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Research Article
Effects of Exogenous Melatonin and Zinc Amino Acid on Male *Clarias macrocephalus* Broodstock

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Abstract

Background and Objective: Melatonin and zinc amino acid is involved in the reproductive processes through the hypothalamus-pituitary-gonadal axis and development process, respectively. The aim of this study was to investigate the effects of melatonin and zinc amino acid (ZnAA) feeding administration on the maturation of male walking catfish, *Clarias macrocephalus*. **Materials and Methods:** The combinations of melatonin and ZnAA at the concentration of 0 ppm ZnAA and 0 mg kg^{-1} melatonin (Control), 100 ppm ZnAA and 50 mg kg^{-1} melatonin (MZn1) and 200 ppm ZnAA and 50 mg kg^{-1} melatonin (MZn2) were to enhance male reproductive system. The testis histology, gonadosomatic index and sperm quality were investigated. **Results:** The melatonin-ZnAA feed was found to be consistent with higher spermatozoa cell counts (Control: 51.3%, MZn1: 75% and MZn2: 70.5%). Furthermore, the feeding treatment increased the gonadosomatic index (0.45-0.96%, p: 0.005), decreased the sperm abnormality (14.5-44%, p: 0.022), increased the sperm motility (3.9-25.5%, p: 0.013) and increased the sperm concentration (109.4-317.5 10^6 mL^{-1}, p: 0.037) after 8 weeks of treatment. **Conclusion:** The present results showed that melatonin-ZnAA (MZn2) helped in enhancing the maturation of male *C. macrocephalus*.

Key words: Melatonin, zinc amino acid, reproduction, male, catfish

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Clarias macrocephalus or walking catfish is widely distributed in Asia. It is a freshwater fish and classified under the family Clariidae. It is mainly found in the wetlands and rivers and is capable of lying buried in mud for lengthy periods if water dries up during the dry seasons[1]. Although the locals prefer *C. macrocephalus*, its production is limited due to the introduction of catfish hybrids and the limited habitat caused by urbanization and over-exploitation[2]. While the development of aquaculture farming of *C. macrocephalus* has resulted in high demands of *C. macrocephalus* broodstock, the broodstock have become limited due to over-exploitation of the natural habitat. Previous study stated that the timing of sexual maturity and successful spawning programme are vital to operate the commercial catfish farming[3]. Several programmes have been developed to accelerate the sexual maturation in cultured catfish, including hormonal and environmental manipulations[3]. It is important to accelerate the maturation in order to enhance the population and increase the production of *C. macrocephalus*.

Melatonin is an in dolamine hormone synthesised in the pineal gland and controlled by photoperiod. During the night cycle, two enzymatic steps allow the formation of serotonin from tryptophan[4]. The tryptophan hydroxylation catalyzed by tryptophan hydroxylase (TpOH) allows the synthesis of hydroxytryptophan. Hydroxytryptophan is then decarboxylated by the aromatic amino acid decarboxylase, leading to the formation of serotonin. Next, the arylalkylamine N-acetyltransferase (AANAT) catalyses the formation of N-acetylserotonin and the hydroxyindole-O-methyltransferase (HIOMT) converts the N-acetylserotonin into melatonin[4,5]. Previous study have suggested that melatonin is involved in the reproductive processes through the hypothalamus-pituitary-gonadal endocrine axis[6]. Previous *in vitro* studies have reported that melatonin induction into the preoptic anterior hypothalamic area (POAH) and the pituitary stimulates the luteinizing hormone (GTH-II) levels[7]. Additionally, melatonin may also stimulate GTH-I by activating the sGnRH synthesis in the brain[6]. Apart from stimulating GTH-I and GTH-II, melatonin also has a direct effect in ovarian functions. Melatonin receptors are found in the ovary, brain and other peripheral tissues. The membrane melatonin receptors are classified as MT1, MT2 and MT3 subtypes while nuclear melatonin receptors are classified as RZR/ROR family[8] where the direct effect of melatonin receptors mechanism is by its metabolites functioning as direct scavengers of free radicals and indirect antioxidant. Zinc is an essential trace element required during developmental processes, being a co-factor for enzymatic activities, such as DNA and RNA polymerases[9]. In addition, zinc is one of the most abundant transition metal, it binds strongly to metalloproteins (cystine- rich proteins) in the liver[10]. Metallothionein that binds with zinc is able to protect cells from free radical groups. Metallothionein stabilizes DNA conformation through its involvement in numerous DNA repair enzymes, especially during the early embryogenesis[10,11]. Vitellogenin from the hepatic cells is abundant with metallothionein. Vitellogenesis transported via the blood circulation to the growing oocytes to form the major yolk proteins for the developing embryo and larvae after spawning[12]. Zinc deficiency may lead to growth impairment and congenital malformation of fetal organs[13]. For male fertility, zinc deficiency may lead to negative effect on serum testosterone concentration and seminal plasma, eventually causing infertility. Metallothionein also acts as free radicals scavenger and stabilizing agent of sperm membrane and nuclear chromatin[14,15].

