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Gonad-stimulating potential of laserpuncture in male African sharptooth catfish (*Clarias gariepinus*)

Abstract:

The African sharptooth catfish (*Clarias gariepinus) telah mendapat perhatian yang luas karena peningkatan global demand. However,* availability of *C. gariepinus* breeding depend on the season, especially during the spawning season. The objective of this study is to investigate the effect of soft laserpuncture induction on testosterone, Androgen Binding Protein (ABP), the number of Sertoli and Leydig cells of male *C. gariepinus*. approximately one year old virgin Ikan dipilih berdasarkan kesehatan, dan body weight of (1000 - 1200 g). Ikan ditreatment dengan Laserpuncture induction setiap 15 hari hingga hari ke 75. The fishes were fed with 38% crude protein content food. This study adopted a completely randomized design. The parameters observed were the levels of testosterone, ABP, the number of Sertoli and Leydig cells measured at various time point intervals, strated form 0, 15, 30, 45, 60, and 75 days. The Sertoli and Leydig cell determinations were analyzed descriptively, while the testosterone and ABP levels were analyzed using analysis of variance. The results showed that induction of Laserpuncture improve male catfish reproductive ability with enhancement testosterone level, ABP, number of Sertoli and Leydig cells. The testosterone level, androgen binding protein content, number of Sertoli and Leydig cells increased and peaked on days 30 and 60 indicate cycle of spermatozoa quality every 30 days.

Keywords: Androgen Binding Protein, testosterone, Sertoli cells, Leydig cells, induction of laserpuncture, broadtock of males *Clarias gariepinus*

1. 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 *Clarias gariepinus* in Indonesia was 2017-2018 increased from 841.75 thousand tons to 1.81 million tons (114.82%) in 2017 –2018 (Marine and Fisheries Ministry, 2018). This fish species has a fast growth rate and highly adapted to environmental factors such as temperature and oxygen changes. *C. gariepinus* species are widely cultivated for the provision of animal protein-based feed (Appelbaum and Kamler, 2000; Jooste et al, 2015; Kusuma et al., 2015; Fisheries Research Institute of Indonesia, 2018). However, there is primary limitations in its production by breeders is the availability of *C. gariepinus* breeding depending 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, in peak dry season during July to August the *C. gariepinus* spawning pause, because of low temperature. The fish breeder in the Pare Kediri area, Indonesia use to overcome this constraint by covering the ponds with paranet and use 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 eva. Furthermore, high quality of spermatozoa is also needed 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* peaks at the age of 12 months with 2.025 ± 0.025 gr body weight (Nwabuisi et al., 2018). In general, after the male catfish spawned, there was a degrade in sperm quality, such as their viability and motility (Mukti et al., 2020). For that, brood stock of *C. gariepinus* need to be given quality feed. The quality of feed for C. *gariepinus* broodstock is one of the main factors that can affect gonads growth and development also affects the quality of spermatozoa such as volume, motility and quantity of spermatozoa, percentage of fertilization and

hatchability of eggs (Chowdhury and Joy,2001; Rurawanga et al.,2004). In addition, it can also used to increase the potency of *C. gariepinus* gonads.

One of the methods to overcome the decrease in spermatozoa quality is laserpuncture. This method has been widely studied to increase gonad maturity since 2015s (Abies et al, 2015; Kusuma et al. 2015). Laserpuncture specifications from Helium-Neon Soft lasers is a safe therapeutic used as a biostimulator of reproductive organs in catfish (Kusuma and Hariani, 2017). Soft lasers are safe to use if they have a wavelength range of 600-900 nm (Karu, 2000; Koutna et al., 2003). Laserpuncture technology has received great attention because it improves the reproductive quality of C gariepinus such as:accelerating growth, development and maturation gonads, also spawning, increasing the production of gonadotropins and steroids broodstocks male and female (Kusuma, 2013; Hariani and Kusuma, 2019; Hariani et al., 2020).

