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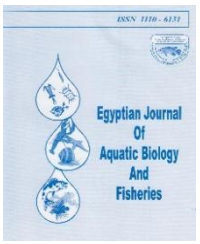

Gonad-stimulating potential of laserpuncture induction on **the** male African sharptooth catfish (*Clarias gariepinus*)

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ABSTRACT

The African sharptooth catfish (*Clarias gariepinus*) has received widespread attention due to increase of global demand. However, availability of *C. gariepinus* breeding depends on the season, especially during the spawning season. The objective of this study was to investigate the effect of soft laserpuncture induction on testosterone level, Androgen Binding Protein (ABP) level, and numbers of Sertoli and Leydig cells of male *C. gariepinus*. An approximately one-year old virgin male fish was selected based on the healthiness and the body weight of 1,000–1,200 g. Fishes were treated by laserpuncture induction every 15 days from 0 to 75 days. The food containing 38% crude protein was used for feeding procedure. This study adopted a completely randomized design. The parameters observed were the levels of testosterone, ABP, and numbers of Sertoli and Leydig cells measured at 0, 15, 30, 45, 60, and 75 days after treatment. The Sertoli and Leydig cell determinations were analyzed descriptively, whereas the testosterone and ABP levels were evaluated using analysis of variance. The results indicated that induction of laserpuncture improves male catfish reproductive ability through enhancement of the testosterone level, ABP level, and numbers of Sertoli and Leydig cells. The testosterone level, ABP level, and numbers of Sertoli and Leydig cells increased and peaked on the30th and 60th days, indicating a cycle of higher spermatozoa quality every 30 days.

INTRODUCTION

The African sharptooth catfish (*Clarias gariepinus)* has been one of the most widely cultivated freshwater fish, especially in the tropics (Prokešová *et al.*, 2017; Das Neves *et al.* 2019), such as in Indonesia (Fisheries Research Institute of Indonesia, 2014). Increasing of market demand has prompted the breeders to improve their production (Mukti *et al.*, 2020). The production of *C. gariepinus* in Indonesia increased from 841,750 tons to 1.81 million tons (114.82%) from 2017 to 2018 (Marine & Fisheries

Ministry, 2018). The catfish species are widely cultivated for the provision of animal protein-based feed (Jooste *et al.*, 2015; Kusuma *et al.*, 2015; Fisheries Research Institute of Indonesia, 2018). This fish species has a high growth rate and adaptable to environmental factors, such as temperature and oxygen changes. However, this fish production remains hampered by limited breeding occasion because it depends on the season, especially during the spawning season. In its natural habitat, the fish reproduces during the rainy season from October to April (Zairin, 2000; Mukti *et al.*, 2020). Meanwhile, during July to August, *C. gariepinus* spawning pauses due to low temperature. Fish breeders in the Pare Kediri area, Indonesia, typically overcome this situation by installing *paranet* for covering ponds and applying the lamp to increase the water temperature (Hariani *et al*., 2010).

Efforts to overcome breeding problems are generally carried out by increasing the quality of the ova. In addition, high quality of spermatozoa is also required to increase the success of artificial fertilization (Mansour *et al.*, 2005; Fauvel *et al.* 2010; Locatello *et al.*, 2018). The productive period of spawning in male *C. gariepinus* starts at the age of 12 months with 2,025 ± 0.025 g body weight (Nwabuisi *et al.*, 2018). In general, after the first spawning, there is degradation in sperm quality, such as their viability and motility (Mukti *et al.*, 2020). To maintain sperm quality, farmers frequently to provide high quality of food. The high food quality is one of the main factors for improving gonad development and the spermatozoa quality indicated by volume, motility, and quantity of spermatozoa, percentage of fertilization, and hatchability of eggs (Chowdhury & Joy, 2001; Rurawanga *et al.*, 2004).

