Effect of different temperatures on GH-transgenesis, testosterone levels and spermatozoa density of G5 transgenic mutiara catfish (*Clarias gariepinus*)

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Abstract

Unstable changes in environmental temperature (during the dry season there are several times of rain) can damage the fish reproductive cycle, leading to inhibition of spawning activity. Low temperatures cause slow fish gonad maturation, while high temperatures cause delays in the spermatogenesis process of male fish. The ideal temperature range can increase fish testosterone (T) levels needed to maintain spermatogenesis and GH expression levels during sperm cell proliferation. The effect of temperature on the presence of exogenous GH (*CgGH*) in G5 transgenic mutiara catfish formed through broodstock breeding GH-transgenesis technology in previous research needs to be evaluated for testicular fertility, especially T levels and spermatozoa (Spz) density during fish gonad maturation. The results showed that the ideal temperature that increases T levels, Spz density and *CgGH* expression levels in G5 transgenic mutiara catfish is 24℃. Temperatures of 28℃ and 32℃ tended to reduce T levels and Spz density in both transgenic and nontransgenic catfish. Besides that, the sperm maturation stage (formation of Spz) was more dominantly induced by 24℃ compared to other temperature treatments. This indication shows that the maximum enzyme activity involved in T production to drive the spermatogenesis process is regulated at 24℃. As a consequence, the use of transgenic male fish broodstock (transgenesis-GH) and temperature regulation for male fish gonad maturation is more profitable in the range of 24℃–28℃.

Keywords: *CgGH*, G5 transgenic mutiara catfish, Steroid and spermatozoa, Temperature

Introduction

Somatic growth in fish broodstock candidate entering the puberty period is more directed towards the development of reproductive organs (testes or ovaries) which is stimulated by light, temperature and rainfall in the aquatic environment (Harvey & Hoar, 1979; Taranger et al., 2010). This external stimulation causes the secretion of reproductive hormones as endogenous (internal) stimulants that regulate (at least in part) the seasonal reproduction of fish (Mañanós et al., 2008). The

effect of temperature on fish reproductive activity can increase or decrease gonad weight. Water temperatures that are too low may cause fish reproductive activity to slow down, while high temperatures cause low fish spawning, as a consequence of inhibiting the spermatogenesis process (Peñaranda et al., 2016; Sundararaj, 1981).

Endogenous factors that are influenced by environmental temperature and are involved in sperm density and Spz development are related to growth hormone (GH), steroid hormones (testosterone, T) which induce sperm cell proliferation and spermatocyte maturation (Baroiller et al., 2014). This indication shows that the regulation of GH expression (including *CgGH* insertions in transgenic catfish) cannot be separated from the influence of environmental temperature. Optimum temperature can increase GH expression levels and has implications for the induction of T levels and Spz density. Previous research results on transgenic G5 mutiara catfish formed through a broodstock breeding program (Buwono et al., 2021, 2023, 2024) showed the potential for broodstock gonad growth after induction of stocking density and light. However, not much is known about the role of temperature in the gonad maturation of male G5 transgenic mutiara catfish, which involves *CgGH* expression which leads to increased T levels and sperm density. The optimum level of temperature that drives *CgGH* expression, T levels and Spz density in G5 transgenic mutiara catfish broodstock needs to be evaluated to increase the number of gonad mature male fish broodstock as brood stock ready to be spawned in mass production of superior catfish fingerlings.

Materials and Methods

Maintenance of G5 male catfish and PCR testing of transgenic fish

The G5 male catfish used came from previous research (results from the spawning of the parent pair of G4 transgenic mutiara catfish) with transmission of *CgGH* inserts in G5 of 89% (Buwono et al., 2024). Furthermore, 18 transgenic fish broodstock candidate and 18 nontransgenic fish broodstock candidate were used in the temperature difference treatment for 60 days. Temperature treatment applied to transgenic catfish (A: 24℃, B: 28℃, C: 32℃) and nontransgenic catfish (A*: 24℃, B*: 28℃, C*: 32℃) with 6 repetitions. Each broodstock rearing tank contains 6 G5 male fish (as replicates) for both transgenic and non-transgenic fish rearing tanks which are equipped with a water heater to regulate the treatment temperature. The volume

of water filled in the round cylinder tank for temperature treatment is 700 L and the male fish are 5 months old with an average initial weight of 400–500 g for transgenic fish and 350–400 g for nontransgenic fish. During 60 days of maintenance, the test fish were given commercial food hiprovite-781 (33% protein), 2 times a day (3% biomass weight) with 50% water changes every week. Identification of G5 transgenic fish was carried out before treatment with a polymerase chain reaction (PCR) test and using primers GH-F (5'-ATGGCTCGAGTTTTGGTGCT-GCT-3') and GH-R (5'-CTACAGAGTGCAGTTGGAATC-CAGGG-3') (Zhang et al., 2009).