Changes in environmental factors like temperature and photoperiod simultaneously with internal signals stimulate the central nervous system to induce the maturation processes, the hypothalamus gland secretes GnRH, that stimulates the release of GtHs from the pituitary gland [7]. In broodstock *C. gariepinus*, the hypothalamic-pituitary-gonadal axis primarily manages reproduction activity. The hypothalamus will release hormone gonadotropin (GnRH) which stimulates the anterior pituitary gland to release follicle-stimulating hormone (FSH) or Gonadotropin Hormone-I (GtH-I) and luteinizing hormone (LH) or Gonadotropin Hormone-II (GtH-II) so that GnRH can be said as a key player for reproductive activity (Chaube et al. 2015; Golan et al, 2015; Honji and Moreira 2019). When broodstock C gariepinus spawning in mature conditions the gonads are influenced by the hypothalamus synthesizes and liberates the gonadotropin releasing hormone, stimulates the anterior pituitary to produce gonadotropin hormone and gonadotropins stimulate steroidogenesis (Zohar et al., 2010 Borella et al., 2020).

Previous study found that the effect of laserpuncture induction every 7 days can spur speed-maturation of gonads and enhance the production of steroid hormones (Kusuma et al, 2015; Mukti 2019). However, the intensive application may lead catfish experiencing stress. Latter study indicated this reduce the sensitivity or block the activity of nerves to stimulate them the hyphotalamus-pituitary anterior-gonad axis in the release of gonadotropin hormones and in the gametogenesis process. Based on the elaboration above, specific methods application to male catfish needs to be evaluated in order to obtain a good quality of spermatozoa. In this research, male sharptooth catfish broodstock with the average body weight of 1000-1200 g/fish with quality feeding 38% crude protein were used and the provision of laser induction was conducted every 15 days to reduce the stress of the broodstock. The objective of this study is to investigate the effect of soft laserpuncture induction on gonads of male *C. gariepinus* that produce superior spermatozoa.

2. 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 Research Ethics Feasibility Commission, Faculty of Dentistry, Hang Tuah University, Surabaya (Protocol Number : EC/003/KEPK-FKGUHT/VII/2020)

Sample preparation

The one years old broodstock male sharptooth catfish (*Clarias gariepinus*) samples were collected from Freshwater Aquaculture Management Unit Kapanjen, Malang, East Java, Indonesia. Forty eight male sharptooth catfish Mutiara variety were selected based on their health, weight (1.000-1.200 g), and virginity.

Broodstock selection male sharptooth catfish (*Clarias gariepinus*)

The selected male sharptooth catfish were acclimated in two cement ponds, each had sized 3.0 m x 3.0 m x 1.5 m. Each pond consisted of 24 individuals. Water level in the pond was adjusted at 70 cm, while temperature and photoperiodic keep as natural condition. During the maintenance, the broodstock were fed with 38% Crude Protein of commercial feed in the morning (8:00 AM) and afternoon (4:00 PM) as much as 3% of their body weight. After

completed the adaptation, all catfish broodstock were fasted 1 x 24 h. All the fish were weighted and collectively taken four individuals for further observation.

Laserpuncture induction male sharptooth catfish

This study used experimental method using control and laserpuncture induction groups with four replicates. The threatment was conducted by applied soft Helium-Neon laserpuncture (output power of 5mW, released from 0.2 cm^2 laser beams, and wavelength of 632.8 nm). Applications were done on two-third of fish ventral part for 15 seconds. Laserpuncture group was induced at various time point intervals, i.e., 0, 15, 30, 45, 60 and 75 day (Hariani et al., 2020). Sampling for both groups was carried out on subsequently four individuals every 15 days until the 75th day. The without laserpuncture (control group) was not treat with laserpuncture.

Enzyme-linked immunosorbent assay (Elisa) kit to determine testosterone dan Androgen Binding Protein levels male sharptooth catfish (Clarias gariepinus)

The catfish blood were collected using an insulin injection needle in the caudal fin vein. The blood was keep in an Ependorf tubes and stored at room temperature with a tilt of 45^0 for 45 - 1 hour for serum and blood cells separation. Furthermore, blood serum was centrifuged at a speed of 1500 - 3000 rpm for 10 minutes at 4ºC. The supernatant was collected using a micropipette and transfer it to the labeled Eppendorf tube. Samples were stored at -20ºC. The supernatant was assayed using Elisa kit to determine testosterone (T) (Elisa Kit Cat. No. CSB-E17554Fh) and androgen binding protein (ABP) (Elisa Kit Cat. No. E0121Fi.) levels. The levels were taken according to the manual and read using ELISA reader at 450 nm wavelength (Sink et al. 2008; (Taghizadeh et al., 2013).