Many studies have been conducted to maintain sperm quality without increasing food quality, such as applying laserpuncture. This method has been widely studied to increase gonad maturity since 2015s (Abies *et al.*, 2015; Kusuma *et al.*, 2015). The technology has received great attention because of its ability to improve the reproductive quality of *C. gariepinus* through acceleration of growth, development and maturation gonads, as well as increasing the production of gonadotropins and steroids in male and female broodstocks (Kusuma, 2013; Hariani & Kusuma, 2019; Hariani *et al.*, 2020). Laserpuncture specifications from helium-neon soft lasers are considered safe therapy for stimulating reproductive organs in catfish (Kusuma & Hariani, 2017).

Changes in environmental factors, such as temperature and photoperiod simultaneously with internal signals stimulate the central nervous system to induce gonad maturation processes. The hypothalamus gland secretes gonadotropin releasing hormone (GnRH), which stimulates the release of gonadotropin hormones (GtHs) from the pituitary gland. In *C. gariepinus* broodstock, the hypothalamic–pituitary–gonad axis primarily manages the reproduction activity. The GnRH is a key player for reproductive activity (Chaube *et al.*, 2015; Golan *et al.*, 2015; Honji *et al.*, 2019). When *C. gariepinus* broodstock spawn, the gonads are influenced by the hypothalamus, as it synthesizes and liberates the GnRH, stimulates the anterior pituitary to produce the gonadotropin

hormone; gonadotropins stimulate steroidogenesis (Zohar *et al.*, 2010; Borella *et al.*, 2020).

A previous study found that laserpuncture induction every 7 days spurs speed-maturation of gonads and enhances the production of steroid hormones (Kusuma *et al.*, 2015; Mukti *et al.*, 2020). However, the intensive application may lead catfish to experiencing stress. The latter study reported that the effect reduces the sensitivity or blocks the activity of nerves to stimulate the hypothalamus–pituitary-anterior–gonad axis, causing inhibition in the release of the gonadotropin hormones and the gametogenesis process. Based on the elaboration above, application of specific methods to male catfish should be evaluated to obtain a high quality of spermatozoa. Therefore, the objective of this study was to investigate the effect of soft laserpuncture induction on the quality of gonads of male *C. gariepinus* that produces superior spermatozoa.

MATERIALS AND METHODS

This study was conducted in dry season from May to July at Freshwater Aquaculture Management Unit Kepanjen, Malang, Indonesia. In this study, the experimental protocols were approved by the Animal Division of Research Ethics Feasibility Commission, Department of Biologi, Universitas Negeri Surabaya.

1. Sample preparation

One-year-old male sharptooth catfish (*Clarias gariepinus*) broodstock samples were collected from Freshwater Aquaculture Management Unit Kapanjen, Malang, and East Java, Indonesia. A total of 48 catfishes (Mutiara variety) were selected based on their fitness, weight (1,000 – 1,200 g), and virginity.

2. Broodstock selection of male sharptooth catfish *(Clarias gariepinus)*

The selected male sharptooth catfish were acclimated in two cement ponds each sized 3.0 × 3.0 × 1.5 m. Each pond consisted of 24 individuals. The water level in the pond was adjusted at 70 cm, whereas temperature and photoperiodic were kept at natural levels. During the maintenance, the broodstock were fed commercial feed with 38% crude protein in the morning (8:00am) and afternoon (4:00pm); the feed was as much as 3% of their body weight. After acclimation was completed, all catfish broodstock were fasted for 24 hours. Fish samples were weighed, and four individuals were taken for further observation.

3. Laserpuncture induction male sharptooth catfish

This study used an experimental method with laserpuncture induction (as treatment groups) and non-treated fishes (as a control group) with four replicates. The treatment was conducted using soft helium-neon laserpuncture (output power of 5 mW, released

from 0.2 cm^2 laser beams, and wavelength of 632.8 nm). Applications were done on two-thirds of fish ventral part for 15 seconds. The treatments were conducted six times as follows 0, 15, 30, 45, 60,for 75 days (Hariani *et al.*, 2020). Sampling for both groups was carried out on subsequent four individuals every 15 days till the 75^{th} day.