Isolated RNA for amplification of *CgGH* (600 bp) using fish tail fins (around 10–25 mg), and then processed using a Quick RNA Miniprep Plus Kit (Zymo Research, Irvine, CA, USA). Several studies have shown that transgene inserts (including *CgGH*) can be found along the head to tail area in transgenic rainbow trout, *Oncorhynchus mykiss* (containing sPRL-lacZ) and coho salmon, *Oncorhynchus kisutch* transgenic (insert On-MTGH1) (Uh et al., 2006; Uzbekova et al., 2003) and is stably inherited in offspring. RT-PCR *CgGH* was carried out using My Taq OneStep RT-PCR (Bioline, London, UK) with the program: 48℃ (20 min) ; 40 cycles of 95℃ (1 min), 95℃ denaturation (10 sec), 60℃ (30 sec), and 72℃ (30 sec); and 72℃ (5 min).

Effect of temperature on testosterone levels and spermatozoa density in G5 fish

The role of temperature during maturation of male broodstock for seed production in spawning activities can be represented by the density of sperm numbers rearing broodstock at the appropriate temperature. Induction of optimum temperature leads to an increase in GH which works together with steroid hormones (T) which is involved in sperm cell proliferation (Schulz et al., 2012). The effect of temperature on transgenic fish can be represented by measuring sperm density and measuring T levels in the blood serum of test fish.

Counting the number of Spz in G5 fish (transgenic and nontransgenic) used a Neubaurer type haemacytometer (GrayMed, Victoria, Australia). A total of 1 μL of fish semen was diluted with 200 μL of 0.9% NaCl solution and then transferred with a micropipette into the haemacytometer counting chamber under a microscope. The multiplication factor for calculating Spz density is 32,000 which is obtained from 16 (the number of small boxes in the haemacytometer counting chamber) × 10 (0.1 mm haemacytometer slit thickness) × 200 (dilution with 0.9% NaCl) (Buwono et al., 2019). Blood sampling was carried out

after the fish were anesthetized with 2-phenoxyethanol, and approximately 1.0 mL of blood was taken. from the tail artery using a 2 mL heparinized syringe. Blood samples were centrifuged at 664×g at room temperature for 20 min. Serum was stored at –20℃ until hormone levels were analyzed. T levels in blood serum were measured using the ELISA method (Fish Estradiol ELISA kit, Bioassay Technology Laboratory, Shanghai, China), following the manufacturer's protocol.

Real-time PCR analysis of the *CgGH* gene and histology of G5 fish sperm

CgGH gene expression in G5 fish (transgenic and non-transgenic) after temperature treatment was carried out to analyze the effect of temperature on GH-transgenesis, density and histological development of fish Spz. RNA samples taken from brain tissue from two male G5 fish were taken and pooled for replicates of each transgenic fish treatment and 6 brain tissue samples were pooled for each nontransgenic fish treatment. Total RNA isolation using Quick-RNA miniprep plus kit (Zymo Research). The ReverTra Ace qPCR RT Master Mix kit with gDNARemover (TOYOBO, Osaka, Japan) was used for cDNA synthesis (as a template in rt-qPCR). *CgGH* gene expression analysis used an Agilent AriaMX Real-time PCR machine (Agilent Technologies, Santa Clara, CA, USA) with the reaction formula: 2× SensiFAST SYBR® NO-ROX (Bioline), with a final concentration of 100 ng/µL cDNA; and primers q*CgGH*-F and q*CgGH*-R to copy the *CgGH* fragment (Table 1). As an internal control for expression, primers CgβAct-Fw and CgβAct-Rv were used to copy the *Clarias gariepinus* β-actin gene and to normalize expression levels (Raghuveer & Senthilkumaran, 2010). The rt-qPCR reaction mixture and thermal profiles were prepared by quantification using 3 µL of cDNA sample in a final reaction volume of 20 µL containing 10 µL 2× SensiFast SYBR Lo-ROX Mix, 400 nmol of each primer, and 5.4 µL ddH2O. Real-time PCR program settings for reaction mixture: 95℃ (120 s), 40 cycles of amplification phase (5 s at 95℃, 30 s at 56℃, and 20 s at 72℃), and melting program (30 seconds at 95℃,

30 seconds at 60℃, and 30 seconds at 95℃). *CgGH* mRNA expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method after normalization with the *C. gariepinus* β-actin gene (Pfaffl, 2001; Schmittgen & Livak, 2001).

The number of G5 fish testes for Spz histology of transgenic and nontransgenic fish was six samples (3 samples of transgenic fish and 3 samples of nontransgenic) representing each temperature treatment of 24℃, 28℃, and 32℃. After taking the test fish's testicles, the sample was sliced 3–5 mm thick using a microtome with an area of 1 cm3 and soaked in Bouin's solution. Furthermore, serial washing with alcohol was carried out with concentrations of 70%, 80%, 90%, 96% and absolute alcohol. The next stage was clearing work using Toluol solution and continued with the infiltration process in an oven (55℃) and making paraffin blocks using paraffinum solidum. The paraffin block is then cut thinly and attached to a glass object and dripped with Mayer's albumin until a thin film is formed. The final stage is staining by dipping the glass object containing the specimen into Xylol I, II, III solutions for 5 min in a row or using hematoxylin-eosin. Histological photo documentation of G5 fish sperm using a Trinocular CX31 Microscope (Olympus, Tokyo, Japan).

