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4 **Variation amount of sperm cells**  
5 **in the first generation of nontransgenic and**  
6 **transgenic Mutiara Catfish hybrid**  
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9 **ABSTRACT**

10 One of the factors that influence the success of catfish spawning, including male broodstock gonad fertility  
11 determine differences in the number of the sperm produced. The use of transgenesis technology (insertion  
12 of the exogenous growth hormone gene) in catfish shows an influence especially multiplication of the sperm  
13 counts. The purpose of this study was to compare the variations in the number of sperm cells of the male  
14 transgenic Mutiara catfish (comprising the African catfish growth hormone gene) with nontransgenic Mutiara.  
15 These research was conducted at the Fish Hatchery, Fisheries Biotechnology Laboratory, and Central  
16 Laboratory of Padjadjaran University. The experimental method was used completely randomized design  
17 and analyzed quantitatively and qualitatively. The male catfish broodstock were used as treatments consists  
18 of the first generation transgenic Mutiara hybrids with nontransgenic Mutiara (G1\_MT-MNT), transgenic  
19 Mutiara hybrids with Sangkuriang (G1\_MTS), and Sangkuriang (G1\_S). The sperm from testes of each  
20 broodstock were used as replications for sperm count test parameters and motility (n = 5). Qualitative  
21 analysis was used for the identification parameters of the transgenic male broodstock using the Polymerase  
22 Chain Reaction (PCR) and sperm volume. Quantitative analysis uses the Sigma Plot 12.0 program for  
23 parameters for sperm cell count and sperm motility. The results showed that the highest number of sperm  
24 ( $6.06 \times 10^6$  cells) and the highest motility (score 4.2) were found in the broodstock of the G1\_MT-MNT male  
25 catfish.  
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27 *Keywords: sperm cells, transgenesis, transgenic Mutiara catfish,*

28 **1. INTRODUCTION**

29 The success of catfish fingerling mass production involves the contribution of quality male broodstocks in  
30 spawning activities. The abundance of the sperm production and sperm motility is one of the important  
31 factors in spawning success (Viveiros *et al.*, 2001). One pair of African catfish (*Clarias gariepinus*) can  
32 produce 50,000 to 60,000 catfish larvae and further rearing was only obtained 32,500-38,000 juveniles  
33 catfish aged 5-6 weeks (mortality ranges from 35-45%) (Steyn and van Vuren, 2011).

34 The frequency of using the same male broodstock causes the quality of male fertility to decreased.  
35 Broodstock quality improvement can be done by the transgenesis (insertion of African catfish growth  
36 hormone gene, *C. gariepinus* Growth Hormone, CgGH) on early generation catfish (G0) and first generation  
37 (G1) of the Mutiara catfish (Buwono *et al.*, 2016). The study of transfer of catfish growth hormone gene  
38 (*Pangasionodon hypophthalmus* Growth Hormone, PhGH) in Dumbo catfish was also carried out by Marnis  
39 *et al.* (2014) to produce transgenic catfish.

40 The male transgenic Mutiara catfish has a fast growth compared to normal catfish (including testicular  
41 growth). GH receptors work on gonadal cells of male fish to induce sperm multiplication in the testes  
42 (Maestro *et al.*, 1997). The exogenous GH expression in the male catfish can increase sperm count and  
43 trigger fish spawning (Devlin *et al.*, 1995). So far it is not known whether of the male transgenic Mutiara  
44 catfish has a different number of sperm cells compared to non-transgenic Mutiara catfish (Buwono *et al.*,  
45 2019).

46 **2. MATERIALS AND METHODS**

47 The study began in December 2018 with a processing time of approximately two months. The parameters  
48 observed included the identification of the hybrid transgenic male catfish, sperm volume, sperm cell count,

49 and sperm motility (G1\_MTS, G1\_MT-MNT, and G1\_S). Identification of the transgenic male catfish using  
50 the RNeasy mini kit (QIAGEN, USA) for RNA isolation and MyTaq OneStep RT-PCR kit (Bioline, UK) for  
51 RT-PCR reactions. Physiological NaCl (0.9%) and immersion oil are used in observing sperm cell count and  
52 sperm motility.