Histology of gonadal male *C. gariepinus*

After blood collection, the fishes were dissected on the abdominal part from anal to ventral and take the gonads for preparation the histological method. This proced was conducted following by McCann (2015) using Hematoxylin-Eosin (HE) staining method. Assessment of the number of Sertoli cells and Leydig cells was conducted according to Baeverfjord and Krogdahl (1996). The number of Leydig and Sertoli cells was counted using IMAGE RASTER 3.0 software.

Data analysis

The data were analyzed using software Statistical Package for Social Science (SPSS) 15.0 for windows. The testosterone and androgen binding protein (ABP) levels were statistically analyzed using analysis of variant (Anova) and Duncan's multiple range test with a confidence level of 95%. Histology of the organ namely the Sertoli and Leydig cells were analyzed descriptively.

3. Results

The testosterone and androgen binding protein (ABP) levels male *C. gariepinus*

This study showed that laserpuncture treatment affected the testosterone level as well as androgen binding protein (ABP) levels in serum of male *C. gariepinus*, as seen in Table. 1. The laserpuncture induction was significantly affected on the levels of the testosterone and ABP significantly ($P < 0.05$). Testosterone levels in fish treated with laserpuncture were significantly higher than that of control fishes. Testosterone levels increased from day 0 and reached a peak on day 30, then decreased on day 45. In treatment these levels increased again on day 60, whereas in control the increase occurred until day 75. The same situation occurred with ABP levels. Laserpiuncture induction increased the ABP levels in the treated fish. ABP levels on treatment experienced two peaks on days 30 and 60. It is proven 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, here it appears that the level of the hormone testosterone is higher than the level of ABP (Table 1).

Number of Sertoli and Leydig cells male *C. gariepinus*

Based on the gonad (testicular) histology of male *C. gariepinus* in Table 2 and Fig. 1 shows that the number of Sertoli cells more than Leydig cells troughs have a similar trend on the 15th, 30th, 45th, 60th and 75th days. The trend is there is a peaking, decreasing and increasing again. For the laserpuncture-induced group, the peak was reached on days 30 and 60, while for the control group (without laserpuncture induction) the peak was reached on days 30 and 75 for the number of Sertoli cells and Leydig cells. The full results can be seen in the following Tabel 2.jumlah sel Sertoli pada ikan treatment meningkat dari hari ke 0 dan mencapai puncak pada hari ke 60 dam 75. jumlah sel Sertoli pada ikan kontrol meningkat dari hari ke 0 dan mencapai puncak pada hari ke 30 dan 75. Similar situation occurred on the number of Leydig Cells. Pada ikan treatment, it meningkat dari hari ke 0 dan mencapai puncak pada hari ke 60 dam 75, sedangkan pada pada ikan kontrol meningkat dari hari ke 0 dan mencapai puncak pada hari ke 30 dan 75 (Figure 2). The number of Sertoli cells on each observation was greater than Leydig cells (Figure 3).

Table 2. The number of Sertoli and Leydig cells in control and laserpuncture-induced group 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± 0.6	21 ±1.7	10 ± 1.3	16 ± 2.2
30	53±6.3	137± 3.6	45 ± 1.8	101± 3.1
45	21±2.2	32 ±2.5	16 ± 2.22	25 ± 1.7
60	44±8.5	197±16.2	39 ± 9.8	170±11.4
75	74±9.2	102±5.9	53 ± 6.2	83 ± 10.7

