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Effects of Zinc Amino Acid in Walking Catfish (*Clarias macrocephalus*) Female Broodstock First Sexual Maturation

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Abstract

This study examines the effects of zinc amino acid (ZnAA) to the first sexual maturity stage in female broodstock of the Walking catfish, *Clarias macrocephalus*. The different ZnAA levels of Control (0 ppm ZnAA), ZnAA1 (100 ppm ZnAA) and ZnAA2 (200 ppm ZnAA) in the diet was applied to the first sexual maturation female catfish (Availa®Zn, Zinpro Corporation, Eden Prairie, MN USA). ZnAA accumulation, broodstock maturation analysis and breeding performance were evaluated. The ZnAA treatment has significant different in serum, meat and ovary ZnAA accumulation. The ZnAA treatment increased the fecundity, gonadosomatic index, egg diameter and development of oocytes at tertiary yolk stage. In comparison, the ZnAA treatment was insignificant in estradiol level. During artificial fertilization, the ZnAA treatment enhanced the fertilization rate and the larval survival rate. During recovery breeding, ZnAA treatment significantly increased the egg production and larval hatching rate. The optimum level to enhance the *Clarias macrocephalus* female broodstock first sexual maturation is ZnAA1.

Keywords: Zinc amino acid; Maturation; Reproduction; Female; Catfish

Introduction

Walking catfish (*Clarias macrocephalus*) is one of the favourite aquaculture species in Southeast Asia especially Thailand. It has a high market value due to its tender flesh and delicious flavour [1,2]. Despite being an important aquaculture species, *C. macrocephalus* population continues to decline due to several issues. *C. macrocephalus* population has become endangered due to the introduction of *Clarias* hybrid species and limited supply of wild broodstock caused by over exploitation in aquaculture farming that claimed the natural habitat of this species [1,3]. As a result, it has taken a toll in the supply of mature broodstock catfish for artificial propagation. Zinc is an essential trace element that plays an important role as a co-factor of enzymes and is a component of many important metallo enzymes. Hence, zinc is fundamentally important for the functioning of reproductive system [4]. Zinc deficiency will cause retarded growth, delayed sexual development, impaired reproduction in males and females, congenital abnormalities, and low hatching rate [4,5]. According to Salgueiro et al. [6], zinc supplementation is able to improve the infertility in female. Supplemented zinc can be absorbed by intestine and delivered to the liver and becomes zinc protein or zinc metallo thionein [7]. The zinc protein in the liver or vitellogenin is transported via the blood to the ovary in order to enhance the oocytes growth for the developing embryo and larvae after fertilization [8]. Zinc is also involved in the production and secretion of luteinizing hormone (GTH-II), follicle-stimulating hormone (GTH-I) and prolactin [6,9]. Zinc amino acid (ZnAA) is zinc that bind to amino acids ligand. According to Formigoni et al. [10], organic minerals including zinc, is capable to bind with ligand such as amino acids, peptides and proteins. In addition, the organic zinc has higher retention, bioavailability and absorption rate compared to inorganic zinc such as $ZnSO_4$ or ZnO [11]. Information on the occurrence and metabolic roles of ZnAA in broodstock maturation and reproductive performance is important in order to initiate and enhance the first sexual maturity of female *C. macrocephalus* ZnAA fundamental mechanism remains unclear even though there are strong evidences of its roles in enhancing the reproductive performance. In order to enhance the maturation and early embryonic development, it is important to investigate the effects of ZnAA administration to the first sexual maturity stage in female broodstock of the *C. macrocephalus*.

Materials and Methods

Fish and culture condition

The maiden *Clarias macrocephalus* female broodstock were obtained from the Fisheries Station of Kham Pheng Phet, Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand. The experiment trial was carried out at the Laboratory of Nutrition and Aquafeed, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. Approximately eighteen weeks old catfish were acclimatized and maintained in 500L tanks at the density of 15 ind/m^2/fish/tank and were fed with control feed for two weeks prior to the experiment. A total of 45 females (initial weight 63.85 ± 4.97 g) were subjected to normal photoperiod (12 hours day light) prior to treatment and fed at a level equivalent to 3% of their body weight. The diet was divided into two equal feedings per day. The fishes were randomly distributed in three treatments (Control, ZnAA1 and ZnAA2) and with three replicates. The experiment tanks were continuously aerated to maintain the oxygen supply and the duration of experiment was for eight week.

Experimental diets

The basal diet was formulated from practical ingredients that contained approximately 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 (Table 1). The diet

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Ingredient	Dry weight (%)
Fishmeal	22.0
Soybean	35.0
Spirulina	1.0
Wheat flour	12.0
Tapioca	11.8
De oil ricebran	5.0
Tuna fish oil	2.0
Soya oil	3.0
Mineral premix	0.5
Soy lecithin	2.0
Calcium phosphate	1.5
MgSO4.7H2O	0.1
KCl	0.6
Attractant	1.0
Binder	2.0
Vitamin premix	0.5

Table 1: Ingredients of the basal diet.