Previously, melatonin and zinc treatment showed significant improvement towards the maturation of female and male *C. macrocephalus*[16,17]. To further the understanding of the influence of melatonin and ZnAA in male *C. macrocephalus,* this study investigated the effects of melatonin-ZnAA feed on the male broodstock of *C. macrocephalus.*

MATERIALS AND METHODS

Animals: The *Clarias macrocephalus* broodstock were obtained from the Fisheries Station of Kham PhengPhet, Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand and the trial experiments were done in the Laboratory of Nutrition and Aqua feed, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The 8 week old catfish were acclimatized in 500 L tanks at the density of 15 ind m^{-2}/fish/tank and fed with control feed for 2 weeks prior to experiment. The water supply for the experiment was aerated to maintain the oxygen supply in the experiment tank.

Experimental diets: A feeding trial comprised of one control and two combinations of melatonin-ZnAA diet. The basal diet was formulated from practical ingredients, containing 22% fishmeal, 35% soybean, 1% spirulina, 12% wheat flour, 11.8% tapioca, 5% ricebran, 2% fish oil, 3% soy oil, 1.2% mineral premix, 2% soy lecithin, 1.5% calcium phosphate, 1% attractant, 2% binder and 0.5% vitamin premix. The diet also consisted of 37% crude protein and 9.3% crude lipid. Diets containing zinc and melatonin were prepared by adding

graded levels of zinc and melatonin to the basal diet. The ZnAA and melatonin concentrations: 0 ppm ZnAA and 0 mg kg^{-1} melatonin (Control), 100 ppm and 50 mg kg^{-1} melatonin (MZn1) and 200 ppm and 50 mg kg^{-1} melatonin (MZn2)[18,19].

Experimental condition: A total of 45 male catfish of mean weight 88.07±9.57 g (Mean±SD) were starved in tanks for 2 days prior to the experiment. All the population was subjected to a normal photoperiod (12L:12D) prior to the treatment. The fish were fed at an amount equivalent to 3% of their body weight and this amount of diet was divided into two equal feedings per day. The fish were randomly distributed in three treatments (Control, MZn1 and MZn2) replicated three times. Following acclimation, the fish were exposed to the treatments for 8 weeks. This study project including experimental trial and laboratory analysis was conducted from early January, 2013 to end of July, 2013 (7 months).

Histology: The testis from each treatment was fixed in 10% buffered formalin. The fixed ovaries were placed in the cassettes for embedding process in paraffin wax. Serial sections were made at four micrometer and were processed using the standard method of histology for sample cutting. For staining method, the samples were stained using Haematoxylin-eosin method. The histology method was according to previous study[20]. The samples' cells were counted by microscope.

