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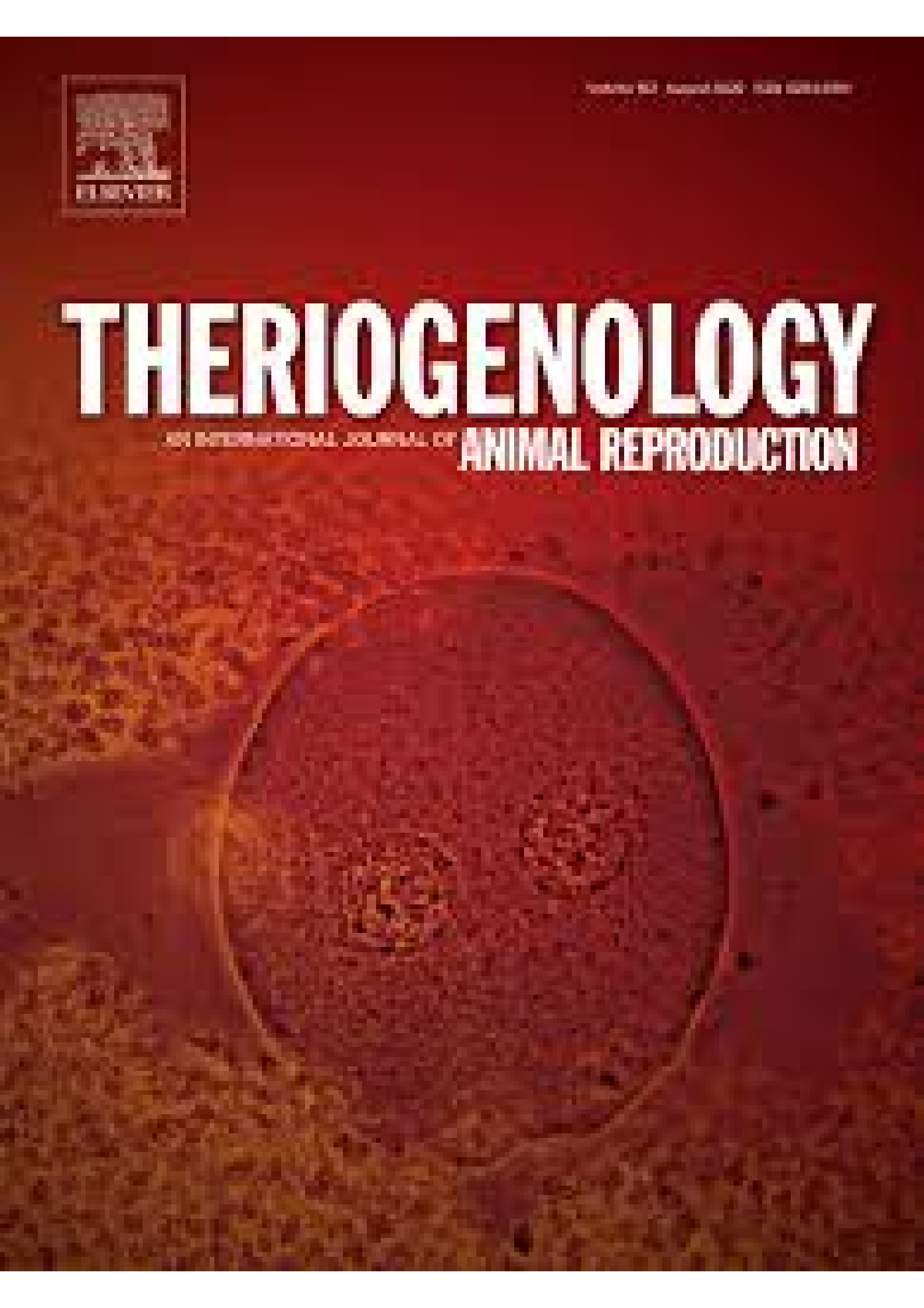
THERIOGENOLOGY

AN INTERNATIONAL JOURNAL OF ANIMAL REPRODUCTION



KORESPONDENSI

Nama Jurnal : Theriogenology

Judul Artikel : The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (*Pangasianodon hypophthalmus*)

No.	Proses	Waktu
1.	Submit manuskrip	23 Mei 2019
2.	Reminder dari editor jurnal	26 Mei 2019
3.	Under review oleh reviewer jurnal	26 Mei 2023
4.	Revisi manuskrip dari reviewer jurnal	31 Juli 2019
5.	Revisi manuskrip dan re-submit	29 September 2019
6.	Revisi manuskrip dari reviewer jurnal	17 Desember 2019
7.	Revisi manuskrip dan re-submit manuskrip revisi	14 Februari 2020
8.	Accepted artikel pada jurnal	19 Februari 2020
9.	Publish artikel di jurnal	21 Februari 2020

Thank you for your submission to Theriogenology

Dari: Theriogenology (eesserver@eesmail.elsevier.com)

Kepada: akhmad-t-m@fpk.unair.ac.id; atm_mlg@yahoo.com

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*** Automated email sent by the system ***

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Dari: Theriogenology (eesserver@eesmail.elsevier.com)

Kepada: akhmad-t-m@fpk.unair.ac.id; atm_mlg@yahoo.com

Tanggal: Minggu, 26 Mei 2019 22.12 GMT+7

Ms. Ref. No.: THERIO-D-19-00521
Title: The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (Pangasianodon hypophthalmus)
Theriogenology

Dear Dr. Mukti,

Your submission entitled "The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (Pangasianodon hypophthalmus)" will be handled by Associate Editor Leonardo Brito, DVM, PhD, DACT.

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is https://ees.elsevier.com/therio/.
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Thank you for submitting your work to this journal.

Kind regards,

Elsevier Editorial System
Theriogenology

Your Submission THERIO-D-19-00521

Dari: Leonardo Brito (eesserver@eesmail.elsevier.com)

Kepada: akhmad-t-m@fpk.unair.ac.id; atm_mlg@yahoo.com

Tanggal: Rabu, 31 Juli 2019 19.59 GMT+7

Ms. Ref. No.: THERIO-D-19-00521
Title: The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (Pangasianodon hypophthalmus)
Theriogenology

Dear Dr. Mukti,

Your manuscript, "The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (Pangasianodon hypophthalmus)" has been examined by two independent reviewers. It has been judged unsuitable for publication in its present form. Substantial revisions and re-review are required before a final decision is made regarding acceptance or rejection. Please view the reviewer's comments appended below.

When revising this paper, please prepare a letter responding to each individual comment made by each reviewer. Please indicate, on a point-by-point basis, how you responded to them (including a brief rebuttal for those points that you chose to not change). It is important that all concerns of all reviewers be addressed completely.

Please spell-check your document, cross-reference literature cited in the text against the bibliography, and ensure that every entry in the bibliography is accurate and has the correct format.

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I hope that you make all the necessary revisions, and look forward to receiving the revised version of your paper within 60 days of the date of this letter. If the revised manuscript is received after the 60-day period, it will be handled as a new manuscript. If there is a valid reason for not resubmitting the revised manuscript within 60 days, a 30-day extension with proper justification may be requested within 30 days of the date of this letter.

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Yours sincerely,

Leonardo Brito, DVM, PhD, DACT
Associate Editor
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Theriogenology

Reviewers' comments:

Reviewer #1:

This MS describes the promotion of testicular maturity in striped catfish by laser puncture method technologies. The low power laser puncture treatments of 0.4 and 0.5-joule accelerates testicular maturation with high spermatozoa qualities. This method seems to be effective for catfish reproduction. The authors describe the effectiveness of this method for female oocyte growth and maturation of this fish without references in the discussion (P8, L181-183). It indicates novelty was not found in this MS. If this methods can apply for other teleost that has been difficult for gonadal maturation, fisheries willing to accept and expand this simple technology with the novelty.

The authors cite the principals of laser puncture to promote the reproduction and metabolite stimulation (i.e. Kusuma et al.[7]), but I cannot easily believe that is true because authors' citation are almost domestic journals I cannot access. In addition, there are many errors in the tables and sentences.

I believe this manuscript does not meet the scientific standards of the Theriogenology.

At first, the authors should arrange the overall discussion with reliable articles.

This manuscript may be unsuitable for publication yet.There are many mistakes in the tables (i.e, Table 4, 58,875 comma to a decimal point)

Ovaprim injection did not induce complete gonadal maturity, therefore it is not called positive control.

P8 L4 References are missing.

The authors success the testicular maturation by laser puncture methods in this study, therefore may discuss in comparison to ovarian maturation by laser puncture of the catfish. Alternatively, the authors had better discuss the only testicular maturation. In this case, remove the paragraphes (L181-208) from the discussion.

P8 L191 Vitelogenin is a precursor of the lipoprotein and phosphoproteins, not a mixture of protein and lipids.

References

There are many domestic journals cited not to be suitable for the international journals in the discussion (i.e. Kusuma [7]).

Reviewer #2:

In the MS, the authors determine the effects of different doses of laserpuncture on gonadal maturity and sperm quality of male striped catfish. They conclude that laserpuncture induction of 0.5-joule dose at the reproductive acupoint increase gonadal maturity and sperm quality of male striped catfish.
In all, the MS is very interesting and is potential for application in aquaculture in the future. The MS is potentially publishable on Theriogenology. However, some questions should be addressed before it is acceptable for publication.

Major points
1. In the MS, the authors claimed that 0.5-joule dose is the optimal dose for laserpuncture induction. However, 0.5-joule dose in the experiment is the maximum dose. Whether the effects is better when the dose is higher than 0.5 in the experiment?
2. An additional study whether administration of laserpuncture has any effect on serum steroid measurements (testosterone and 11-KT) to determine the possible molecular mechanism of laserpuncture on gonad maturity.
3. To evaluate the sperm quality, the authors should include other sperm quality analysis, e.g. morphology abnormality, beat frequency of the flagella, VCL (curvilinear velocity), VSL (straight line velocity). The authors should also compare the fertilization rate and survival rate between the control groups and treat groups.
4. There are numerous grammar errors, sentence structure problems and misspelling in this manuscript. Please rewrite the manuscript and careful editing by native speakers is needed to make the results and discussion more clear. For example, 'That is, almost all sex-related genes are exist in both sexes.', 'Scoring of testis cell development, according to Çek and Yilmas', and 'before was analyzed for microscopically qualities.'.
5. There is not enough description about each panel of Fig.1 and Fig.2 in both document and figure legend. Also, the authors did not include the gonadal morphology and histology of 'Positive +'. And the tagging format of each panel in Fig.1 is not consistent. The tagging of Fig.1 a, c, e was on the right, while the tagging of Fig.1b, d was on the left.
6. Data of 'Positive +' should be included in Table 1 and Table 2.
7. Some recent publications of laserpuncture on female and male reproduction have not been cited in this study. For example, 'Biological study of increasing vitellogenin level and gonadal somatic index by laserpuncture exposure at any protein level of dietary on catfish broodstock (Clarias sp.) ' and 'Effectiveness of low level laser therapy for treating male infertility' . The authors should include the recent study in the references.
8. The section of Introduction and Discussion is very diffused and need to be rewritten. And the authors should include the discussion how the laserpuncture affect the gonadal maturity and sperm quality.