Results

CgGH (600 bp) insert in G5 transgenic catfish

Identification of transgenic G5 catfish (containing *CgGH*) using PCR and primers GH-F and GH-R resulted in 18 potential transgenic male catfish parents (Fig. 1).

The PCR test showed that the *CgGH* gene insert was contained in the transgenic fish (Fig. 1) and the resulting amplicon was 600 bp in size, indicating that the GH-F and GH-R primers could copy *CgGH* in G5 fish samples. Previous research results on transgenic mutiara catfish G1, G2, G3, G4 showed that the size of the GH amplicon in four generations was 600 bp (Buwono et al., 2021, 2023, 2024). Consistency in the size of the *CgGH* insert was also shown in male G5 transgenic mutiara catfish of

Table 1. Primers used for screening of G5 transgenic mutiara male catfish

Primers	Sequence (5' → 3')	Amplicons (bp)	References
q*CgGH*-F	GTCTGATCGAGTCATGGGAGTT	200	Designed from genebank acc no MN249238.1 using Primer3
q*CgGH*-R	CTCAGGGTCTGGTAGAAA TCCTC		
CgβAct-Fw	ACCGGAGTCCATCACAATACCAGT	200	Raghuveer & Senthilkumaran (2010)
CgβAct-Rv	GAGCTGCGTGTTGCCCCTGAC		
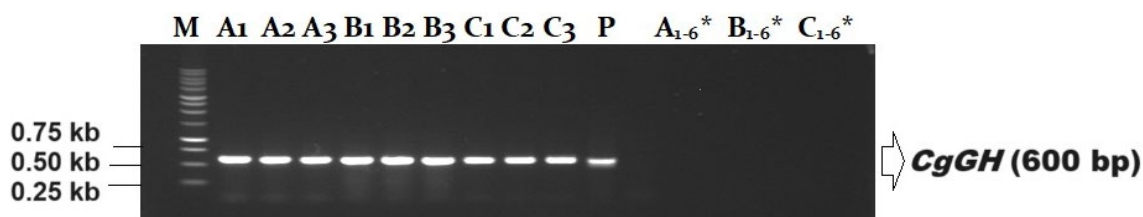
Fig. 1. Electropherogram of *CgGH* gene insert of G5 transgenic mutiara catfsh. M, 1 kb DNA ladder; P, pCMV-*CgGH*; A1-C3, transgenic fish sample number (one well pool of two different fish fins); A1-6*-C1-6*, nontransgenic fish sample number (one well pool of six different fish fins).

600 bp (Fig. 1).

Testosterone (T) levels and spermatozoa density in G5 fish

Low temperature (24℃) caused an increase in serum T levels in G5 transgenic mutiara catfish which was significantly higher than the temperature of 28℃ and 32℃ among transgenic fish, whereas the temperature of 28℃ in nontransgenic fish was higher than the temperature of 24℃ and 32℃ (Fig. 2A). Significant differences in T levels occurred at temperatures of 24℃ and 32℃ and temperatures of 28℃ and 32℃ in nontransgenic fish, and were consistently followed by Spz densities in nontransgenic G5 catfish that were significantly different between temperatures of 24℃ and 32℃ and at temperatures of 28℃ and 32℃ (Fig. 2B). There was no difference in Spz density in nontransgenic fish between temperatures of 28℃ and 32℃. Meanwhile, among G5 transgenic catfish, the density of Spz at 24℃ was higher than at 28℃ and 32℃ and in the three temperature treatments there was a significant difference in Spz density and was consistent with the results of the measured T levels (Fig. 2A and 2B).

CgGH expression levels and spermatozoa profiles of G5 fish

High temperature (32℃) caused an inhibitory effect on *CgGH* expression which led to a decrease in the number of Spz and the spermatogenesis process in G5 fish (Fig. 3A and 3B). In contrast, the optimum temperature (24℃) caused a higher increase in *CgGH* expression levels than 28℃ and 32℃ among G5 transgenic catfish (Fig. 3A and 3C) and was consistent with the T levels and Spz density produced (Fig. 2A and 2B). This indication shows that both the increase in GH expression, T levels and Spz density in transgenic and non-transgenic G5 catfish were influenced by the temperature of 24℃.

In line with the expression of GH (including *CgGH*), there was an indication that relatively low temperature (24℃) in-creased the number of Spz (Fig. 3B) compared to high temperature (32℃) and 28℃. These results indicate that high temperature causes suppression of *CgGH* expression levels and leads to a decrease in the number of Spz (Fig. 3A and 3B).