Gonadosomatic index: The Gonadosomatic Index was determinedas[21]:

$$GSI = 100\ (Gm/Tm)$$

Where:
Gm = Mass of gonad
Tm = Total mass of fish

Characteristics of semen and spermatozoa: Testes from the treated fish were surgically removed and were cut in small pieces to allow sperm release after a gentle squeezing. Semen was stored with extender solution in sterile Appendorf tubes on ice before use. The composition of extenders was as followed: 8.760 g NaCl in 1000 mL distilled water[22].

Live sperm rate and sperm concentration: Eosin-nigrosin stained semen smears were evaluated with brightfield microscopy for the live cells by counting 400 cells per sample. The sperm concentration was determined in duplicate and expressed as number of spermatozoa $\times 10^6$ mL^{-1}. The number of spermatozoa was counted in improved Neuber haemocytometer under a light microscope.

Sperm kinetic characteristic: Kinetic characteristics were evaluated immediately after semen collection. Ten microlitres of the samples were diluted 1:1 with the extender solution and were laid over a pre-warmed Hamilton Thorne Biosciences chamber at 27.8°C. The analyses were done by the Computer-Assisted Sperm Analysis (CASA). The sperm parameters recorded were motility (%) = The percentage of motile spermatozoa, progressive (%) = The percentage of spermatozoa with a progressive motility, path velocity (μm sec^{-1}) = A smoothed path constructed by averaging several neighbouring positions on the track (five points) and joining the averaged positions, which reduced the effect of lateral head displacement, prog. velocity (μm sec^{-1}) = The straightline distance between the first and last tracked points, divided by the acquisition time, track speed or curvilinear velocity (μm sec^{-1}) = the total distance between adjacent points, divided by the time elapsed, lateral amplitude (μm) = The mean width of the head oscillation as the sperm cells swim, beat frequency (Hz) = The frequency of sperm head crossing the average path in either direction, straightness (%) = An index of the departure of the sperm path from a straight line, linearity (%) = An index of the straightness of the path[23,24].

Sperm abnormality: Fresh semen in the extender solution was stained within ten min post-collection. Eosin-nigrosin stained semen smears were evaluated using brightfield microscopy at ×1000 magnification under oil immersion and the percentages of abnormal and normal sperm cells were determined.

Statistical analysis: All data were analysed by one-way ANOVA (analysis of variance), followed by the Tukey's honest significance test to analyse the significance between the treatment means. All means comparisons significance was tested at $p<0.05$ using SPSS software[25].

RESULTS AND DISCUSSION

Histological changes in testes during the melatonin-ZnAA treatment were observed in *C. macrocephalus* (Fig. 1). In the present study, histological analysis showed high spermatogonium percentage in the control treatment (20% (control), 9.9% (MZn1) and 3.8% (MZn2)) and high spermatozoa percentage for both combinations of