Minor points:
1. "GtH-I" and "GtH-II" in the MS should be better changed to "FSH" and "LH", respectively.
2. Line 61, "1,5-2 kg" should be "1.5-2 kg".
3. Line 75, "improve" should be "improves".
4. Line 108, "5×3×1,5" should be "5×3×1.5".
5. Line 184, "has" should be "have".
6. Line 192, "Vitellogenin is aglycophospo lipoprotein which contains 90% protein and 20% lipid" is an inaccurate statement. Actually, vitellogenin is a glycolipoprotein containing approximately 91% protein, 7% lipid, and 2% carbohydrate.
7. Line 231, "state" should be "states".
8. In the Table 1, 2 and 4, sentence"Different superscript the samecolumn show significant differences"should be "Different superscripts the samecolumn show significant differences".
9. In the Table 2 and 4, ","should be changed to ".".
10. In the Table 3, "7.9" in "PH"column should be "7-9"?
11. It is better to examine the serum 11-KT concentration after laserpuncture induction.

Dear Editor-in-Chief of Theriogenology and Reviewers

Thanks for the corrections and suggestions that have been given to our paper. Authors responses on corrections and suggestions of reviewers have mentioned in the article with blue-colored words or sentences.

For Reviewer 1

1. Reviewer comment: This MS describes the promotion of testicular maturity in striped catfish by laser puncture method technologies. The low power laser puncture treatments of 0.4 and 0.5-joule accelerates testicular maturation with high spermatozoa qualities. This method seems to be effective for catfish reproduction. The authors describe the effectiveness of this method for female oocyte growth and maturation of this fish without references in the discussion (P8, L181-183). It indicates novelty was not found in this MS. If this methods can apply for other teleost that has been difficult for gonadal maturation, fisheries willing to accept and expand this simple technology with the novelty.

   Authors response: Thank you very much for the reviewer's correction of the statements listed in the Discussion. We have corrected the statement in accordance with our research theme, as we have mentioned the sentence in the Discussion of article; page 9, line 211-214: "The induction of laserpuncture on the reproductive acupoint of catfish can accelerate GtH formation from the pituitary especially GtH-I or luteinizing hormone (LH) which has a role in the final stage of spermatogenesis or spermatozoa production, testicular maturation, ovulation, and spawning stimulation."

2. Reviewer comment: The authors cite the principals of laser puncture to promote the reproduction and metabolite stimulation (i.e. Kusuma [7]), but I cannot easily believe that is true because authors' citation are almost domestic journals I cannot access. In addition, there are many errors in the tables and sentences.

   Authors response: We have deleted the journal of Kusuma [7]. Thank you very much for the reviewer's correction of some decimal typing errors in the Tables of article. We have revised it, as we have mentioned in the Tables of article.

3. Reviewer comment: There are many mistakes in the tables (i.e, Table 4, 58,875 comma to a decimal point).

   Authors response: Thank you very much for the reviewer's correction of some decimal typing errors in the Tables of article. We have revised it, as we have mentioned in Tables 1 and 4 of the article, pages 23 and 26, respectively.

4. Reviewer comment: Ovaprim injection did not induce complete gonadal maturity, therefore it is not called positive control.

   Authors response: All this time, ovaprim is one of and perhaps the only hormonal material used to induce maturation and spawning of striped catfish, especially males striped catfish because ovaprim contains FSH and LH. Therefore, we used ovaprim as a positive control in this study. However, we revise the use of positive control terms in the article to avoid misunderstanding of meaning, as we have mentioned the sentences in Materials and methods of article; page 5, line 117-121: "...without the laserpuncture and the ovaprim™ treatments as a negative control, and treatment of the ovaprim™ dose of 0.2 mL/kg fish body weight [33] in 1-stage gonadal

maturity of male striped catfish, respectively. The 4-stage gonadal maturity of male striped catfish as positive treatment was treated the ovaprim^{TM} dose of 0.5 mL/kg fish body weight, specifically was only used to observe sperm quality parameters."

5. Reviewer comment: The authors success the testicular maturation by laser puncture methods in this study, therefore may discuss in comparison to ovarian maturation by laser puncture of the catfish. Alternatively, the authors had better discuss the only testicular maturation. In this case, remove the paragraphes (L181-208) from the discussion.

   Authors response: We have revised and deleted some sentences in Discussion of the article that are not quite right according to the reviewer's suggestion.

For Reviewer 2

Major points

1. Reviewer comment: In the MS, the authors claimed that 0.5-joule dose is the optimal dose for laserpuncture induction. However, 0.5-joule dose in the experiment is the maximum dose. Whether the effects is better when the dose is higher than 0.5 in the experiment?

   Authors response: In this study, we stated that laserpuncture dose of 0.5-joule was the treatment that produced the highest value (not the optimum) on all test parameters, both the gonadal maturity and the sperm quality. In the preliminary study, we have actually tested the laserpuncture treatment of 0.5 and 0.6-joule doses. However, observation of testicular morphology indicates that there is no difference between the two treatments, so in this study, we used 0.5-joule as the maximum dose treatment. Although, we have not conducted further studies on other test parameters.

   Based on the results of this study indicate that the highest or maximum gonadal maturity has been achieved using a 0.5-joule dose of laserpuncture treatment, so we assume that a higher dose will never produce a maximum level of maturity (stage 4), at least the same as the 0.5-joule treatment. The use of higher doses with similar results is inefficient and it is feared that there is a feedback mechanism.

   However, further studies on higher doses of laserpuncture are important to know which treatment provides optimum or maximum results on various test parameters of fish reproductive performance. These are our concern for future studies.

2. Reviewer comment: An additional study whether administration of laserpuncture has any effect on serum steroid measurements (testosterone and 11-KT) to determine the possible molecular mechanism of laserpuncture on gonad maturity

   Authors response: Thank you very much for the reviewer's suggestion of this study. This is our concern for further studies and the authors have also stated in Conclusion of the article; page 13, line 299-300: " Further studies on higher doses of laserpuncture and its effects on hormonal mechanisms are very important."

   In this study, we have not made it possible to analyze hormone level due to 1) the limited availability of analytical hormone kits and must be ordered for 2 to 3 months, and limited research grant funds.

3. Reviewer comment: To evaluate the sperm quality, the authors should include other sperm quality analysis, e.g. morphology abnormality, beat frequency of the flagella, VCL (curvilinear velocity), VSL (straight line velocity). The authors should also compare the fertilization rate and survival rate between the control groups and treat groups

   Authors response: Thank you very much for the reviewer's suggestion. The suggestions from reviewers to evaluate the other sperm qualities would be considered in future studies.

   In this study, we actually wanted to do the sperm test and analyze between the treatments on fertilization rate (FR) and hatching rate (HR) of egg (embryo), and survival rate (SR) of larvae, however at the time of this study, there were no available gonad-matured female broodstocks and were ready to be spawned, so we could not observe further.

4. Reviewer comment: There are numerous grammar errors, sentence structure problems and misspelling in this manuscript. Please rewrite the manuscript and careful editing by native speakers is needed to make the results and discussion more clear. For example, 'That is, almost all sex-related genes exist in both sexes.', 'Scoring of testis cell development, according to Çek and Yilmas', and 'before was analyzed for microscopically qualities

   Authors response: Thank you very much for the reviewer's correction of writing to the article. This article has been proofed and read by English native spoken and we have mentioned in Acknowledgments of the article; page 13, line 310-312: "Authors would like to thank and appreciate the comments and corrections given by reviewers, editor, and proofreader to improve this article."

   We have revised and mentioned the sentences in Materials and methods of the article; page 6, line 140-141: "Scoring of testis cell development was conducted according to Çek and Yilmas." and line 145: "...before the analysis of microscopic qualities."

5. Reviewer comment: There is not enough description of each panel of Fig.1 and Fig.2 in both document and figure legend. Also, the authors did not include the gonadal morphology and histology of 'Positive +'. And the tagging format of each panel in Fig.1 is not consistent. The tagging of Fig.1 a, c, e was on the right, while the tagging of Fig.1b, d was on the left

   Authors response: we have mentioned the sentences in Results of the article; page 7-8, line 172-178: "Generally, fish gonad characteristics that have 1-stage gonadal maturity are testicles like threads, smaller, shorter and limited, and visible ends in the body cavity, while the 2-stage gonadal maturity (Figures 1a and 1b) have the testis size and shape that are larger and clearer compared to 1-stage gonadal maturity. The morphology characteristics of 3-stage gonadal maturity are the surface of the testicles appear jagged, bigger, and the whiter color (Figure 1c). The 4-stage gonadal maturity of male striped catfish has testis characteristics of clearer, denser, and milky white color (Figures 1d and 1e)." and page 8-9, line 195-200: "Based on the testicular histology of male striped catfish in Figure 2 shows that the seminiferous tubules are still empty and spermatozoa are not visible (Figures 2a and 2b) as one of the 2-stage gonadal maturity characteristics in the fish. At the 3rd stage of gonadal maturity, seminiferous tubules already contain spermatozoa (Figure 2c), while at the 4-stage gonadal maturity, seminiferous tubules contain more spermatozoa (Figure 2d), even full spermatozoa (Figure 2e)."

   We have revised tagging of Figures 1 and 2. We have also mentioned the sentences in the legend of Figures 1 and 2.

6. Reviewer comment: Data of 'Positive +' should be included in Table 1 and Table 2

   Authors response: We have replaced the positive+ into positive treatment as in Materials and methods of the article; page 5, line 120: "...positive treatment..". In this study, we only used positive treatment to collect and obtain the sperm, and to compare the sperm quality with the laserpuncture treatments due to both negative control and the ovaprim^{TM} treatments did not reach the 4-stage gonadal maturity, so they could not produce sperm for further analysis. We have mentioned the sentences in Materials and methods of the article; page 5, line 119-121: "The 4-stage gonadal maturity of male striped catfish as positive treatment was treated the ovaprim^{TM} dose of 0.5 mL/kg fish body weight, specifically was only used to observe sperm quality parameters."

   On the other hand, this study was conducted during the non-spawning season of striped catfish, so that the stock availability of gonad-matured male striped catfish is very limited. Therefore, we did not observe the testicular morphology and histology in positive treatment, so we only include the positive treatment in Tables 3 and 4 of the article.

7. Reviewer comment: Some recent publications of laserpuncture on female and male reproduction have not been cited in this study. For example, 'Biological study of increasing vitellogenin level and gonadal somatic index by laserpuncture exposure at any protein level of dietary on catfish broodstock (Clarias sp.) ' and 'Effectiveness of low level laser therapy for treating male infertility'. The authors should include the recent study in the references

   Authors response: Thank you very much for the reviewer's suggestion and recommendation of references to improve this article. We have mentioned the sentences in Introduction and Discussion of the article and added in References of article.

8. Reviewer comment: The section of Introduction and Discussion is very diffused and need to be rewritten. And the authors should include the discussion how the laserpuncture affect the gonadal maturity and sperm quality

   Authors response: Thank you very much for the reviewer's correction and suggestion of Introduction and Discussion to improve this article. We have revised and mentioned the sentences in Introduction and Discussion of article.

Minor points

1. "GtH-I" and "GtH-II" in the MS should be better changed to "FSH" and "LH", respectively.

   Revision: page 9, line 212: "…GtH-I or luteinizing hormone (LH) which…", page 11, line 261: "...follicle-stimulating hormone (FSH) and LH [55]. The FSH will...", page 11, line 263: "The LH stimulates...", and page 12, line 279-280: "...release FSH and LH. FSH produces testosterone hormone, and LH produces..."

2. Line 61, "1,5-2 kg" should be "1.5-2 kg".

   Revision: page 3, line 63: "…1.5 to 2.0 kg…"

3. Line 75, "improve" should be "improves".

   Revision: page 4, line 86: "…30 mW improves tissue…"

4. Line 108, "5×3×1,5" should be "5×3×1.5".

   Revision: page 5, line 122: "…of 5.0×3.0×1.5 m^3 …"

5. Line 184, "has" should be "have".

   Revision: page 9, line 214: "…Kert and Rose [46] have proven …"

6. Line 192, "Vitellogenin is aglycophospo lipoprotein which contains 90% protein and 20% lipid" is an inaccurate statement. Actually, vitellogenin is a glycolipoprotein containing approximately 91% protein, 7% lipid, and 2% carbohydrate.