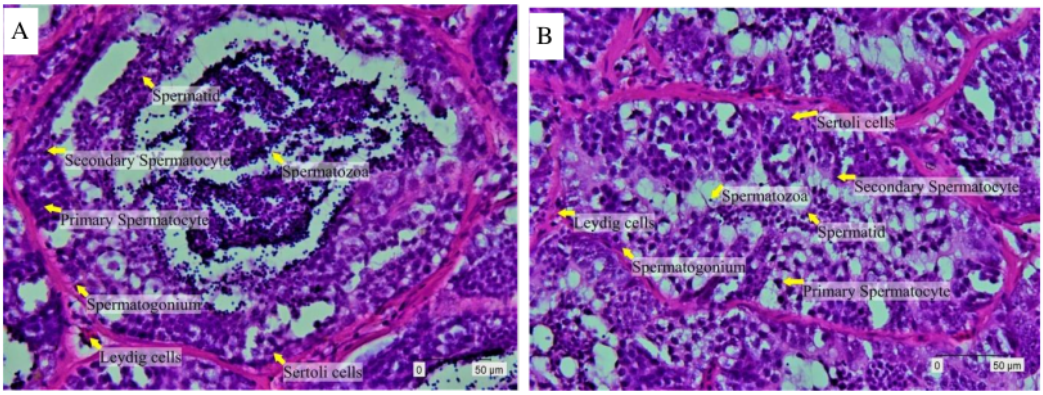

Figure3. Histology section showing Sertoli and Leydig cells Laserpuncture-induced group (A) and in control (B) from day 60.

4. Discussion

The testosterone and androgen binding protein (ABP) levels male *C. gariepinus*

Unlike this research, the broodstock catfish used was about 1 year old with a bodyweight of 1000-1200 g and given a laserpuncture induction every 15 days. It is proven that the level of testosterone produced is 17.97 ± 0.54 ng / ml and

for the control group it is 11.85 ± 0.4 ng / ml. Although this research was conducted for only 75 days, it has shown its influence on the maturation of the gonads. The reproductive activity of the broodfish is greatly influenced by the level of hormones such as the steroid hormone testosterone in their blood. This was to observe the process of development of the gonads in the reproductive cycle of broodstock *C. gariepinus*. This result was consistent with Hilbig et al. (2016) study, who stated that broodstock age affects the gametes quality and reproductive performance. The gamete with sex steroid plasma levels also relates broodstock age (Otoh and Udoh (2019), Chalde et al. (2014) and Rahbar et al. (2012). High or low levels of this steroid hormone can affect the condition of gonads. The results of the research of Buwono et al. (2019) revealed that the testosterone levels produced by transgenic Mutiara catfish of 11.25 ± 0.41 nmol/L are greater than the non-transgenic 6.32 ± 0.11 nmol/L. Mutiara catfish is used in the research was 1.5 years old, body weight 2.2 kg and catfish were given Ovaprim, the catfish quickly demonstrated ripened gonads and high testosterone levels. In this study, the ability of a male catfish to rapidly mature gonads when given a laserpuncture-induction was indicated by the testosterone hormone levels, which increased to a peak. However, the results of this research are still not maximized as the study was conducted for 75 days. In the future, it can be extended to 1-3 years following studies by other researchers. Despite the relatively shorter period of 75 days, the study has shown the influence of laserpuncture-induction on the maturation of the gonads.

The induction of laserpuncture on the reproductive acupoint in male *C. gariepinus* has proven to accelerate the gonad-stimulating potential to produce testosterone which produces two peaks and the highest peak is produced on the 60th day. This study result was consistent with the study conducted by Karu (2000) and Koutna et al. (2003) that laserpuncture induction can stimulate biological organs including the proliferation of somative cells such as gonadal cells and action of hormones. By giving laserpuncture induction at the reproduction point every 15 days until the 75th day, it can be seen that the development of the testes has occurred until the condition is mature, which can be seen from the color of the genital porus until the tip is red to purple. After taking blood once every 15 days until the 75th day. It was proven that the peak was on days 30 and 60. In this condition, the highest testosterone levels compared to the other days showed that the gonads were mature by seeing the color of the porous genitals.