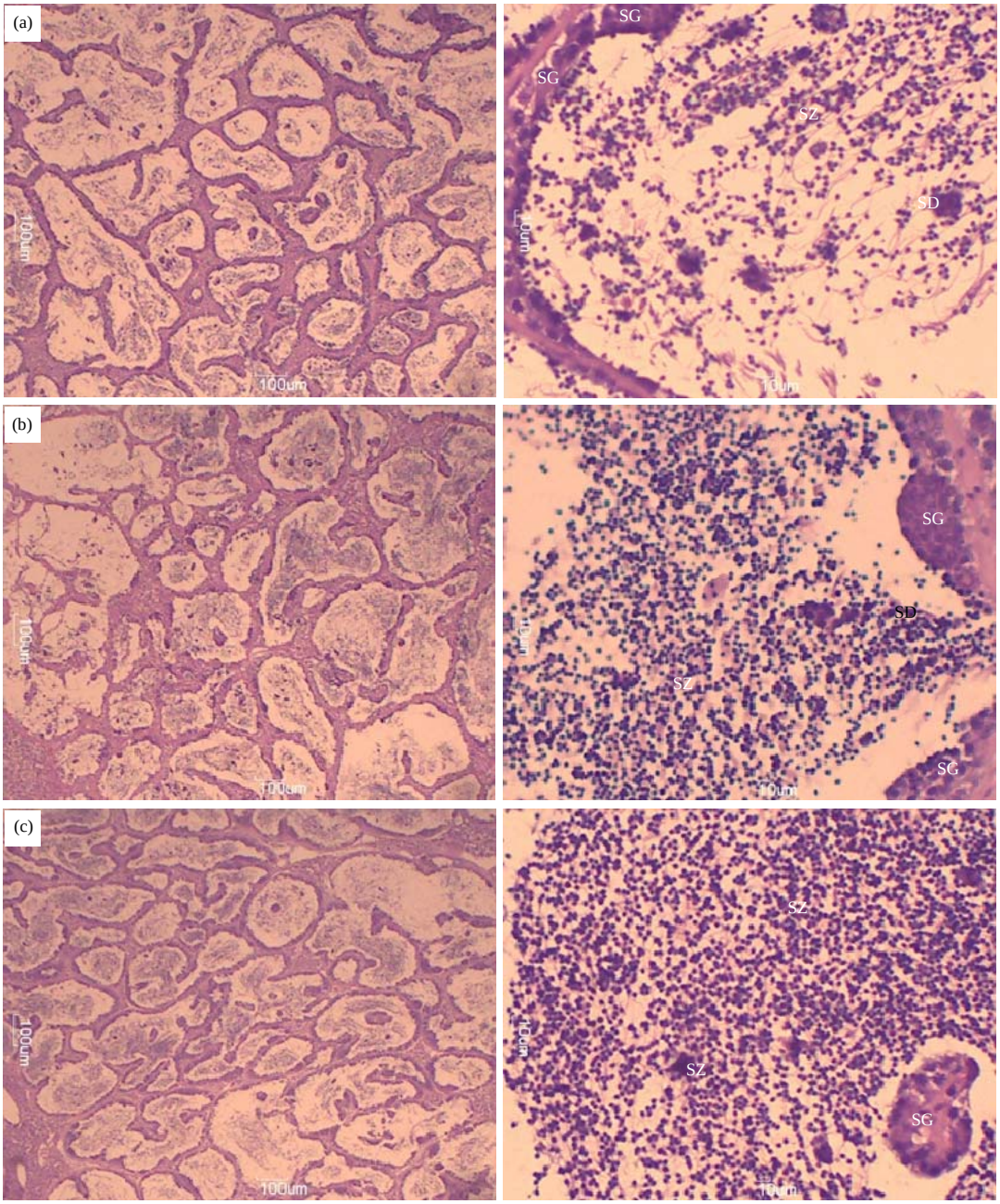

Fig. 1(a-c): Effects of melatonin-ZnAA treatment on testis histology of the *C. macrocephalus*. Cross section of testis treated with ZnAA and control, (a) Control, (b) MZn1 and (c) MZn2. Spermatogonia (SG), spermatids (SD) and spermatozoa (SZ) scale bar: 100 μm

melatonin-ZnAA treatment (51.3% (control), 75% (MZn1) and 70.5% (MZn2)) (Table 1). Spermatogonia and spermatozoa were observed for the testicular development and staging[26]. It also showed that the gonadosomatic index was a significant increase with the mean ranging from 0.45-0.96 %, $p = 0.005$ (Table 1). Spermatozoa cells are the smallest and the most mature cells among the other sperm cells in the sperm developmental stages[27]. In addition, the testicular development is generally divided into four stages: Spermatogonia, spermatocytes, spermatids and spermatozoa[26]. Spermatogonia are the undifferentiated and immature sperm cells, having large ovoid cells, central and vesicular nucleus, distinct nuclear membrane and perinuclear cytoplasmic granules, prominent nucleolus enclosed in a membrane of the spermatogonial nucleus[26]. Meanwhile, spermatozoa are the mature sperm cells and found in