   Authors response: We have deleted the sentences in Discussion of the article based on the first reviewer's suggestion

7. Line 231, "state" should be "states".

   Revision: page 11, line 253: "Zeyl *et al*. [53] states that…"

8. In the Table 1, 2 and 4, sentence"Different superscript the samecolumn show significant differences"should be "Different superscripts the samecolumn show significant differences".

   Revision: pages 23, 24, and 26: "Different superscripts the same column…"

9. In the Table 2 and 4, ","should be changed to ".".

   Revision: pages 23 and 26, Tables 1 and 4

10. In the Table 3, "7.9" in "PH"column should be "7-9"?

    Revision: page 25, Table 3

11. It is better to examine the serum 11-KT concentration after laserpuncture induction.

    Authors response: Thank you very much for the reviewer's suggestion to examine the serum 11-KT concentration after laserpuncture exposure. This suggestion would be considered for our studies in the further.

Thus authors responses on comments, corrections, and suggestions of reviewers, we expect the reviewers and editor were pleased and understand it and we hope that this article will be corrected further. Thank you very much.

Best regards,

Akhmad Taufiq Mukti

The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (*Pangasianodon hypophthalmus*)

Mukti, A.T.[a*], Sari, Y.G.P.[b], Agusdinata, G.S.R.[b], Satyantini, W.H.[a], Mubarak, A.S.[c], Luqman, E.M.[d], Widjiati[d]

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Abstract

Laserpuncture is one of the applicative technologies that is used primarily in animal and fish reproductions. Laserpuncture technology is used for biostimulation of reproduction to rapidly improve gonadal maturity and increase sperm quality in fish. This study was aimed to determine the effects of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish. The 1-stage gonadal maturity of males striped catfish with a body weight range of 800 to 900 g/fish were used. Semi-conductor soft laser was used as laserpuncture tool with treatments of 0.2-, 0.4-, and 0.5-joule doses, while negative control (without the laserpuncture and the ovaprim^{TM}) and only the ovaprim^{TM} were used as comparative treatment, respectively. Laserpuncture was conducted on reproductive acupoint in every week for four weeks, while the ovaprim^{TM} was administered by intramuscular injection (0.2 mL/kg fish) in final rearing. Fish was reared in hapa at the controlled pond. Fish was fed commercial feed of 32% protein content. Gonadal maturity, both morphological and histological, gonadosomatic index (GSI), hepatosomatic index (HSI), and macroscopically and microscopically sperm qualities of male striped catfish were measured in final rearing. The results showed that the laserpuncture at the reproductive acupoint had a significantly high effect ($P<0.01$) on gonadal maturity, GSI, and HSI of male striped catfish. The laserpuncture treatments of 0.4 and 0.5-joule doses accelerated gonadal maturity to reach the 4th stage. The highest levels of GSI and HSI were found in the laserpuncture dose of 0.5-joule, i.e., 2.17% and 1.54%, respectively. The highest sperm qualities were reported in the laserpuncture dose of 0.5-joule, i.e., 81.75% motility, 82.75% viability, and 7.0×10^9 cell/mL concentration. The laserpuncture led to rapid gonadal maturity and increased sperm quality of male striped catfish.

Keywords: Laserpuncture; Semi-conductor soft laser; Gonadal maturity; Sperm quality; Male striped catfish

1. Introduction

In Indonesian, the production of striped catfish commodities in 2015 amounted to 339,060 metric tonnes (MT), which increased rapidly in 2016 amounting to 447,110 MT [1]. The high market demand triggers farmer to increase the amount of striped catfish production, but the supply of quality and sustainable striped catfish seed depends on the spawning season. Zairin [2] states that the reproductive cycle of striped catfish occurred naturally in rain season around October until April months, commonly. Striped catfish has several advantages, such as rapid growth, easy to cultivate, and tolerate in the waters with low oxygen content [3].

The process of gonadal maturity in striped catfish needed a long time and depended on the increase in gonadotropin hormone (GtH) and gonadal steroid. The productive period of spawning in male striped catfish peaks at the age of two years and 1.5 to 2.0 kg body weight [4]. Other constraints of a male are decreased sperm quality, such as motility and viability after the spawn.

Increased production of striped catfish seed requires new technology to improve the reproduction quality using laserpuncture technology. Laserpuncture technology is a stimulation technique on acupuncture points (acupoint) by a laser as a tool that has a stimulatory effect [5, 6]. The use of laserpuncture method could reduce the cost of production [7]. Susan [6] states that in the reproduction organs, application of laserpuncture stimulates arrangement of several reproductive functions of male and female animals. Laserpuncture technology has been shown to accelerate the growth rate, gonadal maturity, and spawning processes and to shorten the reproductive cycles of several species, such as catfish, *Clarias gariepinus* [8-13] and mud crab, *Scylla serrata* [14, 15]. The advantages of laserpuncture

technology as a stimulation method are that it is efficient due to each laser stimulation only takes about 5 to 10 s, does not cause damage to the tissues, and provides maximum response [16], depending on the type of soft-laser that is used.

The low-power laser affects biology system of human [17] and animal [18] include aquatic organisms (fish). The low-power laser used for laserpuncture (5 to 30 mW) proven to increase tissue activity such as increased hormones and enzymes. Some research shows that the laserpuncture improves the vascular and endocrine systems, and various other body systems [19]. The low-power laser also improves the male fertility of human [20, 21], animal [22, 23], and aquatic organisms, such as fish [23] and sea urchin [24, 25]. Kusuma *et al.* [9] found that the use of the low-power laserpuncture technology at the reproductive acupoint precisely at 2/3 ventral parts of the body through induction once a week is optimal for gonadal maturity of catfish. The low-power laser around 5 to 30 mW improves tissue activity, such as an increase in the production of tissue hormones and enzymes [26]. In catfish, the low-power laserpuncture induction alters the cell membrane potential and stimulates the hormone production [26-30]. Moreover, the low-power laserpuncture induction at the reproductive acupoint for 15 seconds increases the production of the GtH [10, 31], which is the regulator for steroidogenesis, oogenesis, and oocyte maturation [32]. On the other hand, induction of laserpuncture also increases the testosterone level in blood serum and the gonadosomatic index (GSI) of male catfish [11]. Several studies show that laserpuncture could improve vascular and endocrine systems and other body systems [19].

If fish are spawned throughout the season, laserpuncture has a significant influence on their reproductive performance, how does laserpuncture induction affect fish whose spawning cycle depends on the season? Therefore, this study was aimed to determine the effects of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish.

2. Materials and methods

This study was conducted at the Fish Breeding Research Centre, Subang, West Java, Indonesia. Histological analysis of fish gonads was held at the Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia. In this study, the experimental protocols were approved by the Scientific Committee, Institute of Research and Innovation, Universitas Airlangga (Protocol Number 886/UN3/2018).

2.1. Animal

Male striped catfish used was sized 800 to 900 g/fish body weight with 1-stage gonadal maturity and never been spawned before. As a precaution, male striped catfish with 4-stage gonadal maturity was also prepared separately (for positive treatment).

2.2. Laserpuncture

In this study, a semi-conductor soft-laser was used, which had a power specification of 20 mW. The preset dosages and timer settings were 0.2, 0.4, and 0.5 with 10, 20, and 25 s, respectively.

2.3. Experimental design

This study used the completely randomized design structure consisting of five treatments with four replicates. Treatments of the laserpuncture doses were 0.2-, 0.4-, and 0.5-joules, without the laserpuncture and the ovaprim^{TM} treatments as a negative control, and treatment of the ovaprim^{TM} dose of 0.2 mL/kg fish body weight [33] in 1-stage gonadal maturity of male striped catfish, respectively. The 4-stage gonadal maturity of male striped catfish as positive treatment was treated the ovaprim^{TM} dose of 0.5 mL/kg fish body weight, specifically was only used to observe sperm quality parameters.

Fish were adapted and reared in hapa of 5.0×3.0×1.5 m^3 size at a controlled pond. Fish were fed commercial feed contain crude protein of 32%. Laserpuncture treatment was done on reproductive acupoint every week for four weeks. Laserpuncture was done at the ova point

(reproductive acupoint), located on the 2/3 ventral part of the body (governoer vessel) measured from the anal to the pectoral fin. Determination of reproductive acupoint was also done using electro-acupuncture device tool. On the other hand, the ovaprim^{TM} was treated by using the intramuscular technique in final rearing stage (week 4), 8 to 10 h before the end rearing of fish. Parameters of gonadal maturity (morphological and histological), gonadosomatic index (GSI), hepatosomatic index (HSI), and sperm qualities, both macroscopically and microscopically were measured.

2.4. Morphology and histology of gonadal maturity, GSI, and HSI

At the end of the rearing process, fish was anesthetized using MS222 (Argent Laboratories, Redmond, Washington, DC) of 100 ppm [34-36] for 10 min. Subsequently, the fish were dissected on the abdominal part from anal to ventral. Observation of morphologically and histologically gonadal maturity was done on the shape, the length, the weight, the color, and the gonadal development through histology preparation. Gonad sample was prepared according to Junqueira and Carneiro [37] to measure the GSI and HSI.

Next, the histological method was conducted by McCann [38] using Hematoxylin-Eosin (HE) staining method according to Genten *et al*. [39]. Scoring of testis cell development was conducted according to Çek and Yilmas [40].

2.5. Sperm qualities

The collection of sperms from the fish was conducted by using the stripping method according to Melo and Godinho [41]. The sperm were stored in 1.5 mL microtubes in the refrigerator at a temperature of 4^{o}C before the analysis of microscopic qualities.

2.5.1. Motility of spermatozoa

Observation and determination of spermatozoa motility were modified according to Muchlisin *et al.* [42]. Motility of spermatozoa was observed microscopically under 100 × and 400 × magnifications using a BH2-RFCA Olympus binocular microscope (Olympus Optical

Ltd. Shinjuku-ku, Tokyo, Japan), which was equipped with a camera. Motile and immotile spermatozoa were calculated using a modified method by Salisbury and VanDemark [43] and Sohouka *et al*. [44], as well as a progressive or active movement forward and non-progressive movements (such as circular, backward or silent).

2.5.2. Viability of spermatozoa

Determination of spermatozoa viability was done by the staining protocol of 2% eosin yellow at sperm preparation. The viability of spermatozoa was observed and counted under 400 × and 1000 × magnifications using the same microscope as the one used to observe motility of spermatozoa. The spermatozoa viability was counted according to Salisbury and VanDemark [43].

2.5.3. Concentration of sperm

The sperm concentration was calculated according to Stoss and Donaldson [45].

2.6. Data analysis

Gonadal maturity stage was statistically analyzed using Kruskal Wallis and Mann Whitney tests, while the morphology and histology of the organ were descriptively analyzed according to Genten *et al*. [39]. The other data were statistically analyzed using analysis of variant (ANOVA) with SPSS ver. 10 software and Duncan's multiple range test with a confidence level of 95%.

3. Results

3.1. Gonadal maturity stage, GSI, and HSI

This study showed that laserpuncture treatment affected the gonadal development of male striped catfish, as seen in Figure 1. Generally, fish gonad characteristics that have 1-stage gonadal maturity are testicles like threads, smaller, shorter and limited, and visible ends in the body cavity, while the 2-stage gonadal maturity (Figures 1a and 1b) have the testis size and

shape that are larger and clearer compared to 1-stage gonadal maturity. The morphology characteristics of 3-stage gonadal maturity are the surface of the testicles appear jagged, bigger, and the whiter color (Figure 1c). The 4-stage gonadal maturity of male striped catfish has testis characteristics of clearer, denser, and milky white color (Figures 1d and 1e).