On each sampled fish, blood was collected using an insulin injection needle in the caudal fin vein. The blood was kept in Ependorf tubes and stored at room temperature with a tilt of 45°Cfor half to an hour for separation of serum. Then, blood was centrifuged at a speed of 1,500–3,000 rpm for 10 minutes at 4°C. The supernatant (serum) was collected using a micropipette and transferred to a labeled Eppendorf tube. Serum samples were stored at −20°C. Serum was assayed using an Enzyme-linked immunosorbent assay (ELISA) kit to determine testosterone (T) (ELISA Kit cat. no. CSB-E17554Fh) and ABP levels (ELISA Kit cat. no. E0121Fi.). The levels were taken according to the manual and read by using an ELISA reader at a 450 nm wavelength (Sink *et al.*, 2008; Taghizadeh *et al.*, 2013).

4. Histology of gonadal male *C. gariepinus*

After blood collection, the fishes were dissected on the abdominal part from anal to ventral, and the gonads were taken for preparation for the histological slides. This procedure was conducted by following **McCann (2015)** procedure using a hematoxylin-eosin staining method. Assessment of the number of Sertoli cells and Leydig cells was conducted according to Baeverfjord and **Krogdahl (1996)**. The cells were observed using optical microscope with 1000 × magnification and counted based on image processing (Miconos, Yogyakarta, Indonesia).

5. Data analysis

The data were analyzed using Statistical Package for Social Science version 15.0 for Windows. The testosterone and ABP levels were statistically analyzed using analysis of variance and Duncan's multiple range tests with a confidence level of 95%. Histology of the organ; namely, the Sertoli and Leydig cells, was determined descriptively.

RESULTS

1. **The testosterone and androgen binding protein levels of male *C. gariepinus***

This study indicated that laserpuncture treatment affected the testosterone level and ABP levels in the serum of male *C. gariepinus*, as seen in Fig. (1 & 2). The laserpuncture induction affected significantly ($P < 0.05$) the levels of the testosterone and ABP. Testosterone levels in fishes treated with laserpuncture were significantly higher than those in control fishes. Male *C. gariepinus* that were induced every 15 days showed an increase in testosterone levels accompanied by ABP levels, specificaly on the 15^{th} and 30^{th} day. Furthermore, it decreased on the 45^{th} day and experienced an increase both on the60^{th} day(induced by laserpuncture) and the 75^{th} day (without laserpuncture). The decrease and increase in levels of testosterone, as well as ABP, were related to the

development of *C. gariepinus* gonads. There were two peaks in the group induced by laserpuncture and the group without laserpuncture induction. The levels that were induced by laserpuncture were higher on the 60th day, and were higher on the 75th day in control group as well. It has beens proved that laserpuncture induction can increase the production of testosterone and ABP compared to the control group, and the second peaks can be accelerated by 15 days. Besides that, the level of the testosterone hormone is higher than the level of ABP (Figs. 1 & 2).

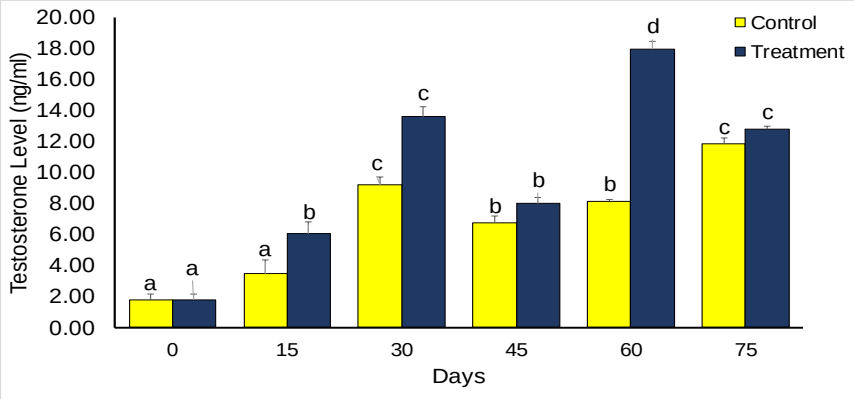

Fig. 1. Testosterone level (ng/mL) from control and laserpuncture-induced group of male sharptooth catfish. Note: Means denoted by a different letter above the error bars indicate significant differences among the treatments and days