Discussion

Detection of *CgGH* inserts in G5 transgenic mutiara male catfish

Identification of G5 transgenic mutiara catfish male broodstock using PCR testing showed 18 transgenic male fish (containing *CgGH*, 600 bp) and 18 nontransgenic fish (negative *CgGH*) to be used as test fish in the temperature difference treatment. Previous research results showed that *CgGH* transmission in G5 transgenic catfish had reached 89% (Buwono et al., 2024), indicating stable transgene inheritance in each generation. Stability of transgene inheritance is required for the production of transgenic fish offspring, as has been observed in mud loach (*Misgurnus mizolepis*) transgenic fish (carrying CMV-H2B-GFP) to produce 50% homozygous G3 progeny (Nam et al., 2000). The presence of the *CgGH* gene in G5 transgenic male catfish broodstock needs to be evaluated to test the effect of temperature differences on GH-transgenesis. Apart from that, it is necessary to compare nontransgenic catfish in testing T levels and sperm density to determine the optimum temperature that increases *CgGH* expression and leads to an increase in T levels and sperm density in G5 fish. The results of research on male transgenic G1 mutiara catfish at a rearing temperature of 26℃ indicated that the T levels and sperm density of transgenic G1 catfish increased 1.78 times (11.25/6.32 ng/mL) with a sperm density of 1.57 times (8.60 / 5.46 × 10⁶ cells/mL semen) from nontransgenic fish (Buwono et al., 2019). This indication shows that GH-transgenesis has the potential to increase T levels and fish sperm density when induced by optimum temperature.
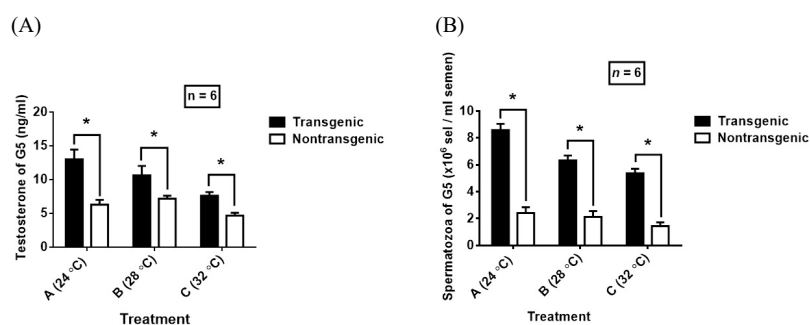
Fig. 2. Effect of temperature on testosterone and spermatozoa density. Testosterone levels of transgenic and non-transgenic G5 catfish at different temperatures (A). Density of G5 fish spermatozoa (transgenic and nontransgenic) (B) at different temperatures. Mean ± SEM values followed by an asterisk indicate significance (*$p < 0.05$).
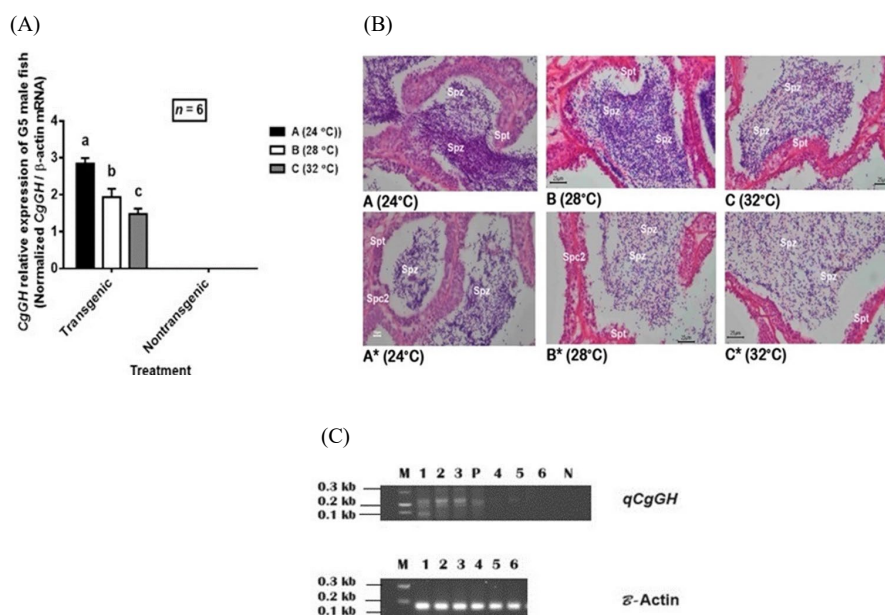
Fig. 3. *CgGH* expression levels and spermatozoa profiles at different temperatures. *CgGH* expression levels of G5 transgenic catfish (A). Histology of G5 fish spermatozoa (A, B, C transgenic and A*, B*, C* nontransgenic) at different temperatures (B). Electropherogram of qCgGH of G5 (200 bp, above) and β-actin of G5 transgenic mutiara catfsh (200 bp, below) (C). M, 100 bp DNA ladder; P, pCMV-CgGH; No. 1–3, fish sample number (one well pool of six different fish fins in transgenic catfish); No. 4–6, fish sample number (one well pool of six different fish fins in non-transgenic catfish); N, a sample PCR product without template. Mean values ± SD followed by different letter notations indicate significance ($p < 0.05$). The scale line represents 25 μm.