Laser is an electromagnetic wave energy when the laser light is induced at the point of reproduction, which is the fastest route because laserpuncture induction can directly penetrate the epidermis to the dermis where it can directly of peripheral nerve endings. The electromagnetic wave energy from this laser beam will be converted into an electrical signal. The electrical signal will cause the depolarization of nerve cell membranes (Kusuma, 2013). As a result of this ion depolarization, the nerve cell membrane experiences an action potential and the membrane responds by opening the extracellular Ca^{2+} ion canal channel. Extracellular Ca^{2+} will enter through the calcium sensing receptor ion canal or through Voltage Gated Calcium Channels (Berridge et al., 2000; Clapham, 2007). Due to the entry of extracellular Ca^{2+} into nerve cells, then Ca^{2+} will increase physiological reactions to the brain tissue. Here a series of physiological reactions will occur by activating the enzyme Glutamic Acid Decarboxylase isoform 65 (GAD-65), this enzyme plays a role in modulating GABAergic neurons to synthesize and release Gama Amino Butiric Acid (GABA) in brain tissue. GABA results from the synthesis will then stimulate the hypothalamus and pituitary neurons (Kusuma et al., 2012; Kusuma and Hariani, 2017). GABA stimulates the hypothalamus neurons to synthesizes and liberates the gonadotropin releasing hormone (GnRH). GnRH will then stimulate the pituitary neurons to release gonadotropin hormones (GtH-I and GtH-II). This can occur because of the relationship between neurons in the *C. gariepinus* broodstock brain. Furthermore, GtH-I and GtH-II are released systemically, so that the levels of the gonadotropin hormones GtH-I and GtH-II in the blood serum increase. GtH-I and GtH-II play a role in steroidogenesis to produce steroid hormones such as androgen hormones, namely testosterone (Cowan et al., 2017; Borella et al., 2020).

The fluctuation in testosterone concentration after the male *C. gariepinus* was adapted, followed by laserpuncture induction treatment every 15 days until the 75th day was related to the activity of the gonadotropin hormone in the sterodogenesis process. Testosterone levels increased on the 15th and 30th days, indicated the role of this hormone, reaching the first peak and synergizing with GtH in gonad maturation. This maturation lead the *C. gariepinus* male to spawn readiness. If spawned did not occur, testosterone levels would drop, which is thought to be reabsorbed which

is reached on day 45. Furthermore, the laserpuncture induction increase again and reach the second peak on the 60th day. Differ trend was occurred in control group because the peak reached on day 75. The spermatogenesis cycle in male C. *gariepinus* broodstock occurs once every 30 days following high levels of testosterone.

The provision of quality feed stimulates metabolic activity in the *C. gariepinus* and activates the work of reproductive hormones regulated by the axis hypothalamus-pituitary-gonad. Ajaji et al. (2018) and Zohar et al. 2010) highlighted that gonads can develop well-regulated hormones. The biosynthesis of androgens takes place in the Leydig cells continuously, where the biosynthesis is regulated by the hypothalamus-hypofisa-testicular (gonad) axis (Ohga et al., 2018). The GtH-I functions to stimulate the development and proliferation of Sertoli cells to produce ABP which stimulate the spermatogonia to start the spermatogenesis process. The GtH-II functions to stimulate Leydig cells to secrete the hormone testosterone (androgens). Intertitial Cell Stimulating Hormone stimulates the development of seminiferous tubules and Sertoli cells to produce ABP, protein responsible for sperm formation (Kusuma et al., 2012). The GtH-I and GtH-II play a role in spermatogenesis which stimulate the gonads to produce steroid hormones, namely testosterone. Testosterone and ABP together 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 indicate that the levels of testosterone and ABP are affected by the condition of the gonads during the male *C. gariepinus* broodstock reproductive cycle. Testosterone hormone levels and ABP reaching the peak indicates a mature gonad condition. The high testosterone levels indicate that the male catfish undergo 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 indicated that there was spermatogenesis process occurring in the fish every 30 days, and the duration of treatment affects the process of spermatogenesis.
Sex hormone-binding globulins (SHBGs) are an example of ABP which is a plasma glycoprotein. This hormone binds and transports steroids in the blood of vertebrates except birds and affects the bioactivity of sex steroids, which access to the tissues (González et al., 2017; Hammond, 2016). The 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 et al., 2010).

Number of Sertoli and Leydig cells male *C. gariepinus*.