Table 1: Maturation analysis of male *C. macrocephalus* with melatonin and different ZnAA levels (Mean±SE)

Treatments	Control	MZn1	MZn2	p-value
Histology spermatogonium cell (%)	20.0	9.9	3.8	-
Histology spermatid cell (%)	28.7	15.1	25.7	-
Histology spermatozoa cell (%)	51.3	75.0	70.5	-
Gonadosomatic index (%)	0.45±0.03^{b}	0.61±0.17^{a}	0.96±0.22^{a}	0.005

^{a,b}Values with different superscripts in a row differ significantly (p<0.05)

Table 2: Sperm analysis for *C. macrocephalus* with melatonin and different ZnAA levels (Mean±SE)

Treatment	Control	MZn1	MZn2	p-value
Sperm abnormality (%)	44.0±34^{b}	20.63±7^{a}	14.5±5.0^{a}	0.022
Sperm concentration (10^{6} mL^{-1})	109.4±82^{b}	197.50±116^{a}	317.5±217^{a}	0.037
Live sperm rate (%)	70.6±29	89.10±4	90.0±6.7	0.063

^{a,b}Values with different superscripts in a row differ significantly (p<0.05)

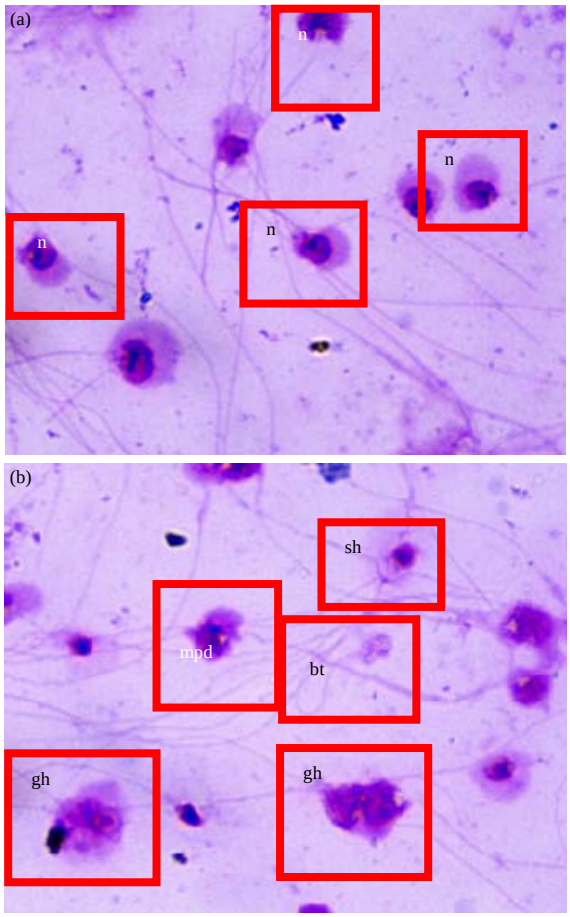

Fig. 2(a-b): Some of the sperm abnormality traits in melatonin-ZnAA treatment of the male *C. macrocephalus*, (a) Normal *C. macrocephalus* spermatozoa and (b) Abnormal *C. macrocephalus* spermatozoa. Normal (n), bend tail (bt), giant head (gh), midpiece defect (mpd) and small head (sh)