53 Total RNA extracted from fish fin tissue was used as a template for amplification of the exogenous GH  
54 using Cg-F (5'-ATGGCTCGAGTTTGGTGCTGCT-3') and Cg-R (5'  
55 CTACAGAGTGCAGTTGGAATCCAGGG-3') primers (Zhang *et al.*, 2009). The RT-PCR reaction mixture  
56 consists of 2x MyTaq OneStep RT-PCR mix (25 µl), Cg-F primers (10 µM) and Cg-R (10 µM) each of 1.25  
57 µl, reverse transcriptase (0.5 µl), ribosafe RNase inhibitors (1.0 µl), DEPC-H<sub>2</sub>O (14.5 µl), and RNA  
58 templates (5 µl). The RT-PCR reaction was carried out using a thermal cycler in 1 cycle for RT reaction at  
59 48°C for 20 minutes. The PCR stage consisted of pre-denaturation at 95°C for 1 min, denaturation at 95°C  
60 for 10s, annealing at 60°C for 20s. Extensions at 72°C for 30s, and replication 40 times. Final extension at  
61 72°C for 5 min, hold at 4°C for 1 min. The amplification product was separated with 1% agarose gel and the  
62 transgenic broodstock was indicated by a 600 bp GH fragment (parallel to the pCMV-CgGH plasmid  
63 fragment as a positive control).

64 The research method used a completely randomized design consisting of the three types of the G1 fish  
65 sperm treatments (G1\_MT-MNT, G1\_MTS, G1\_S) and five replications. Sperm was taken as much as 1µl  
66 from the right testis or left testis in each test fish and repeated five times.

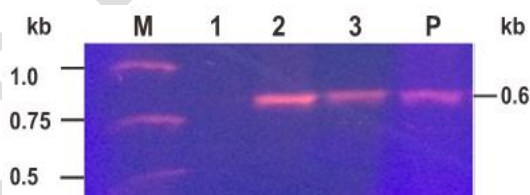
67 Data analysis were used in this study consisted of qualitative and quantitative. The transgenic identification  
68 and sperm volume using qualitative analysis descriptively. Parameters of sperm cell count and sperm  
69 motility were analyzed by Duncan Multiple Range Test at 0.05 level with the help of Sigma Plot 12 software.

### 70 3. RESULTS AND DISCUSSION

#### 71 3.1 Identification of transgenic male catfish broodstock

#### 72 3.2 Sperm Volume

74 Based on the results of the test, it was found that the catfish broodstock of the G1\_MT-MNT and  
75 G1\_MTS were transgenic fish (containing the African catfish GH). These was shown from the  
76 fragments that appear on the catfish broodstock PCR products G1\_MT-MNT and G1\_MTS was 600 bp  
77 (Fig 1).



78 Fig 1. Identificaton of the transgenic male catfish broodstock. M= marker DNA ladder 1 kb (Promega); 1=  
79 G1\_S ; 2= G1\_MT-MNT; 3= G1\_MTS; P= plasmid (pCMV-CgGH, 600 bp)

81 Verification of the African catfish GH fragment size was carried out by Buwono *et al.* (2016) where the  
82 presence of the African catfish GH gene was detected at 600 bp. The primers used were Cg-F and Cg-  
83 R primers (Zhang *et al.*, 2009) specifically designed to detect the African catfish GH gene found from  
84 Mutiara catfish. The DNA fragment that appears is a visual representation of African catfish GH  
85 detected in test fish. The GH African catfish gene insert (600 bp) was also obtained in transgenic  
86 catfish from fish fin tissue samples (Iskandar *et al.*, 2018).

#### 87 3.3 Amount of sperm cells

88 Observation of the sperm volume was carried out objectively or directly, by looking at the size scale on  
89 the 1.5 ml eppendorf tube. Based on observations, the highest volume of the sperm was produced by  
90 G1\_MT-MNT catfish (2.2 ml), then followed by the G1\_MTS (1.6 ml), and G1\_S (1.2 ml). The results  
91 obtained were not different with the Papadaki *et al.* (2008) study which stated that the volume of sperm  
92 produced by the male brooders is generally more than 1 ml in the range 2-4 ml or an average of 3 ml.

93 Increased sperm volume in the male transgenic hybrid catfish broodstock, is associated with the  
94 expression of exogenous GH which induces an increase in testosterone production which stimulate

95 spermatogenesis (Dubey nee Pathak *et al.*, 2015). This activity of spermatogenesis can increase sperm  
96 count so that the volume of sperm also increases. The transgenic fish have rapid growth so that the  
97 process of producing sperm is also more, because of the increase in the growth of the testicular  
98 organs.

99 This **was** also indicated by the research of Iskandar *et al.* (2018) that the growth of transgenic Mutiara  
100 catfish **was** 2-3 times faster than nontransgenic catfish.

101 The reproductive organs of transgenic Mutiara catfish have a larger size than nontransgenic catfish,  
102 can be positively correlated with the amount of sperm volume (cement). The advantages of transgenic  
103 Mutiara catfish can provide benefits in accelerating the maturation of the reproductive organs for  
104 spawning activities. The broodstock sperm volume of catfish G1\_MT-MNT, G1\_MTS, and G1\_S  
105 was shown in Fig 2.