The 4-stage gonadal maturity achieved through laserpuncture treatment of 0.5-, 0.4-, and 0.2-joule doses showed no significant difference between all the joules ($p>0.05$). However, there was significant difference compared to the negative control and the ovaprim^{TM} treatment ($p<0.05$) as seen in Table 2. The results showed that ovaprim^{TM} treatment does not have a significant effect on gonadal development, the gonadal maturity only reached 2nd stage (immature), and could not be stripped to collect sperms, then the laserpuncture-treated male fish compared with ovaprim^{TM}-treated male fish that has a 4-stage gonadal maturity (as positive treatment) on sperm quality.

This study indicated that GSI and HSI had significant differences between treatments ($p<0.05$). The laserpuncture treatment of 0.5-joule had the highest GSI and HSI compared to other treatments, although the HSI is relatively not significantly different between treatments, except compared to the negative control (Table 1).

Similarity, the laserpuncture dose of 0.5-joule had the highest effect on testicular development. This study also showed that the laserpuncture dose of 0.5-joule has the highest scoring of gonadal or testicular histology in male striped catfish (Figure 2) with spermatozoa content of 75 to 100%, as seen in Table 2.

Based on the testicular histology of male striped catfish in Figure 2 shows that the seminiferous tubules are still empty and spermatozoa are not visible (Figures 2a and 2b) as one of the 2-stage gonadal maturity characteristics in the fish. At the 3rd stage of gonadal maturity, seminiferous tubules already contain spermatozoa (Figure 2c), while at the 4-stage

gonadal maturity, seminiferous tubules contain more spermatozoa (Figure 2d), even full spermatozoa (Figure 2e).

3.2. Sperm qualities

This study showed that laserpuncture treatment produced higher sperm quality, both macroscopically and microscopically, as seen in Tables 3 and 4. The laserpuncture treatment of 0.5-joule power resulted in the production of the highest volume of sperms, i.e., 3.00 to 5.25 mL with a creamy color (Table 3). On the other hand, the treatments showed significant differences ($p<0.05$) in microscopic sperm quality. The laserpuncture treatment of 0.5-joule power had the highest microscopic sperm qualities compared to other treatments, as seen in Table 4.

4. Discussion

The induction of laserpuncture on the reproductive acupoint of catfish can accelerate GtH formation from the pituitary especially GtH-I or luteinizing hormone (LH) which has a role in the final stage of spermatogenesis or spermatozoa production, testicular maturation, ovulation, and spawning stimulation. Kert and Rose [46] have proven that if an induction dose counts 0.5 to 1.0 J/cm^2 induced by low power laser of 5 mW with wavelength 632.8 nm improves the nerve regeneration ability located both in central and peripheral sides will be produced to increase the cellular activities, and the ability to produce hormones and enzymes. It indicated that the induction of laserpuncture could increase the performances of the activity of the hormone which takes part in a reproduction control system to accelerate the provision of growth, development, and gonad maturation of catfish. The combination of laserpuncture exposure and high protein feed improves gonadal maturity and GtH, as a study conducted by Kusuma and Hariani [47]. Other studies, the combination of laserpuncture exposure and high protein feed also improves the fertilization, hatching, and survival rates of catfish [48].

Laserpuncture enlarges GtH dynamics [10, 31]. An increase of GSI follows fish gonad development and gonadal maturity. The stage of gonadal maturity is a stage of sexual maturity in fish; most of the metabolic products are used during the gonadal development phase. The reproduction process use some energy for the gonadal development, and the fish gonads would reach maximum when the fish still spawn, then it has decreased rapidly during the spawning process to complete [49]. The gonadal maturity level provides knowledge about conditions of a non-ripe, the beginning of maturity, almost mature, mature, or copied through observable gonadal features.

The laserpuncture treatment on the reproductive acupoint affected the gonadal development of male striped catfish. The condition of fish gonads at the beginning of the study was at the initial gonadal maturity stage with a small gonadal morphology and looked clear. The three treatments showed an effect on the maturity level of the gonads, thus allowing the gonadotropins needed to mature the gonads to be fulfilled. Kusuma [31] states that after six hours of exposure to laserpuncture at the point of reproduction, it can increase the release of the GtH with the gonadotropin-releasing hormone (GnRH) stimulation mechanism.

Kusuma [9] states that with laserpuncture firing, it stimulates active cells in the area governoer vessel (reproduction acupoint) to conduct a series of energies. The formation of energy after laserpuncture shooting in the governoer vessel is related to specific proteins in cells. The results are mostly directed to the development of gonads gradually which is marked by the greater testicles. There are several levels of spermatogenesis in testicular development, namely spermatogonia, spermatocytes, spermatids, and spermatozoa [50].

The laserpuncture exposure at reproductive acupoints in male striped catfish can increase physiological activity in the body. This is indicated by the induction of laserpuncture dose proven to be optimal for increasing the HSI. GSI will continue to increase along with the maturation of the fish gonads and will reach the maximum value when the peak period of

gonad maturity [51]. The relationship between the GSI and gonadal maturity stage is seen by the tendency of the GSI to increase with increasing gonadal maturity stage. Effendie [52] states that increase gonadal development, the value of the GSI will increase until it reaches the maximum when spawning will occur and will fall again after the fish has spawned.

Zeyl *et al*. [53] states that the value of GSI has become a standard protocol for selecting fish in the reproductive process. The development of gonad weight gain in male fish reaches 5 to 10% of fish body weight. Increased gonadal weight is due to increased release of the GtH. The gonads will respond with increasing gonadal weight to the maximum limit when spawning occurs [52]. Kusuma and Hariani [54] also explained that at a higher level of gonad maturity, the number of sperm would increase, affecting the weight of the gonad and the body weight of the fish. The laserpuncture induction at the reproductive acupoint stimulates the hypothalamus to release the GnRH, which stimulates the pituitary to produce the GtH-II or follicle-stimulating hormone (FSH) and LH [55]. The FSH will stimulate Leydig cells and produce testosterone hormone. The testosterone hormone functions to stimulate the division of spermatogonia into spermatocytes. The LH stimulates Leydig cells to produce the 11-ketotestosterone and the 17α 20ß – hydroxyprogesterone hormones. The 11-ketotestosterone hormone plays a role in the process of spermiogenesis, and the 17α 20ß-dihydroxyprogesterone hormone plays a role in the process of spermiation [56].

Factors that play a role in the volume generated in the study include environmental conditions, such as temperature, pH, and oxygen levels that can affect stress on fish so that the fish cannot produce large volumes of cement. The difference in the volume of spermatozoa produced in addition to being influenced by environmental factors also influenced by age, body size, feeding management, and sperm release frequency [43]. Fish spermatozoa motility will decrease if they are at pH below seven [57]. These observations are by Fatmalawati [58]

that the color of striped catfish sperm is milky white and the consistency of catfish sperm is thick.

The low-power laserpuncture has significantly improved semen quality [59], such as volume [59], concentration [21], motility, movement, and viability of spermatozoa [20]. The highest motility value obtained in 0.5-joule treatment was because this treatment can provide a stimulatory effect large enough to stimulate the hypothalamus neuron to release the GnRH, then GnRH stimulates pituitary neurons to release FSH and LH. FSH produces testosterone hormone, and LH produces an androgen-binding protein (ABP). Testosterone hormone and ABP control spermatogenesis and initiate spermatogenic development into motile spermatozoa [48].

Several studies indicate the influence of laserpuncture on improving the sperm quality of animal include the motility [20, 59] and fertility [25] of spermatozoa and increasing the adenosine triphosphate (ATP) content [23, 24, 60-68], Ca^{2+} concentration [65, 69], and cell life [70]. Ca^{2+} stimulates the work of the mitochondria and the ATP synthesis in the cell [71], while mitochondria and ATP plays an important role in supporting spermatozoa motility [72-74].

In this study, the spermatozoa viability percentage after laserpuncture induction was reported to be good according to the research of Rahardhianto *et al*. [75] that the quality of spermatozoa is good based on spermatozoa viability of 80%. Mantayborbir *et al*. [76] found that laserpuncture induction has spontaneous stimulation power and rapidly influences the increase in the number of Leydig cells produced. The function of Leydig cells is to produce testosterone which binds to androgen receptors in Sertoli cells, which secretes ABP and helps form spermatozoa.

5. Conclusion

Laserpuncture induction of 0.5-joule dose at the reproductive acupoint increased gonadal maturity and sperm quality of male striped catfish. Further studies on higher doses of laserpuncture and its effects on hormonal mechanisms are very important.

Conflict of interest

No conflict of interest.

Acknowledgments

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Table 1. Gonadal maturity stage, GSI, and HSI of male striped catfish in different treatments

Treatment	Gonadal maturity stage	GSI (%)	HSI (%)
Negative control	II[a]	0.39 ± 0.15[a]	1.10 ± 0.07[a]
Ovaprim™	II[a]	0.54 ± 0.29[ab]	1.30 ± 0.14[ab]
Ld of 0.2-joule	III to IV[b]	0.70 ± 0.38[ab]	1.32 ± 0.15[ab]
Ld of 0.4-joule	IV[b]	1.09 ± 0.19[b]	1.39 ± 0.26[b]
Ld of 0.5-joule	IV[b]	2.17 ± 0.68[c]	1.54 ± 0.17[b]

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Table 2. Average testicular histology scoring of male striped catfish

Treatment	Score	Description
Negative control	3[a]	Development of cells has reached spermatids
Ovaprim™	4[b]	Development of cells has reached spermatids
Ld of 0.2-joule	5[c]	Already formed spermatozoa of 25.00 to 49.90%
Ld of 0.4-joule	6[d]	Already formed spermatozoa of 50.00 to 74.50%
Ld of 0.5-joule	7[e]	Already formed spermatozoa of 75.00 to 100.00%

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Table 3. Macroscopically sperm quality of male striped catfish in the different treatments

Treatment	Spermatozoa quality parameters			
	Volume (ml)	pH	Color	Consistency
Negative control	Negative	Negative	Negative	Negative
Ovaprim^{TM}	Negative	Negative	Negative	Negative
Positive	0.50 to 1.25	7 to 8	Clear White	Dilute
Ld of 0.2-joule	0.50 to 1.50	7 to 8	White milk	Condensed
Ld of 0.4-joule	0.50 to 2.00	7 to 9	White milk	Condensed
Ld of 0.5-joule	3.00 to 5.25	7 to 9	Creamy	Condensed

Note: Negative control = without the laserpuncture and the ovaprim^{TM} treatments, Positive = the male striped catfish with the 4-stage gonadal maturity treated the ovaprim^{TM} (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose.

Table 4. Microscopically sperm quality of male striped catfish in the different treatments

Treatment	Spermatozoa quality parameters		
	Motility (%)	Viability (%)	Concentration ($\times 10^9$cells/mL)
Negative control	Negative	Negative	Negative
Ovaprim^{TM}	Negative	Negative	Negative
Positive	58.88 ± 1.93^a	58.00 ± 1.58^a	4.31 ± 4.26^a
Ld of 0.2-joule	65.75 ± 2.32^b	66.25 ± 1.75^b	5.25 ± 4.56^b
Ld of 0.4-joule	73.00 ± 2.73^c	73.00 ± 2.27^c	6.06 ± 6.25^c
Ld of 0.5-joule	81.75 ± 1.19^d	82.75 ± 1.84^d	7.00 ± 5.40^d

Note: Negative control = without the laserpuncture and the ovaprim^{TM} treatments, Positive = the male striped catfish with the 4-stage gonadal maturity treated the ovaprim^{TM} (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

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Thanks for the corrections and suggestions that have been given to our paper. Authors responses on corrections and suggestions of reviewers have mentioned in the article with blue-colored words or sentences.