The addition of foreign gene inserts (*CgGH*) has biological benefits related to increasing T levels and sperm count, leading to improvements in the quantity of sperm production in male fish and has implications for improving the reproductive quality of male fish. The results of research on catfish, *Clarias batrachus*, show that the GH receptor (GHR) found in Leydig cells plays a role in binding GH which is important in regulating steroidogenesis. GH stimulates the growth factor IGF-1 (insulin like growth factor-1) of Leydig cells. Next, Leydig cells are activated to secrete T which maintains the spermatogenesis process of spermatocyte cells until Spz are formed (nee Pathak et al., 2015). This explanation shows that GH (including *CgGH*) has a positive effect on improving the reproductive performance of male fish and the effect of GH-transgenesis in transgenic fish is

more beneficial than in non-transgenic fish. Transgenesis makes it possible to introduce beneficial traits or phenotypes that cannot be realized by conventional selective breeding (Hallerman et al., 2007).

Correlation of temperature with testosterone levels and sperm density

Among exogenous cues, water temperature has a major influence on gametogenesis of fish, especially tropical fish species and has implications for the spawning of brood fish during the reproductive season. In addition, research has shown that higher water temperatures cause inhibition of spermatogenesis, considering that the regulation of spermatogenesis stages is controlled by steroids (T), the production of which depends on the activity of enzymes involved in steroid production and whose activity is influenced by temperature. Maximum T production is obtained when the temperature is in the range of 22℃–30℃ in *Sarotherodon mossambicus* Tilapia fish (Kime & Hyder, 1983). Like the results shown in Tilapia fish, in transgenic G5 mutiara catfish, temperature treatment determined the T levels produced over 60 days (Fig. 2A), indicating that a temperature of 24℃ increased T levels higher than temperatures of 28℃ and 30℃. in transgenic fish and nontransgenic fish. The optimum temperature (24℃) has a higher effect on T levels in G5 transgenic catfish than other treatments, related to the temperature range that induces enzyme activity involved in converting the precursor 17,20β-dihydroxypregn-4-en-3-one into T such as which was studied in male fish (Scott et al., 2010). On the other hand, high temperatures (32℃) had the effect of reducing T levels, both shown in transgenic and nontransgenic G5 mutiara catfish. These results confirms that the effect of temperature which produces maximum T levels in Tilapia fish was in the range of 22℃–30℃, while temperatures above 30℃ reduce T levels as shown in test fish. Besides that, it can be shown that the use of GH-transgenesis in G5 transgenic catfish provides higher average T levels compared to nontransgenic fish in all temperature treatments. This indication explains that exogenous GH (insertion *CgGH*) plays a role in increasing Leydig cell activity in T production, where the effect is enhanced higher at 24℃ than other temperature treatments. This indication has been shown in research on the fish *C. batrachus*, that GH is a mediator for Leydig cells for T production (nee Pathak et al., 2015). Previous studies in tilapia showed that increasing GH levels increased Leydig cell activity for T production (Tokalov & Gutzeit, 2005; Yamaguchi et al., 2006).

A decrease in T levels of 6 month old African catfish (*C. gariepinus*) to 0.73 ng/mL was found at a temperature of 34℃ (Okoye et al., 2018). Confirmation of these results was also shown by the effect of high temperature treatment (32℃) causing a decrease in T levels in both transgenic G5 catfish (average 7.60 ng/mL) and nontransgenic (average 4.66 ng/mL), indicating a negative effect of temperature. high levels reduced the T levels of test fish (Fig. 2A). As explained previously, it shows that androgen (T) production is regulated by enzymes involved in T production (11-ketotestosterone, 11 KT and DHP, 17α,20β-dihydroxy-4-pregnen-3-one) and maximum work activity. depending on the optimum temperature (Kime & Hyder, 1983; Quirino et al., 2021; Schulz et al., 2010). The results showed that the optimum temperature of 24℃ increased the T levels of G5 transgenic mutiara catfish (average 12.99 ng/mL) higher than other temperature treatments between transgenic and nontransgenic fish, but in non-transgenic fish, the temperature of 28℃ increased the T levels. (average 7.17 ng/mL) was higher than the temperature treatment among nontransgenic fish. This difference is likely due to the activity of enzymes involved in the production of maximum T levels in nontransgenic fish found at a temperature of 28℃.