As is the case with testosterone and ABP levels after the induction of laserpuncture on the reproductive acupoint in male C. gariepinus has proven to accelerate the gonad-stimulating potential. Likewise, this happens to the number of Sertoli cells and Leydig cells produced. It can be seen that the sharptooth catfish broodstock groups induced by laserpuncture show flkuctuation trend in the number of Sertoli and Leydig cells. The peak number of Sertoli cells and Leydig cells was reached on 30 and 60 days after induction, while in the control group the peak was reached on days 30 and 75. Here, it can be seen that the advantages of the second peaks laserpuncture induction is achieved 15 days faster and the number of Sertoli cells and Leydig cells produced is more than the control group. Likewise, the number of Sertoli cells was greater than Leydig cells. The increasing number of Leydig cells on these days is related to the increasing levels of testosterone and ABP. The peak indicated the maximum number required when the gonad condition was mature. The moment the level of testosterone reached the peak indicated the time where many sperm were released in the spawning condition. The time the number of Leydig cells decreasing on the 45th day indicated the condition of the development in the reproductive cycle back to the initial position. Previously, Weltzein et al. (2002; 2004) asserted that H. nemurus males achieving a high level of testosterone indicate spermatogenesis, spermyogenesis, and spermiation process. This is confirmed by Tessaro et al. (2019) as well as Chatakondi and Davis (2011), 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 a mature gonads condition. As found by Kobayashi et al. (1996) as well as Gazola and Borella (1997), the significantly decreased levels of testosterone show a transition towards a regression phase. It is undeniable that there is a link between the fluctuation of steroid hormone levels with the reproductive process in fish as reported by Miura and Miura (2003) as well as Munakata and Kobayashi (2010)

Sertoli cells play an important role in nourishing spermatogenesis. Both Sertoli and Leydig cells regulate the activity of spermatogenesis (Schulz et al. 2010; 2019). Sertoli cells secrete ABP (Grover et al. 2004), while Leydig cells secrete testosterone (Ahmed et al. 2013). The ABP is responsible for transporting steroids in the blood in the teleosts group of fish and influencing the bioactivity of sex steroids (Hammond, 2016). These activities are all regulated by the hypothalamus-hypofisa-gonads axis. The results of the study strengthened the theory that the hypothalamus-hypofisa-gonads axis regulates the activity that occurs in the process of maturation of gonads on the fish broodstock. Hypothalamus to release Gonadotropin Releasing Hormone (GnRH). The existence of this GnRH to stimulate the release of the Gonadotropin Hormone (GtH) such as GtH-I or Follicle-Stimulating Hormone (FSH) for the development and proliferation of the Sertoli cells where these cells to produce ABP. Besides that GtH-I was playing a role in the process of spermatogenesis through taking the role of the Sertoli. The GtH-II or Luteinizing Hormone (LH) plays a role in the maturation of gonads and GtH-II stimulated Leydig cells for androgen production such as testosterone (Hammond, 2016; Ohga et al. 2018; Schulz et al. 2019). The GtH-II acts to stimulate Leydig cells to produce testosterone and plays a role in regulating the activity of Sertoli cells in the seminiferous tubules. Furthermore, testosterone with Sertoli cells regulates spermatogenesis and finally sperm products and stimulates the secretion of ABP (Amer et al. 2001; Meachem et al. 2005; Ohta et al. 2007; Cheng, et al. 2010). With this series of GtH-I and Gt-II activities, it can cause gonad maturation of broodstock fish just like broodstock sharptooth catfish. This is in line with the research of Kusuma and Hariani (2017) that the induction of laserpunctures on broodstock sharptooth catfish can accelerate the process of maturation of gonads

Both types of cells are required for hormonal-regulated steroidogenesis activity on those days when there is an increase in the number of Sertoli cells and Leydig cells. The hypothalamus-pituitary-gonad axis commands this hormonal activity. The Sertoli cell plays a role in feeding the sperm and there are more in number than Leydig cells. Leydig cells plays in producing testosterone hormones. Also, it is necessary that the role of hypothalamus to release Gonadotropin Realising Hormone (GnRH). The existence of this GnRH to stimulate the release of the GtH-I for the development and proliferation of the Sertoli cells to produce ABP. Similarly, it takes GtH-II to stimulate Leydig cells to produce testosterone. Both testosterone and ABP cooperate in the Sertoli cell controlling the process of spermatogenesis.

CONCLUSION

This research found that laserpuncture induced at the reproductive point with a duration of 15 seconds every 15 days accelerate the maturation of gonads with indicators involve testosterone levels and ABP indicators, number of Seroli cells and Leydig cells. `Testosterone level, androgen binding protein content, number of Sertoli and Leydig cells` increased on days 30 and 60 indicating peaking spermatozoa quality every 30 days.

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