Proximate analysis (%)	Control	ZnAA1	ZnAA2
Moisture	3.6	3.0	3.0
Crude protein	37.7	38.0	37.8
Crude Fiber	9.3	9.3	9.3
Ash	1.5	1.7	1.6
Calcium	11.6	11.7	11.8
Phosphorus	1.8	1.9	1.9
Ether extract	1.2	1.3	1.4

Table 2: Proximate analysis for different ZnAA level in the diet.

also consisted of 37% crude protein and 9.3% crude lipid (Table 2). Diets containing ZnAA (one zinc ion bound to one amino acid ion) were prepared by adding different levels of ZnAA (Availa®Zn, Zinpro Corporation, Eden Prairie, MN USA) to the basal diet. These ZnAA concentrations were 0 ppm (control), 100 ppm (ZnAA1) and 200 ppm (ZnAA2) per kilogram in the diet according to NRC [12].

Zinc analysis

The ZnAA analysis was performed by using Inductive Couple Plasma–Optical Emission Spectrophotometer (ICP–OES) at Central Laboratory (Thailand) Company Limited. 0.25-2 g of samples were prepared for the analysis. All determinations were made in three replicates. Weighted triplicate of the samples were mixed with 7 ml nitric acid and 1 ml hydrogen peroxide in each flask. Microwave digestion was applied at 220°C for 45 minutes. After cooling, the resulting solutions were diluted up to 25 ml in volumetric flasks with deionised water. Blanks were prepared in the same way as the sample but excluding the samples. The prepared samples were injected and analysed in ICP–OES machine. The results were compared with standard curve to determine the ZnAA concentration [13].

Growth performance

All female *C. macrocephalus* were anaesthetised with clove oil and were individually weighted prior and after the experiment to measure the fish growth by using the following formula:

Weight gain (%) = (Final body weight – Initial body weight) ÷ Initial body weight × 100

Histology

The histology method was in accordance to Drury and Wallington [14]. The ovary samples were fixed in 10% buffered formalin and dehydrated in graded alcohol series. The ovaries were embedded in paraffin wax, cut to four micrometer, stained with hematoxylin-eosin and observed under Motic microscope (Motic BA210 Digital Laboratory Microscope with Moticam 1000 camera). Staging of female oocytes was performed in accordance to Lubzens et al. [15].

Gonadosomatic index

The Gonadosomatic Index was determined: GSI (%) = 100 × ovary weight ÷ body weight

Estradiol analysis

Chemiluminometric enzyme immunoassay of estradiol was determined by accordance to Ibrahim and Harabawy [16]. Estradiol analysis was conducted using commercially available immunoassay kit (IMMULITE® Estradiol, Siemens Medical Solution Diagnostic and United Kingdom).

Artificial breeding

Artificial breeding was conducted with the remaining female *C. macrocephalus* from each treatment to evaluate the reproductive performance of ZnAA experiment. The female broodstock were lightly anaesthetized with clove oil and were individually inspected to observe matured fish indication including papilla colouring, abdominal swelling and swollen papilla. A mixture of 30 µg buserelin acetate (LHRH analogue) and 10 mg domperidone (dopamine analogue) were induced into one kilogram female broodstock (0.1ml mixture for 100 g females) [17]. Male African catfish milt was obtained from dissected testis as the control male to standardize the male semen quality. The eggs and milt were mixed for fertilization and the fertilized eggs were then transferred to the hatching tanks for incubation.

Fish reproductive characteristics

Egg production, fertilization rate, hatching rate, gonadosomatic index (GSI) and survival rate (seven days old) of the larvae were investigated after the artificial fertilization. Egg production was estimated by direct counting of sub-sample of fertilized eggs in the female ovaries [18]. Oocytes diameters were measured from fresh ovarian tissue with Motic microscope (Motic BA210 Digital Laboratory Microscope with Moticam 1000 camera). Fertilization rate, larval hatching rate and larval survival rate were determined in accordance to Unuma et al. [19]. A recovery breeding was conducted a month later to assess the recovery breeding performance with similar parameters used in the initial breeding session.

Statistical analysis

Statistical analysis was performed using SPSS software. Data were analysed by one-way ANOVA (analysis of variance) and by the Duncan test which analyses the significant differences among means. The means comparisons significance was tested at $P<0.05$ [20].