The density of Spz produced by male fish determines the number of larvae produced, because the number of sperm produced contributes to the success of fish spawning (Miura et al., 2011). The process of Spz formation cannot be separated from the induction of the steroid T which maintains spermatogenesis. The correlation between T levels and Spz density could indicate that steroid hormones play a role in spermatocyte cell proliferation and this correlation is influenced by water temperature during the maturation of male broodstock (Miura et al., 2011; Yamaguchi et al., 2006). The results of research on transgenic G5 mutiara catfish showed that a temperature of 24℃ increased Spz density higher than temperatures of 28℃ and 32℃ between transgenic fish and nontransgenic fish (Fig. 2B). These results indicate that the optimum temperature affects transgenesis-GH in G5 transgenic male parents induces an increase in T levels, and GH works together with the steroid hormone T to activate testicular tissue to stimulate the production of male gametes (sperm) which are needed for fish spawning activities (Maestro et al., 1997; Miura et al., 2011). A temperature of 24℃ increased the number of Spz in G5 transgenic catfish higher (an average of 8.64×10^6 cells/mL semen) than other treatments, which is the optimum temperature that maximizes the effect of exogenous GH (*CgGH*) to stimulate sperm cell proliferation as

compensation. Spz density of G5 transgenic fish (Fig. 2A and 2B). In contrast, in nontransgenic catfish, at both temperatures of 24℃ and 28℃, the Spz density was lower (2.42×10^6 cells/mL semen and 2.13×10^6 cells/mL semen) than in transgenic catfish, indicating that the presence of *CgGH* inserts in the fish increased the density. Spz. This temperature-influenced GH activity effect in sperm production and T steroids was similarly demonstrated in studies of *Fundulus heteroclitus* fish and zebrafish (*Danio rerio*) (Negatu et al., 1998; Nóbrega et al., 2015), explaining the role of temperature in T production and male fish sperm production.

CgGH expression and spermatozoa histology

Over-expression of GH in male transgenic G1 mutiara catfish mediated by IGF-1 causes an increase in the number of Spz due to the influence of exogenous GH (*CgGH*) inducing sperm cell proliferation (Buwono et al., 2019). The GHR located in Leydig cells suggests a potential function of GH in the gonads of male fish (Yamaguchi et al., 2006). The interaction of GH with T steroids was also demonstrated in the testes of catfish, *C. batrachus* increasing T levels produced by Leydig cells. The steroidogenic action of GH on testicular tissue causes an increase in sperm cell proliferation (nee Pathak et al., 2015). *CgGH* expression levels in G5 transgenic catfish showed that high expression was found at 24℃ treatment among transgenic fish, consistent with the temperature effect shown by T levels and Spz density of transgenic fish (Fig. 2A and 2B). This confirmation is closely related to the optimum enzyme activity at 24℃, as studied in male tilapia (Baroiller et al., 2014), increasing T production and Spz density. Previous research on G1 transgenic male mutiara catfish at a temperature of 26℃ (a relatively close temperature), showed that increasing *CgGH* expression caused G1 transgenic T levels to be higher than in G1 nontransgenic fish (Buwono et al., 2019). Exogenous GH expression in male transgenic catfish caused *CgGH* expression levels in the testes of transgenic fish to be higher than in non-transgenic fish and T production in transgenic males was higher than in non-transgenic fish. This consistency was shown from the results of research on G5 transgenic catfish, that the expression level of *CgGH* at 24℃ (average 2.85 times higher) than at temperatures 28℃ (average 1.93 times) and 32℃ (average 1.48 times) than nontransgenic fish (Fig. 3A), explaining that GH-transgenesis in transgenic fish is more beneficial for increasing the number of Spz and T levels compared to nontransgenic fish. These results confirms previous studies that optimum temperature regulation induces

an increase in exogenous GH levels which leads to increased GHR expression in tilapia Leydig cells and T production also increases (Tokalov & Gutzeit, 2005; Yamaguchi et al., 2006).

The microanatomy of G5 fish Spz shown in Fig. 3B explains that in all temperature treatments in G5 transgenic catfish, the number of Spz formed was higher than in nontransgenic fish, indicated by the density of the number of Spz that filled the lumen of the seminiferous tubules. The multiplication of Spz shown by the histology results of testicular tissue of test fish at 24℃ (Fig. 3B) was higher than at other temperature treatments and nontransgenic fish, consistent with the results of Spz density measurements (Fig. 2B). Likewise, the number of Spz at 28℃ in transgenic fish was greater than at 32℃ and nontransgenic fish. Research on catfish, *Lophiosilurus alexandri*, shows that a temperature of 23℃ increases the number of sperm cells compared to a temperature of 27℃ with the proportion of Spz dominating the seminiferous tubules (Melo et al., 2022). The sperm development stage in the test fish moreover showed an abundance of Spz at a temperature of 24℃, followed by temperatures of 28℃ and 32℃. The density of Spz of transgenic fish was higher than that of nontransgenic fish as shown by the abundance of Spz in Fig. 3B, indicating that high temperature (32℃) reduced T levels and as a consequence suppressed the number of spermatocytes that developed into Spz. Histologically, it was shown that in nontransgenic fish (not containing *CgGH*), the development of secondary spermatocytes was still found at 24℃ and 28℃ treatments, indicating that the effect of GH-transgenesis in transgenic fish stimulated faster spermatocyte development in both treatments. temperature compared to nontransgenic fish (Fig. 3B). The 24℃ temperature treatment had a positive impact on the development of spermatocytes into Spz in G5 transgenic fish in abundance, as indicated by the dominance of Spz development which filled the entire lumen space of the seminoferous tubules. These results, indicate that GH-transgenesis increases the number of Spz and increases the formation of Spz.