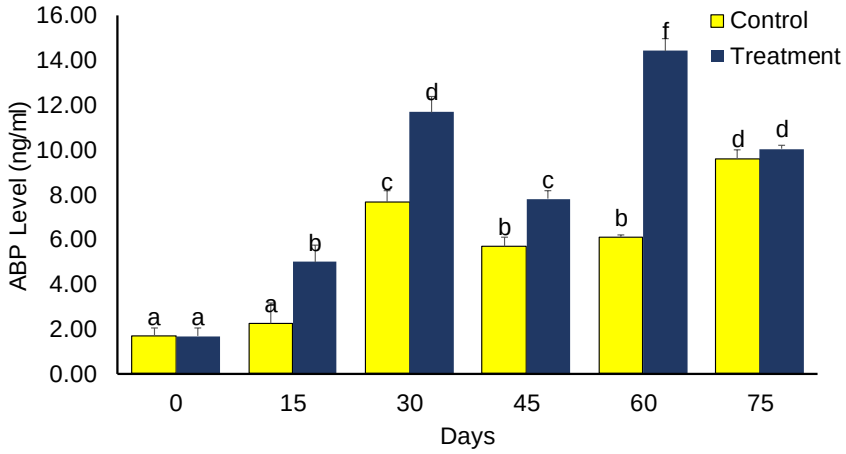

Fig. 2. Androgen Binding Protein (ABP) level (ng/mL) from control and laserpuncture-induced group of male sharptooth catfish. Note: Means denoted by a different letter above the error bars indicate significant differences among the treatments and days

Testosterone levels increased from 0 day, reached a peak on the30th day, and then decreased on the 45th day. Under treatment, those levels increased again on the60th day, whereas in the control, the increase occurred on the 75th day. The same situation occurred with ABP levels. Laserpuncture induction increased the ABP levels in the treated fishes.

The ABP levels on treatment experienced two peaks on the30th and the 60th days. Laserpuncture induction has increased the production of testosterone and ABP compared to the control group; and it can accelerate the second peaks by 15 days.

2. Numbers of Sertoli and Leydig cells in male *C. gariepinus*

Based on the gonad (testicular) histology of male *C. gariepinus*, the number of Sertoli cells was higher than Leydig cells (Table 1). The number of cells was fluctuated, and in treatment group there were two peaks onn the 30th and the 60th days, and this situation was consistent between the Sertoli and Leydig cells. Meanwhile, in control the peaks occurred in the 30th and the 75th days. The number of Sertoli cells on each observation was greater than that of Leydig cells (Fig. 3).

Table 1. The number of Sertoli and Leydig cells in the control and in laserpuncture-induced group of male C. *gariepinus*

Days	Number of Sertoli cells		Number of Leydig cells	
	Control	Laserpuncture Induction	Control	Laserpuncture Induction
0	8.0 ± 1.3	8.0 ± 1.3	7.1 ± 1.3	7.1 ± 1.3
15	15.0 ± 0.6	21.0 ± 1.7	10.0 ± 1.3	16.0 ± 2.2
30	53.0 ± 6.3	137.0 ± 3.6	45.0 ± 1.8	101.0 ± 3.1
45	21.0 ± 2.2	32.0 ± 2.5	16.0 ± 2.2	25.0 ± 1.7
60	44.0 ± 8.5	197.0 ± 16.2	39.0 ± 9.8	170.0 ± 11.4
75	74.0 ± 9.2	102.0 ± 5.9	53.0 ± 6.2	83.0 ± 10.7