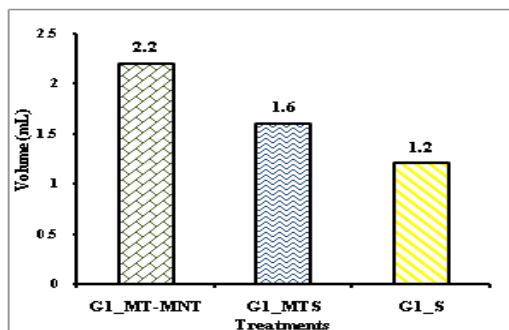


Fig 2. Sperm volume

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108 Visually, the form of the broodstock G1\_MT-MNT was large and milky white, the broodstock G1\_MTS was  
109 medium size and creamy white, while G1\_S was small and pale white (Fig 3).

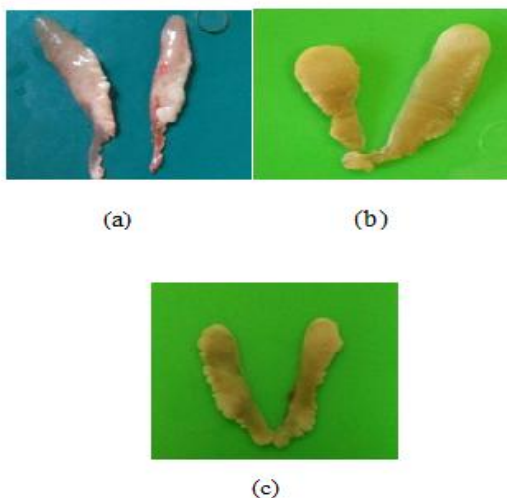


Fig 3. Profile of testes (a).G1\_MT-MNT,  
(b).G1\_MTS, dan (c).G1\_S

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### 113 3.4 Amount of sperm cells

114 Based on the results of the research that has been done, the highest number of **the** sperm cells was  
115 obtained by the catfish broodstock G1\_MT-MNT ( $6.06 \times 10^6$  sperm/ml), then followed by G1\_MTS ( $4.92 \times$   
116  $10^6$  sperm/ml), and G1\_S ( $1.97 \times 10^6$  sperm/ml). The results of statistical analysis showed that the number of  
117 sperm cells G1\_MT-MNT with G1\_MTS was not significantly different, but the number of sperm cells  
118 G1\_MT-MNT with G1\_S and the number of sperm cells G1\_MTS with G1\_S was significantly different.

119 The calculation of the number of sperm cells in this study is not much different from that obtained by Steyn  
120 and van Wren (1986) in African catfish (*C. gariepinus*) of  $6.2 \times 10^6$  sperm/ml and Nayak *et al.* (2016) in *C.*  
121 *batrachus* ( $6.0 \times 10^6$  sperm/ml). The MT-MNT male broodstock containing GH African catfish was derived  
122 from *C. gariepinus* as Mutiara catfish so the number of sperm cells **was** not much different.

123 The number of sperm cells in the study of Hu *et al.* (2011) in European catfish (*Ictalurus punctatus*) is  $2.9 \times 10^6$  sperm/ml. This amount is lower when compared to MT-MNT male catfish ( $6.06 \times 10^6$  sperm/ml) and MTS  
124 male catfish ( $4.92 \times 10^6$  sperm/ml). This shows that transgenesis has an influence on the production of the  
125 number of sperm cells.  
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127 The G1 male Sangkuriang catfish was used as a control in these study to represent the presence of catfish  
128 in the community. The G1\_MT-MNT catfish broodstock produces more sperm than G1\_S because the  
129 catfish broodstock was a hybrid of transgenic Mutiara and nontransgenic Mutiara (Fig. 4).

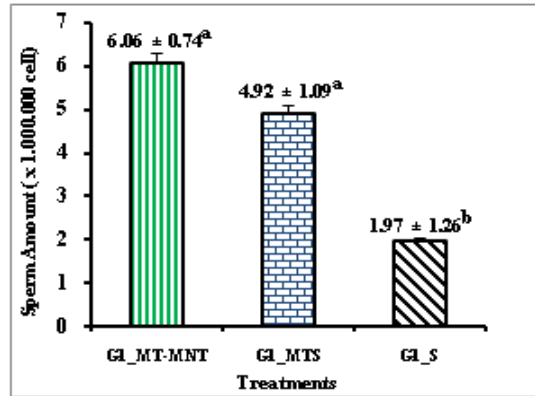


Fig 4. Amount of sperm cells

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132 Spermatogonia cells in transgenic fish can increase in number due to over-expression of exogenous  
133 insertion of growth hormone (GH) (Caelters *et al.*, 2005). This causes an increase in growth hormone that  
134 can induce multiplication of the spermatogonia cells. The production of sperm counts is induced by growth  
135 hormone controlled by the GH gene. In nontransgenic fish the production of sperm counts is less than that of  
136 transgenic fish (only containing endogenous GH), whereas transgenic fish contain exogenous GH and  
137 endogenous GH which causes over-expression which promote the production of sperm of fish broodstocks  
138 (Dewi *et al.*, 2013).