Increasing the temperature above 30℃ causes inhibition of androgen formation in the biosynthesis of T which is shown in the testes of *S. mossambicus* (Kime & Hyder, 1983). In line with the effect of a temperature of 32℃ on the testes of G5 transgenic mutiara catfish, it caused a decrease in T levels and as a result the number of Spz decreased (Fig. 2A and 2B). The results of the analysis of calculating the proliferation of tilapia testicular cells (*Oreochromis niloticus*) at a temperature of 35℃, showed a decrease in the proliferation index of somatic testicular cells

(both Leydig cells and Sertoli cells) leading to a decrease in sperm count compared to a temperature of 30℃. Based on this study, it can be explained that the decrease in sperm count treated at 32℃ in this study is similar to the discussion at 35℃ in *O. niloticus* (de Alvarenga & França, 2009).

The decrease in GH expression levels (including *CgGH*) in transgenic catfish at 32℃ (Fig. 3A) followed by a decrease in T levels (Fig. 2A) indicated the inhibitory effect of GH at the pituitary level. Regulation of GH secretion by this T negative feedback mechanism was also demonstrated in trout testes when spermatogenesis had reached the final stage of spermatid formation (Le Gac et al., 1992). This confirmation shows that the high temperature (32℃) in this study caused an inhibitory effect on T secretion (T levels decreased) and T steroids provided negative feedback to the pituitary which suppressed *CgGH* expression and as an acceleration the number of Spz also decreased.

Conclusion

Increases in *CgGH* expression, T levels and Spz density in G5 transgenic mutiara catfish were optimally induced by a temperature of 24℃. GH-transgenesis in G5 transgenic catfish increased the number of Spz formation when induced at 24℃.

Competing interests
No potential conflict of interest relevant to this article was reported.

Availability of data and materials
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate
Research procedures was approved by the Ethical Commission of Universitas Sebelas Maret (UNS) (Number: 282/UN27.14/TU.00/2024).

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References

Baroiller JF, D'cotta H, Shved N, Berishvili G, Toguyeni A, Fostier A, et al. Oestrogen and insulin-like growth factors during the reproduction and growth of the tilapia *Oreochromis niloticus* and their interactions. Gen Comp Endocrinol. 2014;205:142-50.

Buwono ID, Iskandar I, Grandiosa R. *CgGH* and IGF-1 expression level and growth response of G4 transgenic mutiara strain catfish (*Clarias gariepinus*) reared at different stocking densities. Aquac Int. 2023;31:827-46.

Buwono ID, Iskandar I, Grandiosa R. Growth hormone transgenesis and feed composition influence growth and protein and amino acid content in transgenic G_3 mutiara catfish (*Clarias gariepinus*). Aquac Int. 2021;29:431-51.

Buwono ID, Junianto J, Iskandar I, Alimuddin A. Reproduction performance of transgenic mutiara catfish (G_1) comprising the growth hormone gene. J Biotech Res. 2019;10:199-212.

Buwono ID, Grandiosa R, Mulyani Y, Pratiwy FM. GnRHr, LHr, and Vg gene expression levels and ovarian development of G5 transgenic mutiara female catfish (*Clarias gariepinus*) after exposure photoperiod induction. Discover Appl Sci. 2024;6:63.

de Alvarenga ÉR, França LR. Effects of different temperatures on testis structure and function, with emphasis on somatic cells, in sexually mature Nile tilapias (*Oreochromis niloticus*). Biol Reprod. 2009;80:537-44.

Hallerman EM, McLean E, Fleming IA. Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: a review identifying research needs. Appl Anim Behav Sci. 2007;104:265-94.

Harvey BJ, Hoar WS. The theory and practise of induced breeding in fish. Ottawa, ON: International Development Research Centure [IDRC]; 1979.

Kime DE, Hyder M. The effect of temperature and gonadotropin on testicular steroidogenesis in Sarotherodon (Tilapia) *mossambicus in vitro*. Gen Comp Endocrinol. 1983;50:105-15.

Le Gac F, Ollitrault M, Loir M, Le Bail PY. Evidence for binding and action of growth hormone in trout testis. Biol Reprod. 1992;46:949-57.

Maestro MA, Planas JV, Moriyama S. Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. Gen Comp Endocrinol. 1997;106:189-201.