For Reviewer

1. Reviewer comment: However, the numerous sentence and structure problem are still present in the discussion.

   Authors response: We have corrected and revised the numerous sentences and structure in the article, especially in the Discussion of the article.

2. Reviewer comment: At first, bring the message home with a summary of the key findings of the paper, then listing a selection of the most relevant work in the field, finally, may explain implications of the study, what might be further work in the area.

   Authors response: We have read and analyzed again of the our article and we have corrected and revised of the article, starting from the Abstract to the Conclusion of the article.

3. Reviewer comment: The authors only explain testicular development with numerous citation, GSI (P10, L224-L231, P11, L249-260) also steroidogenesis (L261-266) and so on. They are not called discussion. I don't believe this discussion are edited or rewritten by the native speakers.

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Thus authors responses on comments, corrections, and suggestions of reviewers, we expect the reviewers and editor were pleased and understand it and we hope that this article will be corrected further. Thank you very much.

Best regards,

Akhmad Taufiq Mukti

The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (*Pangasianodon hypophthalmus*)

Mukti, A.T.[a*], Sari, Y.G.P.[b], Agusdinata, G.S.R.[b], Satyantini, W.H.[a], Mubarak, A.S.[c], Luqman, E.M.[d], Widjiati[d]

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Abstract

Laserpuncture is one of the applicative technologies used mainly in animal and fish reproductions. Laserpuncture technology used to improve gonadal maturity and sperm quality in fish rapidly. This study aimed to determine the effects of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish. Males striped catfish have a body weight range of 800 to 900 g/fish and I gonadal maturity stage were used. Semi-conductor soft laser was used with doses of 0.2-, 0.4-, and 0.5-joule, while the negative control (without the laserpuncture and the ovaprim^{TM}) and only the ovaprim^{TM} were used as a comparison treatment, respectively. The soft-laser was treated on reproductive acupoint every week for four weeks, while the ovaprim^{TM} was administered by intramuscular injection of 0.2 mL/kg fish in final rearing. Fish was reared on hapa at the controlled pond. Fish was fed commercial feed containing 32% crude protein. Gonadal maturity (morphology and histology), gonadosomatic index (GSI) and hepatosomatic index (HSI), and sperm quality macroscopic and microscopic of male striped catfish were measured in the final rearing. The results showed that the laserpuncture on the reproductive acupoint had a highly significant effect ($P<0.01$) on gonadal maturity, GSI, and HSI of male striped catfish. The laserpuncture doses treatment of 0.4 and 0.5-joule accelerate gonadal maturity to reach the IV stage. The highest levels of GSI and HSI were found in 0.5-joule of laserpuncture dose, which was 2.17% and 1.54%, respectively. The highest sperm qualities were reported in 0.5-joule of laserpuncture dose, namely 81.75% motility, 82.75% viability, and 7.0×10^9 cell/mL concentration. The laserpuncture causes rapid gonadal maturity and improved sperm quality in male striped catfish.

Keywords: Laserpuncture; Semi-conductor soft laser; Gonadal maturity; Sperm quality; Male striped catfish

1. Introduction

In Indonesian, the production of striped catfish commodities in 2015 amounted to 339,060 metric tonnes (MT), which increased rapidly in 2016, amounting to 447,110 MT [1]. The high market demand triggers farmers to increase the amount of striped catfish production, but the supply of quality and sustainable striped catfish seed depends on the spawning season. Zairin [2] stated that typically, the reproductive cycle of striped catfish occurs naturally in the rainy season around October to April month. Striped catfish has several advantages, such as fast growth, easy cultivation, and tolerate in the waters with low oxygen content [3].

The process of gonadal maturity in striped catfish needed a long time and depended on the increase in gonadotropin hormone (GtH) and gonadal steroid. The productive period of spawning in male striped catfish peaks at the age of two years and 1.5 to 2.0 kg body weight [4]. Other constraints of a male fish are the decrease in sperm quality, such as motility and viability after spawning.

Increased production of striped catfish seed requires new technology to improve the quality of reproduction using laserpuncture technology. Laserpuncture technology is a stimulation technique on acupuncture points (acupoint) by a laser beam [5]. The use of the laserpuncture method could reduce production costs [6]. Susan [5] states that in the reproductive organs, the application of laserpuncture stimulates the arrangement of several reproductive functions of male and female animals. Laserpuncture technology has been proven to accelerate the growth rate, gonadal maturity, and spawning processes and to shorten the reproductive cycles of several species, such as catfish, *Clarias gariepinus* [7, 8] and mud crab, *Scylla serrata* [9]. The advantage of laserpuncture technology as a stimulation method is that it is efficient due to each laser stimulation only takes about 5 to 10 s, does not cause

tissue damage, and provides a maximum response [10], depending on the type of soft-laser used.

The low-power laser affects the biology system of humans [11] and animals [12], including aquatic organisms (fish). The low-power laser around 5 to 30 mW improves tissue activity, such as increased production of hormones and enzymes [13]. The low-power laser gives a biological stimulus, such as changing the cell membrane's potential and permeability. On the other hand, it improves the nerve regeneration ability located both in central and peripheral sides will be produced to increase the cellular activities, and the ability to produce hormones and enzymes [13, 14].

Several studies have shown that the laserpuncture improves the vascular and endocrine systems, and various other body systems [15]. The low-power laser also improves the male fertility of humans [16], animals [17], and aquatic organisms, such as fish [17] and sea urchins [18]. Kusuma et al. [7, 8] found that the use of the low-power laserpuncture technology on the reproductive acupoint precisely in 2/3 ventral parts of the body through induction once a week is optimal for the maturation of catfish gonads. Moreover, the low-power laser induction at the reproductive acupoint for 15 seconds increases the production of the GtH [10], which is the regulator for produce steroid, oogenesis, and oocyte maturation [19]. On the other hand, induction of laserpuncture also increases the testosterone level in blood serum and the gonadosomatic index (GSI) of male catfish [8].

The low-power laser has a significant effect on the reproductive performance of fish whose spawning cycle occurs throughout the season, however, does laserpuncture induction affect fish whose spawning cycle depends on the season? Therefore, this study was aimed to determine the effect of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish.

2. Materials and methods

This study was conducted at the Fish Breeding Research Centre, Subang, West Java, Indonesia. Histological analysis of fish gonads was held at the Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia. In this study, the experimental protocols were approved by the Scientific Committee, Institute of Research and Innovation, Universitas Airlangga (Protocol Number 886/UN3/2018).

2.1. Animal

Male striped catfish used was sized 800 to 900 g/fish body weight and the I gonadal maturity stage and never been spawned before. As a precaution, male striped catfish with the IV gonadal maturity stage were also prepared separately (for positive treatment).

2.2. Laserpuncture

In this study, a semi-conductor soft-laser was used, which had a power specification of 20 mW. The preset doses and timer set were 0.2, 0.4, and 0.5 with 10, 20, and 25 s, respectively.

2.3. Experimental design

This study was used the completely randomized design structure consisting of five treatments with four replicates. Treatments used were doses of 0.2-, 0.4-, and 0.5-joule, without laserpuncture and ovaprim^{TM} treatments as a negative control, and treatment of the ovaprim^{TM} dose of 0.2 mL/kg fish body weight in male striped catfish with the I gonadal maturity stage. The IV gonadal maturity stage of male striped catfish as positive treatment was treated the ovaprim^{TM} dose of 0.5 mL/kg fish body weight, correctly was only used to observe sperm quality parameters.

Fish were adapted and reared in a hapa sized of 5.0×3.0×1.5 m^3 at a controlled pond and fed commercial feed containing 32% crude protein. Laserpuncture treatment was performed on reproductive acupoint every week for four weeks. Reproductive acupoint located on the 2/3 ventral part of the body (governoer vessel) was measured from the anal to the pectoral fin.

The determination of reproductive acupoint was also done using an electro-acupuncture device tool. On the other hand, the ovaprim^{TM} was treated by using the intramuscular technique in the final rearing stage (week 4), 8 to 10 h before the end rearing of fish. Gonadal maturity (morphology and histology), gonadosomatic index (GSI) and hepatosomatic index (HSI), and sperm qualities, both macroscopic and microscopic, were measured.

2.4. Morphology and histology of gonadal maturity, GSI, and HSI

At the end of the rearing process, fish was anesthetized using MS222 (Argent Laboratories, Redmond, Washington, DC) of 100 ppm [20] for 10 min. Subsequently, the fish were dissected on the abdominal part from anal to ventral. Morphological and histological gonadal maturity was observed on the shape, the length, the weight, the color, and the gonadal development through histology preparation. Gonad sample was prepared according to Junqueira and Carneiro [21] to measure the GSI and HSI.

Next, the histological method was conducted by McCann [22] using Hematoxylin-Eosin (HE) staining method according to Genten et al. [23]. Scoring of testis cell development was conducted according to Çek and Yilmas [24].

2.5. Sperm qualities

The collection of sperms from the fish was conducted by using the stripping method. The sperm were stored in 1.5 mL microtubes in the refrigerator at a temperature of 4^{o}C before the analysis of microscopic qualities.

2.5.1. Motility of spermatozoa

Microscopic spermatozoa motility was observed under 100 × and 400 × magnifications using a BH2-RFCA Olympus binocular microscope (Olympus Optical Ltd. Shinjuku-ku, Tokyo, Japan), which was equipped with a camera. Motile and immotile of the spermatozoa were calculated using a modified method by Sohouka et al. [25], as well as a progressive or

active movement forward and non-progressive movements (such as circular, backward or silent).

2.5.2. Viability of spermatozoa

The determination of spermatozoa viability was done by the staining protocol of 2% eosin yellow at sperm preparation. The viability of the spermatozoa was observed and counted under 400 × and 1000 × magnifications using the same microscope like the one used to observe motility of the spermatozoa. The spermatozoa viability was counted according to Sohouka et al. [25].

2.5.3. Concentration of sperm

The sperm concentration was calculated according to Stoss and Donaldson [26].

2.6. Data analysis

Gonadal maturity stage was statistically analyzed using Kruskal Wallis and Mann Whitney tests, while the morphology and histology of the organ were descriptively analyzed according to Genten et al. [23]. The other data were statistically analyzed using analysis of variant (ANOVA) with SPSS ver. 10 software and Duncan's multiple range test with a confidence level of 95%.

3. Results

3.1. Gonadal maturity stage, GSI, and HSI

This study showed that laserpuncture treatment affected the gonadal development of male striped catfish, as seen in Figure 1. In general, gonad characteristics of fish with the I gonadal maturity stage are testicles like threads, smaller, shorter and limited, and visible ends in the body cavity. The II gonadal maturity stage (Figures 1a and 1b) have more significant and more apparent the testis size and shape compared to the I gonadal maturity stage. The morphology characteristics of the III gonadal maturity stage are the surface of the testicles

appear jagged, more prominent, and the whiter color (Figure 1c). The IV gonadal maturity stage of male striped catfish has testis characteristics of more definite, denser, and milky white color (Figures 1d and 1e).