139 The sperm count of G1\_MTS was lower compared to the G1\_MT-MNT broodstock even though both  
140 were transgenics, due to the G1\_MT-MNT has better hybrid quality. The low sperm of Sangkuriang catfish  
141 (nontransgenic) due to cell multiplication is only caused by endogenous GH itself. In transgenic catfish (MT-  
142 MNT and MTS), sperm production is higher due to over-expression of exogenous and endogenous GH  
143 (Schulz *et al.*, 2012)

### 144 3.5 Sperm Motility

145 Sperm quality can be measured by sperm motility parameters. There are various methods that can be used  
146 to measure sperm motility scores, one of which is using a method developed by McMaster (1992). The  
147 lowest score is represented by number 1 and the highest score is represented by number 5.

148 The highest sperm motility was found in the G1\_MT-MNT broodstock with a score of 4.2 (progressive  
149 moves). Based on its movements, almost 85% of sperm cells move to all sides (Fig 5). These results are  
150 close to the range of the Kovacs *et al.*, (2010) study on the sperm motility test of the African catfish (*C.*  
151 *garipepinus*) showing maximum sperm movement can reach 94% ( $80 \pm 14\%$ ).

152 Superior transgenic and hybrid factors also determine sperm motility. Transgenic fish have an exogenous  
153 GH insertion which results in a large number of spermatogonia cell production so that the number of motile  
154 sperm is relatively higher than other sperm which is indicated by a score of 4.2.

155 The subsequent effects of transgenesis and hybrid effects caused sperm count and sperm motility in the  
156 broodstock crossing of MT-MNT to be higher than those of MTS or S broodstock. The broodstock sperm  
157 motility of G1\_MTS was not significantly different from the broodstock G1\_S. Due to, the hybrid broodstock  
158 of the G1\_MTS is a transgenic Mutiara catfish crossing (medium sperm motility) and Sangkuriang (low  
159 sperm motility) which causes G1\_MTS motility at a score of 3.4 which is relatively similar to the Sangkuriang  
160 motility (score 3) (Fig 5).

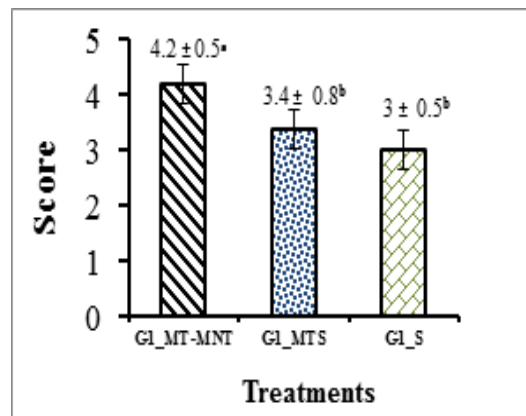


Fig 5. Sperm motility

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163 Based on its movement, the G1\_MTS and G1\_S sperm motility is quite progressive about 50% moves to all  
164 sides. This shows that sperm motility is quite high. The Toelihere study (1981) states that the percentage of  
165 motility of spermatozoa is low (40%) causing the ability to fertilize the egg decreases.

166 The results of the study showed that the number of spermatozoa in G1 transgenic Mutiara catfish (MT-MNT  
167 and MTS) was higher than nontransgenic G1 catfish. As compensation, more larvae are produced and the  
168 use of the G1 transgenesis-GH Mutiara catfishes is needed to overcome the reduced reproductive  
169 performance of catfish in conventional spawning.  
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171 This study discovered the CgGH that can be beneficial for fertility of the male catfish. This study will help the  
172 researchers to uncover the critical areas of selection of the male broodstock quality that many researchers  
173 were not able to explore. Thus a new theory on transgenesis and hybridization may be arrived at.

#### 174 4. Conclusion

175 The G1\_MT-MNT male catfish broodstock produces higher sperm cell count and sperm motility than other  
176 test fish. The sperm volume of G1\_MT-MNT male catfish is greater than G1\_MTS and G1\_S. The results of  
177 molecular identification showed that the male catfish G1\_MT-MNT and G1\_MTS were transgenic fish  
178 (containing of 600 bp exogenous GH).  
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