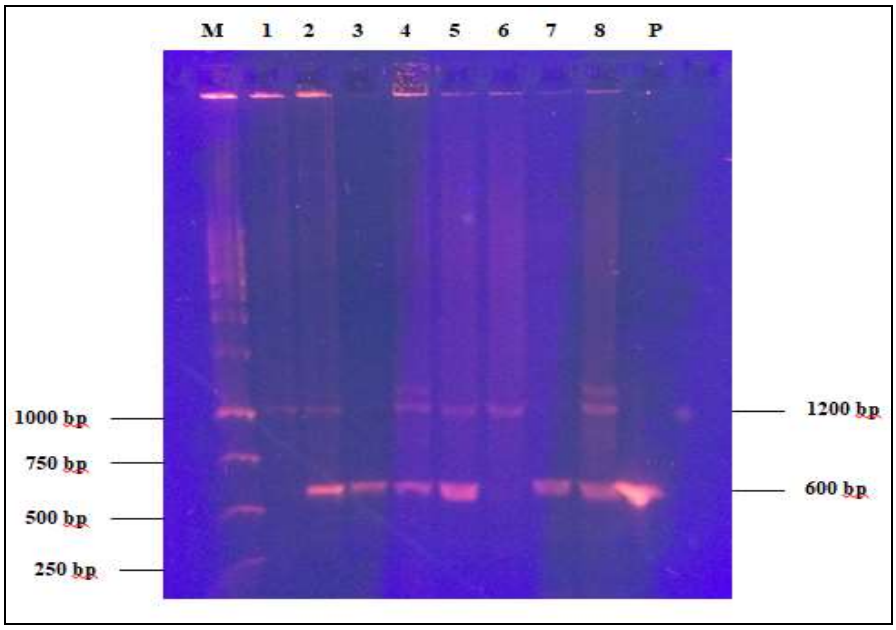


Fig 2: Identification of G_2 transgenic Mutiara catfish broodstock

M: 1 kb DNA ladder, 1: male non-transgenic, 2: female-1 transgenic, 3: male-1 transgenic,
4: female-2 trangenic, 5: male-2 transgenic, 6: female non-transgenic, 7: female-3 transgenic,
8: male-3 transgenic, 9: pCMV-*Cg*GH plasmid (control positive)

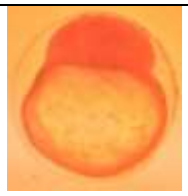
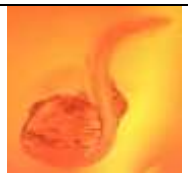
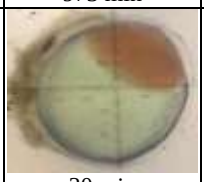
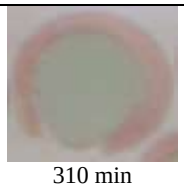
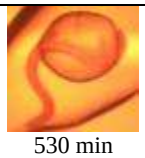
Verification of the PCR results of the G_2 broodstock in Figure 2 above showed the difference between transgenic and non-transgenic Mutiara catfish indicated by the presence of the *Cg*GH gene (600 bp) in the genome of the transgenic fish broodstock, while in non-transgenic fish there is no band size 600 bp. This confirmation was used for the broodstock crosses of G_2 transgenic Mutiara catfish in treatments A, B and C (Figure 1) for the G_3 transgenic Mutiara catfish egg production.

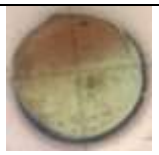
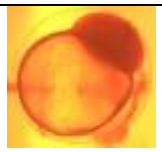
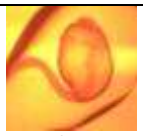
Embryogenesis of the G_3 transgenic Mutiara catfish egg

The stages of development of G_3 eggs produced by G_2 transgenic females in each treatment were observed microscopically to determine the duration for hatching eggs at room temperature. This observation is useful to determine whether the hatching of transgenic fish eggs is accelerated at room temperature and whether there is a negative effect caused by the effect of transgenesis. The developmental phase of the egg embryo starts from the stage of cell division (cleavage), cell multiplication (morula), blastula (formation of blastomeres resembling germ rings that cover almost 95% of the fertilized egg), gastrula (expansion of blastoderm development that covers the entire yolk). eggs), organogenesis (formation of head cells, eyes, tails, somites or vertebrae, heart and other organs) to form larvae and hatch [6, 10]. Microscopic observations showed that the development of the G_2 transgenic Mutiara catfish egg embryos from female-1 (A), female-2 (B) and female-3 (C) showed differences in the rate of development, which was thought to be related to the level of expression of the transgene (*Cg*GH) inherited by each of the G_2 female broodstock. The rate of development of egg embryos from female A was faster than B and C, presumably because over-expression of *Cg*GH stimulated faster oocyte growth and could induce the rate of embryogenesis in G3 eggs from female A. This phenomenon is similar to the growth rate of oocytes from G_2 transgenic Mutiara catfish eggs [3], and in egg embryogenesis from female A indicates the rapid development of cleavage, morula, blastula, gastrula, organogenesis phases compared to B and C (Table 2). On the other hand, the development of G_3 embryos from female B and C was slower than A, which suspected to be related to the low level of *Cg*GH expression in these eggs causing late oocyte growth [8].