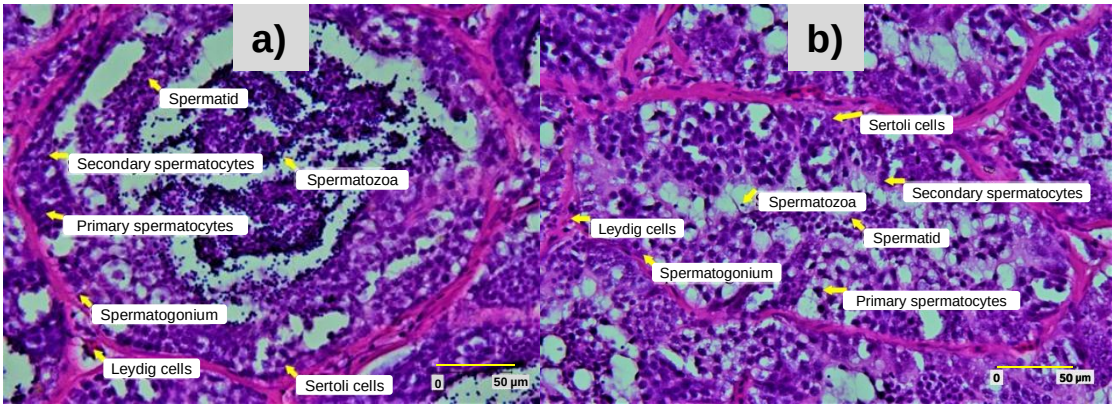

Fig. 3. Histology section showing Sertoli and Leydig cells in the treatment (a) in the control (b) on the 60th day.

DISCUSSION

The results of the present study revealed the influence of laserpuncture induction on the level of testosterone, ABP, number of Sertoli and Leydig cells. The reproductive activity of the broodstock fish is greatly influenced by the level of hormones such as the

testosterone in their blood. The fluctuation of testosterone concentration after treatment by laserpuncture induction might be related to the activity of the gonadotropin hormone in the steroidogenesis process. Laserpuncture induction can affect the hypothalamus gland to secrete gonadotropin-releasing hormone (GnRH). This hormone stimulates the release of gonadotropin hormones from the pituitary gland. Testosterone levels increased and, reaching the first peak on the 30th day, indicating the role of this hormone, and synergizing with gonadotropin hormones in gonad maturation. This maturation led the *C. gariepinus* male ready to spawn. If spawning is halt, testosterone levels would drop, and is reabsorbed, hence the testosterone concentration dropped on the45th day. Furthermore, the laserpuncture induction re-increased and reached the second peak on the 60th day. A different trend occurred in the control group because the peak was reached on the75th day. The spermatogenesis cycle in male *C. gariepinus* broodstock occurs every 30 days, after high levels of testosterone.

The induction of laserpuncture on the reproductive organ in male *C. gariepinus* has accelerated the gonad-stimulating potential to produce testosterone. This result was consistent with studies conducted by **Karu (2000)** and **Koutna (2013)**. Both studies found that, laserpuncture induction may stimulate biological organs through the proliferation of somatic cells, gonadal cells, and action of hormones. Laserpuncture induction affects the mitochondrial respiratory chain by changing the electric potential of cell membranes and improve their selective permeability for sodium, potassium and calcium ions, or by increasing the activity of certain enzymes such as cytochrome oxidase and adenosine triphosphatase. This induction also increases DNA synthesis, collagen and pro-collagen production, and may increase the cell proliferation **(Koutna, 2013)**.

Several studies highlighted that gonads can develop well-regulated hormones **(Zohar *et al.*, 2010; Ajayi *et al.*, 2018).** The biosynthesis of androgens takes place continuously in the Leydig cells, where the biosynthesis is regulated by the hypothalamus–hypofisa–testicle (gonad) axis **(Ohga *et al.*, 2018)**. The GtH-I stimulates the development and proliferation of Sertoli cells to produce ABP, which stimulates the spermatogonia to start the spermatogenesis process. The GtH-II stimulates Leydig cells to secrete the hormone testosterone. The interstitial cell stimulating hormone stimulates the development of seminiferous tubules and Sertoli cells to produce ABP, a protein responsible for sperm formation **(Kusuma *et al.*, 2012)**. The GtH-I and GtH-II play a role in spermatogenesis, which stimulates the gonads to produce steroid hormones, namely testosterone. Testosterone and ABP control the formation of sperm in the process of spermatogenesis and stimulate the initiation of spermatogenic development.