Mañanós E, Duncan N, Mylonas C. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In: Cabrita E, Herraez P, Robles V, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton, FL: CRC Press; 2008. pp. 3-66.

Melo RMC, Cruz CKF, Weber AA, Luz RK, Bazzoli N, Rizzo E. Effects of temperature manipulation on gamete development and reproductive activity in the farmed catfish *Lophiosilurus alexandri*. Anim Reprod Sci. 2022;247:107100.

Miura C, Shimizu Y, Uehara M, Ozaki Y, Young G, Miura T. Gh is produced by the testis of Japanese eel and stimulates proliferation of spermatogonia. Reprod Res. 2011;142:869-77.

Nam YK, Cho YS, Chang YJ, Jo JY, Kim DS. Generation of transgenic homozygous line carrying the CAT gene in mud loach *Misgurnus mizolepis*. Fish Sci. 2000;66:58-62.

nee Pathak ND, Kumar P, Lal B. Endocrine regulation of testosterone production by Leydig cells in the catfish, *Clarias batrachus*: probable mediators of growth hormone. Anim Reprod Sci. 2015;154:158-65.

Negatu Z, Hsiao SM, Wallace RA. Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. Fish Physiol Biochem. 1998;19:13-21.

Nóbrega RH, Morais RDVS, Crespo D, Waal PP, França LR, Schulz RW, et al. Fsh stimulates spermatogonial proliferation and differentiation in zebrafish via Igf3. Endocrinology. 2015;156:3804-17.

Okoye CN, Udoumoh AF, Igwebuike UM, Okereke CT. Ovarian morphology and development of 3 to 8 months old African catfish, *Clarias gariepinus*. Comp Clin Pathol. 2018;27:887-91.

Peñaranda DS, Morini M, Tveiten H, Vílchez MC, Gallego V, Dirks RP, et al. Temperature modulates testis steroidogenesis in European eel. Comp Biochem Physiol A Mol Integr Physiol. 2016;197:58-67.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 2001;29:2002-7.

Quirino PP, da Silva Rodrigues M, da Silva Cabral EM, de Siqueira-Silva DH, Mori RH, Butzge AJ, et al. The influence of increased water temperature on the duration of spermatogenesis in a neotropical fish, *Astyanax altiparanae* (*Characiformes*, Characidae). Fish Physiol Biochem. 2021;47:747-55.

Raghuveer K, Senthilkumaran B. Cloning and differential expression pattern of *vasa* in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comp Biochem Physiol A Mol Integr Physiol. 2010;157:79-85.

Schmittgen T, Livak K. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2001;3:1101-8.

Schulz RW, de França LR, Lareyre JJ, LeGac F, Chiarini-Garcia H, Nobrega RH, et al. Spermatogenesis in fish. Gen Comp Endocrinol. 2010;165:390-411.

Schulz RW, Dijk W, Chaves-Pozo E, García-López Á, França LR, Bogerd J. Sertoli cell proliferation in the adult testis is induced by unilateral gonadectomy in African catfish. Gen Comp Endocrinol. 2012;177:160-7.

Scott AP, Sumpter JP, Stacey N. The role of the maturation-inducing steroid, 17,20β-dihydroxypregn-4-en-3-one, in male fishes: a review. J Fish Biol. 2010;76:183-224.

Sundararaj BI. Reproductive physiology of teleost fishes: a review of present knowledge and needs for future research. Rome: Food and Agriculture Organization of the United Nations [FAO]; 1981.

Taranger GL, Carrillo M, Schulz RW, Fontaine P, Zanuy S, Felip A, et al. Control of puberty in farmed fish. Gen Comp Endocrinol. 2010;165:483-515.

Tokalov SV, Gutzeit HO. Spermatogenesis in testis primary cell cultures of the tilapia (*Oreochromis niloticus*). Dev Dyn. 2005;233:1238-47.

Uh M, Khattra J, Devlin RH. Transgene constructs in coho salmon (*Oncorhynchus kisutch*) are repeated in a head-to-tail fashion and can be integrated adjacent to horizontally-transmitted parasite DNA. Trans Res. 2006;15:711-27.

Uzbekova S, Amoros C, Cauty C, Mambrini M, Perrot E, Hew CL, et al. Analysis of cell-specificity and variegation of transgene expression driven by salmon prolactin promoter in stable lines of transgenic rainbow trout. Trans Res. 2003;12:213-27.

Yamaguchi S, Gen K, Okuzawa K, Matsuyama M, Kagawa H. Influence of estradiol-17β, testosterone, and 11-ketotestosterone on testicular development, serum steroid hormone, and gonadotropin secretion in male red sea bream *Pagrus*

major. Fish Sci. 2006;72:835-45.

Zhang MQ, Chen CX, Guo YJ, Guo J, Wang XM. Cloning and sequence analysis of full-length growth hormone cDNA from *Clarias gariepinus*. ACTA Agric Bor Sin. 2009;24:27-32.