The IV gonadal maturity stage achieved through laserpuncture treatment of 0.5-, 0.4-, and 0.2-joule doses showed no significant difference between all the joules ($p>0.05$). However, there was significant difference compared to the negative control and the ovaprim^{TM} treatment ($p<0.05$) as seen in Table 2. The results showed that ovaprim^{TM} treatment does not have a significant effect on gonadal development; the gonadal maturity only reached the II stage (immature). It could not be stripped to collect sperms, then the laserpuncture-treated male fish compared with ovaprim^{TM}-treated male fish that has the IV gonadal maturity stage (as positive treatment) on sperm quality.

This study indicated that GSI and HSI had significant differences between treatments ($p<0.05$). The laserpuncture treatment of 0.5-joule had the highest GSI and HSI compared to other treatments, although the HSI is relatively not significantly different between treatments, except compared to the negative control (Table 1).

Similarity, the laserpuncture dose of 0.5-joule had the highest effect on testicular development. This study also showed that the laserpuncture of 0.5-joule dose has the highest scoring of gonadal or testicular histology in male striped catfish (Figure 2) with testis containing spermatozoa of 75 to 100%, as seen in Table 2.

Based on the testicular histology of male striped catfish in Figure 2 shows that the seminiferous tubules are still empty and the spermatozoa are not visible (Figures 2a and 2b) as one of the II gonadal maturity stage characteristics in the fish. In the III gonadal maturity stage, seminiferous tubules already containing spermatozoa (Figure 2c), while the IV gonadal maturity stage, seminiferous tubules containing more spermatozoa (Figure 2d), even full spermatozoa (Figure 2e).

3.2. Sperm qualities

This study showed that laserpuncture treatment produces higher sperm quality, both macroscopic and microscopic, compared to negative and positive treatments, as seen in Tables 3 and 4. The laserpuncture treatment of 0.5-joule power resulted in the production of the highest volume of sperms, i.e., 3.00 to 5.25 mL with a creamy color (Table 3). On the other hand, the treatments showed significant differences ($p<0.05$) in microscopic sperm quality. The laserpuncture treatment of 0.5-joule power had the highest microscopic sperm qualities compared to other treatments, as seen in Table 4.

4. Discussion

The induction of laserpuncture on the reproductive acupoint in fish has proven to increase the development and maturity of the gonad. The induction of laserpuncture on the reproductive acupoint accelerates FSH and LH from the pituitary, which has a vital role in development and maturity of the gonad. Although we did not measure FSH and LH levels in this study, however, other studies [7] have indicated that the laserpuncture exposure increases FSH and LH levels in catfish.

The induction of laserpuncture also accelerates LH formation which has a role in the final stage of spermatogenesis or spermatozoa production, testicular maturation, ovulation, and spawning stimulation [7]. It indicated that the induction of laserpuncture could increase the performances of the activity of the hormone, which takes part in a reproduction control system to accelerate the provision of growth, development, and gonad maturation of fish.

Induction of laserpuncture on the tissue increases gonadotropin-releasing hormone (GnRH) released by the hypothalamus and it stimulates the anterior pituitary to secrete FSH and LH which act on theca cells in the male gonad (testis) to produce testosterone hormone [27]. Furthermore, Chang et al. [28] explained that laserpuncture stimulates the release of

neurotransmitters. Next, the laser beam transduced into chemical signals to be received by various ion channels, such as G-proteins (GTP-binding protein)-coupled receptors subunit α and VGCC (voltage-gated Ca2+ channels), or through calcium receptors, such as calcium-sensing receptor (CaSR), located in the nervous membrane cells. Then, ligands binding to specific receptors trigger the release of second messengers causing a chain reaction and bringing changes in the cell. Electrical signals caused by depolarization of the nerve cell membrane propagated from cell to cell along the axon, then insert the pre-synapse membrane into the post-synapse membrane and trigger the release of neurotransmitter molecules in the synapse. The same mechanism, the electrical signals are transmitted to the brain. Intracellular and extracellular Ca2+ ions mediated through changes in spontaneous membrane potential play an essential role in stimulating the release of GnRH from the hypothalamus and it stimulates the pituitary to release FSH and LH. Next, FSH and LH are then channeled into the bloodstream towards the gonads (testis), which enable various activities. This process repeats when the nerve cell membrane is depolarized [29].

Anglade et al. [29] stated that the release of neurotransmitters such as gamma-aminobutyric acid (GABA) from GABAergic neurons depends on nerve cell membrane depolarization, action potential, calcium ions, decarboxylation of glutamate, and glutamic acid decarboxylase (GAD). The anterior pituitary directly innervates GABAergic neurons, so it has a stimulatory effect on the release of LH [30].

The laserpuncture treatment on the reproductive acupoint affected the gonadal development of male striped catfish. The condition of fish gonads at the beginning of the study was at the initial gonadal maturity stage with a small gonadal morphology and looked clear. The three treatments showed an effect on the maturity level of the gonads, thus allowing the gonadotropins needed to mature the gonads to be fulfilled.

Kusuma [7] stated that laserpuncture stimulates active cells in the area governoer vessel (reproductive acupoint) to conduct a series of energy. The formation of energy after laserpuncture exposure in the reproductive acupoint is related to specific proteins in cells. The results are mostly directed to the development of gonads gradually, which is marked by the greater testicles. There are several levels of spermatogenesis in testicular development, namely spermatogonia, spermatocytes, spermatids, and spermatozoa.

The laserpuncture exposure at reproductive acupoints in male striped catfish can increase physiological activity in the body. The induction of laserpuncture dose indicates this has proven to be optimal for increasing the HSI and GSI. GSI will continue to increase along with the maturation of the fish gonads and will reach the maximum value when the peak period of gonad maturity [31].

GSI has been used as one of the indicators of the development and maturity of gonad [32] in both sexes. In general, GSI increases with the increasing gonadal maturity stage as well as a show in this study (Table 1). Increased GSI followed the bigger size of the gonad (testis), as shown in Figure 1, and increase the number of spermatozoa produced by testis (Figure 2).

The FSH will stimulate Leydig cells and produce testosterone hormone. Testosterone hormone as a part of an androgen steroid hormone plays a vital role in the reproductive tissue development and the secondary sexual characteristics expression of male. GnRH promotes the secretion of LH by stimulating the pituitary gland. Then, LH promotes the synthesis of testosterone hormone by stimulating the Leydig cells of the testis [33]. Alves et al. [34] also have proven that the induction of low-level laser improves the testosterone hormone level in males.

In males, increased 11-ketotestosterone hormone level was related to spermatogenesis, which is consistent with several reports about 11-ketotestosterone hormone stimulating spermatogenesis in fish species rather than testosterone hormone [35]. The concurrent

elevation of 11-ketotestosterone and testosterone hormones level observed is another typical pattern in male fish [36]. In the future, the measurement of 11-ketotestosterone and testosterone hormones is our concern for further laserpuncture studies in fish, especially in striped catfish.

Laserpuncture improved protein synthesis, cell growth, differentiation and motility, membrane potential, binding affinities, neurotransmitter release, phagocytosis, and prostaglandin and ATP synthesis [37]. Factors that play a role in the sperm volume generated in the study include environmental conditions, such as temperature, pH, and oxygen levels that can affect stress on fish so that the fish cannot produce large volumes of sperm. The difference in sperm volume produced being influenced by environmental factors, age, body size, feeding management, and sperm release frequency [38]. These observations are the color of striped catfish sperm is milky white and the consistency of striped catfish sperm is thick.

The low-power laser has significantly improved sperm quality [39], such as volume [39], concentration [40], motility, movement, and viability of spermatozoa [41]. The highest motility obtained in 0.5-joule treatment because this dose can provide a stimulatory effect large enough to stimulate the hypothalamus neuron to release the GnRH. GnRH stimulates pituitary neurons to release FSH and LH. FSH produces testosterone hormone, and LH produces an androgen-binding protein (ABP). Testosterone hormone and ABP control spermatogenesis and initiate spermatogenic development into motile spermatozoa [42]. Mantayborbir et al. [49] also found that laserpuncture induction has spontaneous stimulation power and rapidly influences the increase in the number of Leydig cells produced. The function of Leydig cells is to produce testosterone, which binds to androgen receptors in Sertoli cells, which secretes ABP and helps form spermatozoa.

Several studies indicate the influence of laserpuncture on improving the sperm quality of animals including motility [39, 41] and fertility [39] of spermatozoa, the adenosine

triphosphate (ATP) content [43], Ca^{2+} concentration [44], and cell life [45]. Ca^{2+} stimulates the work of the mitochondria and the ATP synthesis in the cell [46], while mitochondria and ATP play an important role in supporting spermatozoa motility [47].

In this study, the spermatozoa viability after laserpuncture treatment was reported to be good according to Rahardhianto et al. [48], who stated that the quality of spermatozoa is good based on spermatozoa viability of 80%. Spermatozoa viability also determines success and quality of fertility including in the fish.

5. Conclusion

Laserpuncture induction of 0.5-joule dose at the reproductive acupoint increase gonadal maturity and sperm quality of male striped catfish. Further studies on higher doses of laserpuncture and its effects on hormonal mechanisms are critical, especially in the fish.

Conflict of interest

No conflict of interest.

Acknowledgments

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Table 1. Gonadal maturity stage, GSI, and HSI of male striped catfish in the different treatments

Treatment	Gonadal maturity stage	GSI (%)	HSI (%)
Negative control	II[a]	0.39 ± 0.15[a]	1.10 ± 0.07[a]
Ovaprim™	II[a]	0.54 ± 0.29[ab]	1.30 ± 0.14[ab]
Ld of 0.2-joule	III to IV[b]	0.70 ± 0.38[ab]	1.32 ± 0.15[ab]
Ld of 0.4-joule	IV[b]	1.09 ± 0.19[b]	1.39 ± 0.26[b]
Ld of 0.5-joule	IV[b]	2.17 ± 0.68[c]	1.54 ± 0.17[b]

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Table 2. Average testicular histology scoring of male striped catfish in the different treatments

Treatment	Score	Description
Negative control	3[a]	Development of cells has reached spermatids
Ovaprim™	4[b]	Development of cells has reached spermatids
Ld of 0.2-joule	5[c]	Already formed spermatozoa of 25.00 to 49.90%
Ld of 0.4-joule	6[d]	Already formed spermatozoa of 50.00 to 74.50%
Ld of 0.5-joule	7[e]	Already formed spermatozoa of 75.00 to 100.00%

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Table 3. Macroscopically sperm quality of male striped catfish in the different treatments

Treatment	Spermatozoa quality parameters			
	Volume (ml)	pH	Color	Consistency
Negative control	Negative	Negative	Negative	Negative
Ovaprim^{TM}	Negative	Negative	Negative	Negative
Positive	0.50 to 1.25	7 to 8	Clear White	Dilute
Ld of 0.2-joule	0.50 to 1.50	7 to 8	White milk	Condensed
Ld of 0.4-joule	0.50 to 2.00	7 to 9	White milk	Condensed
Ld of 0.5-joule	3.00 to 5.25	7 to 9	Creamy	Condensed

Note: Negative control = without the laserpuncture and the ovaprim^{TM} treatments, Positive = the male striped catfish with the IV gonadal maturity stage treated the ovaprim^{TM} (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose.