Table 2: Embryogenesis of the G_3 eggs produced by female-1 (A), female-2 (B), female-3 (C) of the G_2 transgenic broodstock

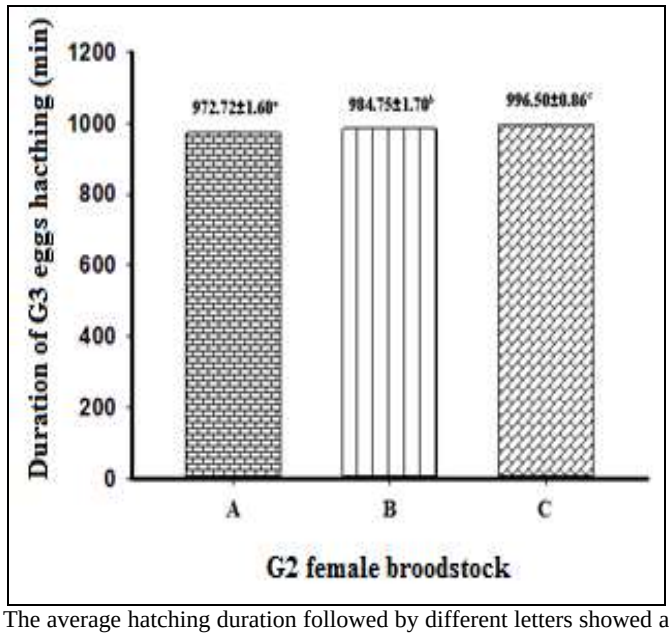
G2 transgenic broodstock	Embryogenesis step	Time (min) and embryo profile	Development description
Female A	Cleavage	 25 min	Multi-cell development

	Morula	 120 min	Multiplication of cells to form a serving hood
	Blastula	 185 min	The thickening of the sprout ring forms a blastosul (empty space covered by blastoderm)
	Gastrula	 305 min	Closure of the entire yolk by the blastoderm
	Organogenesis	 525 min	Formation of the head, tail, somites, liver, etc
	Larvae hatch	 973 min	Newly hatched larvae
Female B	Cleavage	 30 min	Multi-cell development
	Morula	 130 min	Multiplication of cells to form a serving hood
	Blastula	 195 min	The thickening of the sprout ring forms a blastosul (empty space covered by blastoderm)
	Gastrula	 310 min	Closure of the entire yolk by the blastoderm
	Organogenesis	 530 min	Formation of the head, tail, somites, liver, etc
	Larvae hatch	 985 min	Newly hatched larvae

Female C	Cleavage	 35 min	Multi-cell development
	Morula	 135 min	Multiplication of cells to form a serving hood
	Blastula	 200 min	The thickening of the sprout ring forms a blastosul (empty space covered by blastoderm)
	Gastrula	 315 min	Closure of the entire yolk by the blastoderm
	Organogenesis	 540 min	Formation of the head, tail, somites, liver, etc
	Larva menetas	 997 min	Newly hatched larvae

Hatching rate of the G_3 Mutiara catfish egg

The results of observations of the hatching time of G_3 transgenic Mutiara catfish eggs in each G_2 broodstock spawning treatment showed differences in hatching duration (Figure 3).



The average hatching duration followed by different letters showed a significant difference ($p<0.01$)

Fig 3: Duration (minutes) of the G_3 eggs hatching from G_2 transgenic Mutiara catfish female.

The embryological development of G_3 transgenic Mutiara catfish eggs in Table 2 and Figure 3 above, showed that G_3 eggs produced by G_2 female A were faster than eggs produced by female B and C. The rate of development of the embryonic stages of G_3 eggs produced by each G_2 transgenic female broodstock differed at each stage. The difference in cleavage development time of G_3 egg embryos between female A and B was 5 minutes, while between B and C was 5 minutes and A and C was 15 minutes. Meanwhile, the development of morula between the three female broodstock is 5 minutes. The difference in development time of the embryonic G_3 blastula produced between females A and B was 10 minutes, while between females B and C was 5 minutes (Table 2). Meanwhile, the difference in the development time of the G3 embryo gastrula between the three G_2 transgenic females was 5 minutes. The difference in the rate of development of the organogenesis stage of G_3 embryos produced by females A and B was 5 minutes, on the contrary between females B and C was 10 minutes. Furthermore, for the larval hatching stage, the average time required for G_3 embryos to hatch from female A was 973 minutes (16.22 hours), female B was 985 minutes (16.42 hours) and C 996 minutes (16.60 hours) (Figure 3). Whereas, hatching of non-transgenic catfish (*C. gariepinus*) eggs was 1040 minutes (17.33 hours) and for Egyptian African catfish (*C. gariepinus*) eggs it was 1080 minutes (18 hours) [11]. Comparison of hatching duration of catfish eggs between transgenic and non-transgenic fish showed that the hatching rate of transgenic fish eggs was 1-1.5 hours faster than that of non-transgenic fish, indicating that GH-transgenesis (*Cg*GH) was induced embryo

development and hatching of fish eggs. The development of each stage of the G_3 embryo produced by female A is faster than B and C is the influence of the level of exogenous GH expression by broodstock A which suspected to be relatively higher than B and C. This is also shown by the research of Russian sturgeon fish (*Acipenser gueldenstaedtii*) that GH stimulates the ovaries which induce IGF-1 to stimulate the production of estradiol which plays a role in the development of embryogenesis of fish eggs [12]. This study also showed similar results, that exogenous GH (*Cg*GH) stimulated the development process of the G_3 fish embryo until the larvae hatched which was inherited by each G_2 transgenic female broodstock.

Conclusions
The rate of embryo development and hatching of G_3 transgenic Mutiara catfish eggs was faster by the female broodstock of treatment A (973 minutes). Transgenic Mutiara catfish eggs hatch 1-1.5 hours (90 minutes) faster than non-transgenic catfish.

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Authors' contributions
IDB take samples in the hatchery and the eggs incubation in the aquaria and collect research data. IDB compiles research articles and embriogenesis analysis and article submissions. Author also critically reviewed the manuscript for final approval to be published.

Competing interests
The authors declare no competing interest.

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