The results from both the control group and the laserpuncture-induced group indicate that levels of testosterone and ABP are affected by the condition of the gonads during the male broodstock *C. gariepinus* reproductive cycle. Testosterone hormone levels and ABP reaching the peak indicate a mature gonad condition. The high testosterone levels indicate

that the male catfish undergoes sperm maturation process, such as spermatogenesis, spermyogenesis, and spermiation. Conversely, low testosterone levels indicate that the gonads are at the initial condition, where the male *C. gariepinus* is in the proliferation phase of spermatogonia, which is the resting phase. This indicates that there was a spermatogenesis process occurring in the fish every 30 days, and the duration of treatment affects the process of spermatogenesis.

Sex hormone-binding globulins are an example of ABP. This hormone binds and transports steroids in the blood of vertebrates, except birds, and affects the bioactivity of sex steroids, which have access to the tissues **(Hammond, 2016; González *et al.*, 2017)**. Sex hormone-binding globulins in fish are produced in the liver and work on testes, kidneys, stomach, and brain **(Miguel-Queralt *et al.*, 2009)**. The concentration of this sex steroid in fish plasma has an important role in development and reproduction **(Bobe & Labbe, 2010)**.

Previously, **Weltzein *et al.* (2004)** asserted that *H. nemurus* males achieving a high level of testosterone. This was confirmed by **(Tessaro *et al.*, 2019)** and **(Chatakondi & Davis, 2011)**, who demonstrated that levels of sex steroids such as testosterone in plasma can be used as indirect indicators of fish reproductive capacity. A time of high testosterone levels indicates mature gonads. As found by **Gazola and Borella(1997)**, the significantly decreased levels of testosterone indicate a transition toward a regression phase. It is undeniable that there is a link between the fluctuation of steroid hormone levels and the reproductive process in fish, as reported by **Munakata and Kobayashi (2010)**.

This study indicates an association between the increase of testosterone and ABP levels and an increase in the numbers of Sertoli cells and Leydig cells. Additionally, increased the numbers of Sertoli cells and Leydig cells also occurred in the same day of observation (on the 30^{th} and 60^{th} days), whereas in the control group, the peak was reached on the30^{th} and 75^{th} days. Both Sertoli and Leydig cells regulate the activity of spermatogenesis **(Schulz & Nóbrega, 2011; Schulz *et al.*, 2019)**. Sertoli cells secrete ABP **(Grover *et al.*, 2004)**, whereas Leydig cells secrete testosterone **(Ahmed *et al.*, 2013)**. The ABP is responsible for transporting steroids in the blood in a teleost group of fish and influencing the bioactivity of sex steroids **(Hammond, 2016)**. The results of the study strengthened the theory that the hypothalamus–hypofisa–gonad axis regulates the activity that occurs in the process of maturation of gonads on the broodstock fish.

The existence of this GnRH to stimulate the release of GtH, such as GtH-I or FSH for the development and proliferation of the Sertoli cells, are used to produce ABP, besides the fact that GtH-I played a role in the process of spermatogenesis by taking the role of the Sertoli. Remarkably, GtH-II or LH plays a role in the maturation of gonads, and GtH-II stimulates Leydig cells for production of androgens such as testosterone **(Hammond, 2016; Ohga *et al.*, 2018; Schulz *et al.*, 2019)**. Furthermore, the GtH-II stimulates Leydig cells to produce testosterone and plays a role in regulating the activity of Sertoli cells in

the seminiferous tubules. Additionally,, testosterone with Sertoli cells regulates spermatogenesis and sperm products and stimulates the secretion of ABP **(Cheng *et al.*, 2010; Ohta *et al.*, 2007)**. This series of GtH-I and Gt-II activities can cause gonad maturation for broodstock fish such as broodstock sharptooth catfish. This result is in line with that of **(Kusuma & Hariani, 2017)** who stated that the induction of laserpunctures in broodstock sharptooth catfish can accelerate the process of maturation of gonads.

CONCLUSION

This research found that laserpuncture induction in the reproductive point of male African sharptooth catfish (*Clarias gariepinus*) accelerates the maturation of gonads. The testosterone level, ABP level, numbers of Sertoli and Leydig cells increased on the30th and 60th days, indicating peaking spermatozoa quality every 30 days.

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