Table 4. Microscopically sperm quality of male striped catfish in the different treatments

Treatment	Spermatozoa quality parameters		
	Motility (%)	Viability (%)	Concentration ($\times 10^9$cells/mL)
Negative control	Negative	Negative	Negative
Ovaprim^{TM}	Negative	Negative	Negative
Positive	58.88 ± 1.93^a	58.00 ± 1.58^a	4.31 ± 4.26^a
Ld of 0.2-joule	65.75 ± 2.32^b	66.25 ± 1.75^b	5.25 ± 4.56^b
Ld of 0.4-joule	73.00 ± 2.73^c	73.00 ± 2.27^c	6.06 ± 6.25^c
Ld of 0.5-joule	81.75 ± 1.19^d	82.75 ± 1.84^d	7.00 ± 5.40^d

Note: Negative control = without the laserpuncture and the ovaprim^{TM} treatments, Positive = the male striped catfish with the IV gonadal maturity stage treated the ovaprim^{TM} (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

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The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (*Pangasianodon hypophthalmus*)

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Abstract

Laserpuncture is one of the applicative technologies used mainly in animal and fish reproductions. Laserpuncture technology has been used to improve gonadal maturity and sperm quality in fish rapidly. This study aimed to determine the effects of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish. Males striped catfish (800 to 900 g/fish body weight) and I gonadal maturity stage were used. Semi-conductor soft laser was used with doses of 0.2-, 0.4-, and 0.5-joule, while the negative control (without the laserpuncture and the ovaprim^{TM}) and only the ovaprim^{TM} were used as a comparison treatment, respectively. The soft-laser was treated on reproductive acupoint every week for four weeks, while the ovaprim^{TM} was administered by intramuscular injection at dose of 0.2 mL/kg fish in final rearing period. Fish was reared in hapa at the controlled pond. Fish was fed with a commercial feed containing 32% crude protein. Gonadal maturity, gonadosomatic index (GSI), hepatosomatic index (HSI), and sperm quality of male striped catfish were measured in the final rearing period. The results showed that the laserpuncture on the reproductive acupoint had a highly significant effect ($P<0.01$) on the gonadal maturity, GSI, HSI, and sperm quality of male striped catfish. In terms of the gonadal maturity, laserpuncture doses treatment of 0.4 and 0.5-joule gave the most mature IV stage. While the highest levels of GSI and HSI were found in 0.5-joule of laserpuncture dose, which was 2.17% and 1.54%, respectively. In addition, the best sperm qualities were observed in 0.5-joule of laserpuncture dose, which were 81.75% motility, 82.75% viability, and 7.0×10^9 cell/mL concentration. These results suggest that the laserpuncture can accelerate a gonadal maturity and improve sperm quality in male striped catfish.

Keywords: Laserpuncture; Semi-conductor soft laser; Gonadal maturity; Sperm quality; Male striped catfish

1. Introduction

Striped catfish has been considered as one of the main cultured fish in Indonesia due to several advantages, such as fast growth, easy cultivation, and high tolerance to low dissolved oxygen content in the rearing water [1]. The production of striped catfish in Indonesia was 339,060 metric tonnes (MT) in 2015 and increased rapidly in 2016 become 447,110 MT [2]. The high market demand triggers farmers to increase the amount of striped catfish production. However, one of the main problems faced by farmers was the supply of striped catfish seed, which depends on the spawning season. Zairin [3] stated that the reproductive cycle of striped catfish occurs naturally during the rainy season from October to April every year.

The process of gonadal maturity in striped catfish needed a long time and depended on the increase in gonadotropin hormone (GtH) and gonadal steroid. The productive period of spawning in male striped catfish peaks at the age of two years or about 1.5 to 2.0 kg body weight [4]. Other constraints of a male fish are the decrease in sperm quality, such as motility and viability after spawning.

To increase production of striped catfish seed requires new technology to improve the quality of reproduction using laserpuncture technology. Laserpuncture technology is a stimulation technique on acupuncture points (acupoint) by a laser beam [5]. The use of the laserpuncture could reduce production costs [6]. Susan [5] stated that the application of laserpuncture in the reproductive organs could stimulate several reproductive functions of male and female animals. Laserpuncture technology has been proven to accelerate gonadal maturity, spawning processes, and to shorten the reproductive cycles of several aquatic species, such as catfish, *Clarias gariepinus* [7, 8] and mud crab, *Scylla serrata* [9]. Other author explained that the main advantages of laserpuncture technology as a stimulation

method are requiring a short time takes only 5 to 10 s, does not cause tissue damage, and provides a maximum response [10], depending on the type of soft-laser used.

The low-power laser affects the biology system of humans [11] and animals [12], including aquatic organisms (fish). The low-power laser around 5 to 30 mW improves tissue activity, such as increased production of hormones and enzymes [13]. The low-power laser gives a biological stimulus, such as changing the cell membrane's potential and permeability. On the other hand, it improves the nerve regeneration ability located both in central and peripheral sides will be produced to increase the cellular activities, and the ability to produce hormones and enzymes [13, 14].

Several studies have shown that the laserpuncture improves the vascular and endocrine systems, and various other body systems [15]. The low-power laser also improves the male fertility of humans [16], animals [17], and aquatic organisms, such as fish [17] and sea urchins [18]. Kusuma et al. [7, 8] found that the use of the low-power laserpuncture technology on the reproductive acupoint precisely in 2/3 ventral parts of the body through induction once a week is optimal for the maturation of catfish gonads. Moreover, the low-power laser induction at the reproductive acupoint for 15 seconds increases the production of the GtH [10], which is the regulator for produce steroid, oogenesis, and oocyte maturation [19]. On the other hand, induction of laserpuncture also increases the testosterone level in blood serum and the gonadosomatic index (GSI) of male catfish [8].

The low-power laser has a significant effect on the reproductive performance of fish whose spawning cycle occurs throughout the season, however, does laserpuncture induction affect fish whose spawning cycle depends on the season? Therefore, this study was aimed to determine the effect of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish.

2. Materials and methods

This study was conducted at the Fish Breeding Research Centre, Subang, West Java, Indonesia. Histological analysis of fish gonads was held at the Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia. In this study, the experimental protocols were approved by the Scientific Committee, Institute of Research and Innovation, Universitas Airlangga (Protocol Number 886/UN3/2018).

2.1. Animal

Male striped catfish with the average body weight of 800 to 900 g/fish and the I gonadal maturity stage and never been spawned before was used in the present study. As a precaution, male striped catfish with the IV gonadal maturity stage were also prepared separately for positive treatment.

2.2. Laserpuncture

In this study, a semi-conductor soft-laser was used, which had a power specification of 20 mW. The preset doses and timer set were 0.2, 0.4, and 0.5 with 10, 20, and 25 s, respectively.

2.3. Experimental design

This study was used the completely randomized design structure consisting of five treatments with four replicates. Treatments used were doses of 0.2-, 0.4-, and 0.5-joule, without laserpuncture and ovaprim^{TM} treatments as a negative control, and treatment of the ovaprim^{TM} dose of 0.2 mL/kg fish body weight in male striped catfish with the I gonadal maturity stage. The IV gonadal maturity stage of male striped catfish as positive treatment was treated the ovaprim^{TM} dose of 0.5 mL/kg fish body weight, correctly was only used to observe sperm quality parameters.

Fish were adapted and reared in a hapa sized of 5.0×3.0×1.5 m^3 at a controlled pond and fed commercial feed containing 32% crude protein. Laserpuncture treatment was performed on reproductive acupoint every week for four weeks. Reproductive acupoint located on the

2/3 ventral part of the body (governoer vessel) was measured from the anal to the pectoral fin. The determination of reproductive acupoint was also done using an electro-acupuncture device tool. On the other hand, the ovaprim^{TM} was treated by using the intramuscular technique in the final rearing stage (week 4), 8 to 10 h before the end rearing of fish. Gonadal maturity (morphology and histology), gonadosomatic index (GSI) and hepatosomatic index (HSI), and sperm qualities, both macroscopic and microscopic, were measured.

2.4. Morphology and histology of gonadal maturity, GSI, and HSI

At the end of the rearing process, fish was anesthetized using MS222 (Argent Laboratories, Redmond, Washington, DC) of 100 ppm [20] for 10 min. Subsequently, the fish were dissected on the abdominal part from anal to ventral. Morphological and histological gonadal maturity was observed on the shape, the length, the weight, the color, and the gonadal development through histology preparation. Gonad sample was prepared according to Junqueira and Carneiro [21] to measure the GSI and HSI.

Next, the histological method was conducted by McCann [22] using Hematoxylin-Eosin (HE) staining method according to Genten et al. [23]. Scoring of testis cell development was conducted according to Çek and Yilmas [24].

2.5. Sperm qualities

The collection of sperms from the fish was conducted by using the stripping method. The sperm were stored in 1.5 mL microtubes in the refrigerator at a temperature of 4^{o}C before the analysis of microscopic qualities.

2.5.1. Motility of spermatozoa

Microscopic spermatozoa motility was observed under 100 × and 400 × magnifications using a BH2-RFCA Olympus binocular microscope (Olympus Optical Ltd. Shinjuku-ku, Tokyo, Japan), which was equipped with a camera. Motile and immotile of the spermatozoa were calculated using a modified method by Sohouka et al. [25], as well as a progressive or

active movement forward and non-progressive movements (such as circular, backward or silent).

2.5.2. Viability of spermatozoa

The determination of spermatozoa viability was done by the staining protocol of 2% eosin yellow at sperm preparation. The viability of the spermatozoa was observed and counted under 400 × and 1000 × magnifications using the same microscope like the one used to observe motility of the spermatozoa. The spermatozoa viability was counted according to Sohouka et al. [25].

2.5.3. Concentration of sperm

The sperm concentration was calculated according to Stoss and Donaldson [26].

2.6. Data analysis

Gonadal maturity stage was statistically analyzed using Kruskal Wallis and Mann Whitney tests, while the morphology and histology of the organ were descriptively analyzed according to Genten et al. [23]. The other data were statistically analyzed using analysis of variant (ANOVA) with SPSS ver. 10 software and Duncan's multiple range test with a confidence level of 95%.

3. Results

3.1. Gonadal maturity stage, GSI, and HSI

This study showed that laserpuncture treatment affected the gonadal development of male striped catfish, as seen in Figure 1. In general, gonad characteristics of fish with the I gonadal maturity stage are testicles like threads, smaller, shorter and limited, and visible ends in the body cavity. The II gonadal maturity stage (Figures 1a and 1b) have more significant and more apparent the testis size and shape compared to the I gonadal maturity stage. The morphology characteristics of the III gonadal maturity stage are the surface of the testicles

appear jagged, more prominent, and the whiter color (Figure 1c). The IV gonadal maturity stage of male striped catfish has testis characteristics of more definite, denser, and milky white color (Figures 1d and 1e).

The IV gonadal maturity stage achieved through laserpuncture treatment of 0.5-, 0.4-, and 0.2-joule doses showed no significant difference between all the joules ($p>0.05$). However, there was significant difference compared to the negative control and the ovaprim^{TM} treatment ($p<0.05$) as seen in Table 2. The results showed that ovaprim^{TM} treatment does not have a significant effect on gonadal development; the gonadal maturity only reached the II stage (immature). It could not be stripped to collect sperms, then the laserpuncture-treated male fish compared with ovaprim^{TM}-treated male fish that has the IV gonadal maturity stage (as positive treatment) on sperm quality.

This study indicated that GSI and HSI had significant differences between treatments ($p<0.05$). The laserpuncture treatment of 0.5-joule had the highest GSI and HSI compared to other treatments, although the HSI is relatively not significantly different between treatments, except compared to the negative control (Table 1).

Similarity, the laserpuncture dose of 0.5-joule had the highest effect on testicular development. This study also showed that the laserpuncture of 0.5-joule dose has the highest scoring of gonadal or testicular histology in male striped catfish (Figure 2) with testis containing spermatozoa of 75 to 100%, as seen in Table 2.

Based on the testicular histology of male striped catfish in Figure 2 shows that the seminiferous tubules are still empty and the spermatozoa are not visible (Figures 2a and 2b) as one of the II gonadal maturity stage characteristics in the fish. In the III gonadal maturity stage, seminiferous tubules already containing spermatozoa (Figure 2c), while the IV gonadal maturity stage, seminiferous tubules containing more spermatozoa (Figure 2d), even full spermatozoa (Figure 2e).