abundance in the lumen of the seminiferous lobules. In male teleost, melatonin plays a significant role in regulating the testicular event. Melatonin treatment may act through interneurons to influence the GnRH neural activity and regulate follicle stimulating hormone (GTH-I) in male teleost[6]. On the other hand, zinc plays an important role in the synthesis of GTH-I and GTH-II gonadotropins hormone in male teleost. Even though the zinc mechanism on synthesizing the gonadotropins is not fully understood, it is known that zinc deficiency may lead to decreasing GTH-I and GTH-II production[28]. In the teleost testicular event, GTH-I regulates Leydig cell and Sertoli cell activities[28]. Another pituitary hormone that regulates the testicular physiology is gonadotropin GTH-II that co-regulates Sertoli cell function[28]. Sertoli cells' main roles are to sustain germ cell survival, growth and physiological function. The development of sperm cells mostly depends on the Sertoli cells to provide the somatic elements of the testis[28]. In the current study, melatonin and ZnAA mechanism in the testicular event indicated that melatonin and zinc were essential components for spermatogenesis.

In the current study, sperm abnormality was observed (Fig. 2) and it was noted that it significantly decreased in the melatonin-ZnAA treatment (p = 0.022, Table 2) and some sperm kinetic parameters were found highly significant (motility p = 0.013, sperm concentration p = 0.037, sperm lateral amplitude p = 0.037) (Table 2, 3). Both melatonin and zinc was effective in protecting the sperm cells from free radical (reactive oxygen species) and lipid peroxidation via antioxidative properties[29]. The unsaturated fatty acids (PUFA) in the plasma membranes of the spermatozoa are highly sensitive to free radicals that may lead to lipid peroxidation[30]. In addition, cell proliferation, differentiation and migration for intracellular signaling may benefit from the activity of free radicals[31,32]. However, high levels of free radicals especially the reactive oxygen species (ROS) may produce excessive peroxides that affect the spermatozoa membrane. Lipid peroxidation may lead to membrane damage, especially in the acrosomal area. The membrane damage may cause low motility and viability of the spermatozoa and oxidize the DNA,

Table 3: Motility parameters measured by CASA for male *C. macrocephalus* with melatonin and different ZnAA levels (Mean±SE)

Parameters	Control	MZn1	MZn2	p value
Motility (%)	13.9±5.8[b]	21.8±9.3[a]	25.5±6.1[a]	0.013
Progressive (%)	3.0±2.0	3.3±1.7	4.0±2.0	0.5
Path velocity (μm sec^{-1})	40.6±5.5	41.5±5.5	44.6±4.9	0.3
Prog. velocity (μm sec^{-1})	33.6±3.8	34.6±4.0	35.4±6.0	0.7
Track speed (μm sec^{-1})	60.2±3.4	62.5±10.3	63.8±2.6	0.5
Lateral amplitude (μm)	3.7±0.7	5.5±2.0	4.3±1.0	0.037
Beat frequency (Hz)	32.9±3.8	33.8±3.4	34.2±3.7	0.7
Straightness (%)	82.4±7.9	82.5±5.9	84.1±2.5	0.8
Linearity (%)	60.9±8.1	63.1±7.7	64.0±3.2	0.6

[a,b]Values with different superscripts in a row differ significantly ($p<0.05$)

ultimately resulting to defective sperm cells that are unable to fertilise the oocytes[33]. In the current study, melatonin and ZnAA showed that melatonin and zinc were able to reduce abnormality rate and increase the sperm kinetic in male *C. macrocephalus*.

CONCLUSION

For the first time, this study showed that a combined melatonin and zinc amino acid feeding treatment to male *C. macrocephalus* has significantly improved the maturation in MZn2 treatment. The results indicated that this feeding treatment could be applicable to teleost reproductive biology.

SIGNIFICANCE STATEMENT

This study discovers that combined melatonin and zinc amino acid in the feed able to enhance the maturation of male *Clarias macrocephalus* broodstock with improved sperm qualities instead of conventional feeds. This study will help the researchers uncover the mechanism of melatonin and zinc amino acid in reproductive physiology of *Clarias macrocephalus* and finally, the improved quality of male *Clarias macrocephalus* may increase the production of this species due to high demand by the local consumers.

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