3.2. Sperm qualities

This study showed that laserpuncture treatment produces higher sperm quality, both macroscopic and microscopic, compared to negative and positive treatments, as seen in Tables 3 and 4. The laserpuncture treatment of 0.5-joule power resulted in the production of the highest volume of sperms, i.e., 3.00 to 5.25 mL with a creamy color (Table 3). On the other hand, the treatments showed significant differences ($p<0.05$) in microscopic sperm quality. The laserpuncture treatment of 0.5-joule power had the highest microscopic sperm qualities compared to other treatments, as seen in Table 4.

4. Discussion

The induction of laserpuncture on the reproductive acupoint in fish has proven to accelerate the development and maturity of the gonad including male gonad of striped catfish from I stage to IV stage, while in the control treatments (negative control and ovaprim^{TM} treatment), the maturity of the gonad developed from I stage to only II stage during one month. This study result was consistent with the study conducted by Matayborbir et al. [27] in the catfish that laserpuncture exposure accelerate gonadal maturity from II stage to IV stage of male rapidly. The condition of fish gonads at the beginning of the study was at the initial gonadal maturity stage with a small gonadal morphology and appeared clear. Then, after the laserpuncture treatment, the gonadal maturity reached IV stage (fully maturated).

GSI has been used as one of the indicators of the development and maturity of gonad [28] in both sexes. In general, GSI increases with the increasing gonadal maturity stage as well as a show in this study (Table 1). Increased GSI followed the bigger size of the gonad (testis), as shown in Figure 1, and increase the number of spermatozoa produced by testis (Figure 2). Kusuma [7] stated that laserpuncture stimulates cell activations in the area governoer vessel (reproductive acupoint) to produce energy. The formation of energy after laserpuncture

exposure in the reproductive acupoint related to specific proteins in cells. As the results, the development of gonads gradually from spermatogonia, spermatocytes, spermatids, and spermatozoa, which are marked by the bigger testicles. The laserpuncture exposure at reproductive acupoints in male striped catfish increased GSI and HSI. GSI will continue to increase along with the maturation of the fish gonads and will reach the maximum value when the peak period of gonad maturity [29].

The exposure of laserpuncture on the reproductive acupoint stimulate FSH and LH from the pituitary, which has a vital role in the development and maturity of the gonad. Although FSH and LH levels were not measured in this study, a previous study [7] have reported that laserpuncture exposure to reproductive acupoint increased FSH and LH levels and also accelerated LH formation which play a role in the final stage of spermatogenesis or spermatozoa production, testicular maturation, ovulation, and spawning stimulation in catfish. It indicated that the induction of laserpuncture could increase the performances of the activity of the hormone, which takes part in a reproduction control system to accelerate the provision of growth, development, and gonad maturation of fish.

Furthermore, the induction of laserpuncture on the tissue increased gonadotropin-releasing hormone (GnRH) released by the hypothalamus and it stimulated the anterior pituitary to secrete FSH and LH which act on theca cells in the male gonad (testis) to produce testosterone hormone [30]. Similarly, a study by Chang et al. [31] showed that laserpuncture stimulated the release of neurotransmitters. The laser beam transduced into chemical signals band being received by various ion channels, such as G-proteins (GTP-binding protein)-coupled receptors subunit α and VGCC (voltage-gated Ca2+ channels). Other possible mechanisms is through calcium receptors, such as calcium-sensing receptor (CaSR), located in the nervous membrane cells. Then, ligands binding on the specific receptors triggers the release of second messengers causing a chain reaction and bringing changes in the cell.

Electrical signals caused by depolarization of the nerve cell membrane were propagated from cell to cell along the axon. Next, the electrical signals were inserted from the pre-synapse membrane into the post-synapse membrane and trigger the release of neurotransmitter molecules in the synapse. The same mechanism, the electrical signals were transmitted to the brain. intracellular and extracellular Ca2+ ions mediated the electrical signals through changes in spontaneous membrane potential play an essential role in stimulating the release of GnRH from the hypothalamus and it stimulates the pituitary to release FSH and LH. Next, FSH and LH are then channeled into the bloodstream towards the gonads (testis), which enable various activities. This process repeats when the nerve cell membrane is depolarized [32].

Other study by Anglade et al. [32] explained that the release of neurotransmitters such as gamma-aminobutyric acid (GABA) from GABAergic neurons depends on nerve cell membrane depolarization, action potential, calcium ions, decarboxylation of glutamate, and glutamic acid decarboxylase (GAD). The anterior pituitary directly innervates GABAergic neurons, so it has a stimulatory effect on the release of LH [33].

Factors which determined the volume of sperm production include environmental conditions, such as temperature, pH, and dissolved oxygen levels. Fish which are in stress condition due to environmental condition cannot produce large volumes of sperm. The same factors have been confirmed by a study of Salisbury and VanDenmark [34] where the volume of sperm production was influenced by environmental factors, age, body size, feeding management, and sperm release frequency.

The low-power laser has significantly improved sperm quality, such as volume [35], concentration [36], motility, movement, and viability of spermatozoa [37]. The highest motility obtained in 0.5-joule treatment because this dose can provide a stimulatory effect large enough to stimulate the hypothalamus neuron to release the GnRH. GnRH stimulates

pituitary neurons to release FSH and LH. FSH produces testosterone hormone, and LH produces an androgen-binding protein (ABP). Testosterone hormone and ABP control spermatogenesis and initiate spermatogenic development into motile spermatozoa [38]. Mantayborbir et al. [27] also found that laserpuncture induction has spontaneous stimulation power and rapidly influences the increase in the number of Leydig cells produced. The function of Leydig cells is to produce testosterone hormone, which binds to androgen receptors in Sertoli cells, which secretes ABP and helps form spermatozoa.

Several studies indicate the influence of laserpuncture on improving the sperm quality of animals including motility [35, 37] and fertility [35] of spermatozoa, the adenosine triphosphate (ATP) content [39], Ca^{2+} concentration [40], and cell life [41]. Ca^{2+} stimulates the work of the mitochondria and the ATP synthesis in the cell [42], while mitochondria and ATP play an important role in supporting spermatozoa motility [43]. Furthermore, laserpuncture improved protein synthesis, cell growth, differentiation and motility, membrane potential, binding affinities, neurotransmitter release, and ATP synthesis [44].

The best spermatozoa viability obtained in the present study after laserpuncture treatment was 82.75% at dose of 0.5-joule. The result was considered to be good according to Rahardhianto et al. [45], who stated that the quality of spermatozoa is good based on spermatozoa viability of 80%. Spermatozoa viability also determines successfullness of fertility including in the fish.

The FSH will stimulate Leydig cells to produce testosterone hormone. Testosterone hormone as a part of an androgen steroid hormone plays a vital role in the reproductive tissue development and the secondary sexual characteristics expression of male. GnRH promotes the secretion of LH by stimulating the pituitary gland. Then, LH promotes the synthesis of testosterone hormone by stimulating the Leydig cells of the testis [46]. Alves et al. [47] also

have proven that the induction of low-level laser improves the testosterone hormone level in males by increasing 11-ketotestosterone hormone level in spermatogenesis.

The result was consistent with a study by Schulz et al. [48] in which 11-ketotestosterone hormone influenced more in the spermatogenesis than testosterone hormone. The concurrent elevation of 11-ketotestosterone and testosterone hormones level is another typical pattern in male fish [49]. Therefore, the measurement of 11-ketotestosterone and testosterone hormones will be investigated for further laserpuncture studies of fish.

5. Conclusion

Laserpuncture induction of 0.5-joule dose at the reproductive acupoint increase gonadal maturity and sperm quality of male striped catfish. Further studies on higher doses of laserpuncture and its effects on hormonal mechanisms are critical, especially in the fish.

Conflict of interest

No conflict of interest.

Acknowledgments

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Table 1. Gonadal maturity stage, GSI, and HSI of male striped catfish in the different treatments

Treatment	Gonadal maturity stage	GSI (%)	HSI (%)
Negative control	II[a]	0.39 ± 0.15[a]	1.10 ± 0.07[a]
Ovaprim™	II[a]	0.54 ± 0.29[ab]	1.30 ± 0.14[ab]
Ld of 0.2-joule	III to IV[b]	0.70 ± 0.38[ab]	1.32 ± 0.15[ab]
Ld of 0.4-joule	IV[b]	1.09 ± 0.19[b]	1.39 ± 0.26[b]
Ld of 0.5-joule	IV[b]	2.17 ± 0.68[c]	1.54 ± 0.17[b]

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Table 2. Average testicular histology scoring of male striped catfish in the different treatments

Treatment	Score	Description
Negative control	3[a]	Development of cells has reached spermatids
Ovaprim™	4[b]	Development of cells has reached spermatids
Ld of 0.2-joule	5[c]	Already formed spermatozoa of 25.00 to 49.90%
Ld of 0.4-joule	6[d]	Already formed spermatozoa of 50.00 to 74.50%
Ld of 0.5-joule	7[e]	Already formed spermatozoa of 75.00 to 100.00%

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Table 3. Macroscopically sperm quality of male striped catfish in the different treatments

Treatment	Spermatozoa quality parameters			
	Volume (ml)	pH	Color	Consistency
Negative control	Negative	Negative	Negative	Negative
Ovaprim^{TM}	Negative	Negative	Negative	Negative
Positive	0.50 to 1.25	7 to 8	Clear White	Dilute
Ld of 0.2-joule	0.50 to 1.50	7 to 8	White milk	Condensed
Ld of 0.4-joule	0.50 to 2.00	7 to 9	White milk	Condensed
Ld of 0.5-joule	3.00 to 5.25	7 to 9	Creamy	Condensed

Note: Negative control = without the laserpuncture and the ovaprim^{TM} treatments, Positive = the male striped catfish with the IV gonadal maturity stage treated the ovaprim^{TM} (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose.

Table 4. Microscopically sperm quality of male striped catfish in the different treatments

Treatment	Spermatozoa quality parameters		
	Motility (%)	Viability (%)	Concentration ($\times 10^9$cells/mL)
Negative control	Negative	Negative	Negative
Ovaprim^{TM}	Negative	Negative	Negative
Positive	58.88 ± 1.93^a	58.00 ± 1.58^a	4.31 ± 4.26^a
Ld of 0.2-joule	65.75 ± 2.32^b	66.25 ± 1.75^b	5.25 ± 4.56^b
Ld of 0.4-joule	73.00 ± 2.73^c	73.00 ± 2.27^c	6.06 ± 6.25^c
Ld of 0.5-joule	81.75 ± 1.19^d	82.75 ± 1.84^d	7.00 ± 5.40^d

Note: Negative control = without the laserpuncture and the ovaprim^{TM} treatments, Positive = the male striped catfish with the IV gonadal maturity stage treated the ovaprim^{TM} (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p<0.05$).

Your Submission THERIO-D-19-00521R3

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Ms. Ref. No.: THERIO-D-19-00521R3
Title: The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (Pangasianodon hypophthalmus)
Theriogenology

Dear Dr. Mukti,

I am pleased to confirm that your manuscript, "The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (Pangasianodon hypophthalmus), has been accepted for publication in Theriogenology.

Your accepted manuscript will now be transferred to our production department and work will begin on creation of the proof. If we need any additional information to create the proof, we will let you know. If not, you will be contacted again in the next few days with a request to approve the proof and to complete a number of online forms that are required for publication.

Thank you for submitting your work to this journal.

Sincerely,

Leonardo Brito, DVM, PhD, DACT
Associate Editor
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