Results

The *C. macrocephalus* female broodstock were subjected to ZnAA accumulation analysis at the end of the experiment trial. ZnAA concentrations in serum, meat and ovary were significantly different between the treatments with P value of 0.006, 0.013 and 0.03 respectively (Figure 1 and Table 3). However, there were no statistical differences for liver, bone, egg and total tissue with the P value at 0.07, 0.9, 0.1, and 0.8, respectively (Table 3). Dietary ZnAA treatment did not significantly affect the weight gain with the mean weight gain

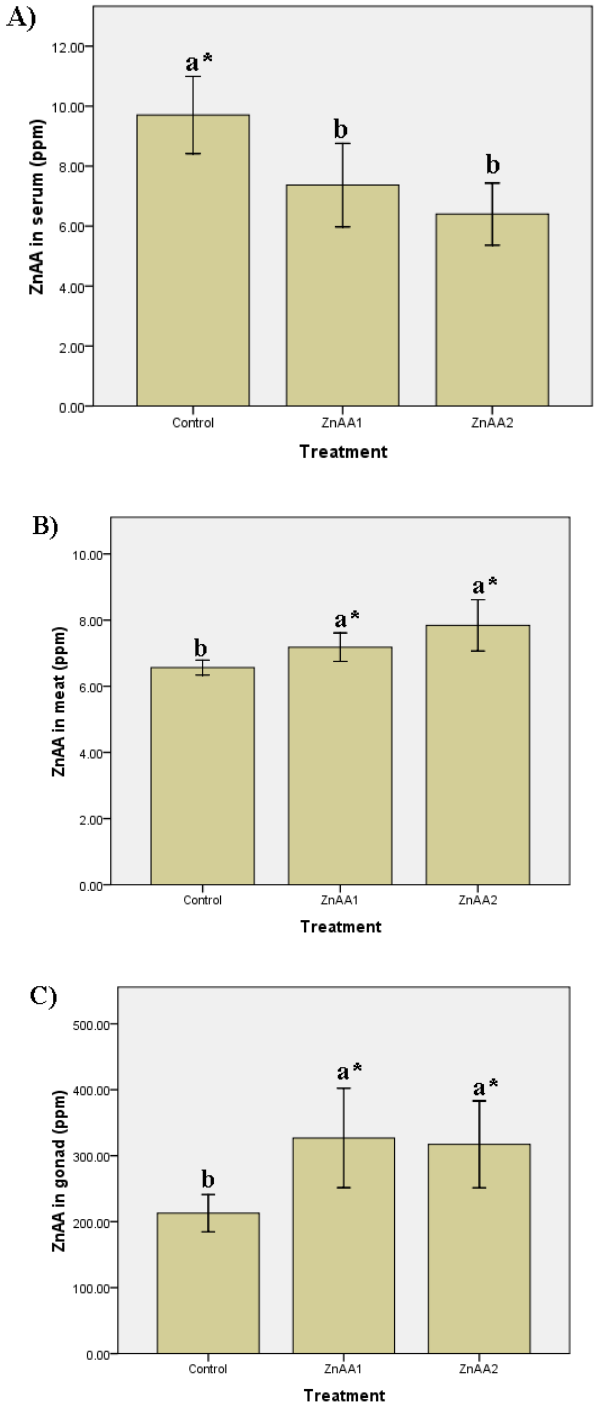

Figure 1: ZnAA concentration in serum (A), meat (B) and ovary (C) of female *C. macrocephalus* after eight weeks of ZnAA treatment. Values are expressed as mean ± SEM p<0.05.

ranged from 10.3–14.2% with the value at p=0.6. Similar result was found in estradiol profile (p=0.4) where the estradiol profile was not significantly different between treatments (Table 4). In histological analysis, most ovaries in the control group contained the highest percentage of peri-nucleolus stage (PNS) where the percentage was 51.5% (control), 28.7% (ZnAA1) and 20.8% (ZnAA2) (Figure 2 and Table 4). Differences in ovarian histology between the ZnAA treatments after eight weeks were highest in the ZnAA2 group for tertiary yolk stage (TYS). The well-developed oocytes at tertiary yolk stage (TYS) were 15.5% (control), 50.5% (ZnAA1) and 54.6% (ZnAA2) (Figure 2 and Table 4). After the experiment trial, there were significant increase in the gonadosomatic index (GSI), fecundity, and egg diameter in the presence of ZnAA treatment with the P value at 0.002, 0.025, and 0.001, respectively (Figure 3 and Table 4). The significant difference in GSI, fecundity, egg diameter and also the prominent evidence in histology

Control	ZnAA1	ZnAA2	*P* value
9.70^{a} ± 1.5	7.37^{b} ± 1.7	6.40^{b} ± 1.2	0.006
6.56^{b} ± 0.3	7.18^{a} ± 0.5	7.84^{a} ± 0.9	0.013
25.34 ± 0.9	29.60 ± 6.5	23.51 ± 3.2	0.07
30.43 ± 4.4	31.07 ± 3.2	30.83 ± 17.8	0.9
55.09 ± 7.0	52.65 ± 2.8	49.44 ± 2.4	0.1
212.81^{b} ± 34.5	326.88^{a} ± 92.2	317.20^{a} ± 80.6	0.03
140.35 ± 29.6	142.42 ± 36.2	133.35 ± 35.7	0.8

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

Table 3: ZnAA concentration in serum, meat, liver, bone, egg and ovary with different ZnAA levels (mean ± SD).

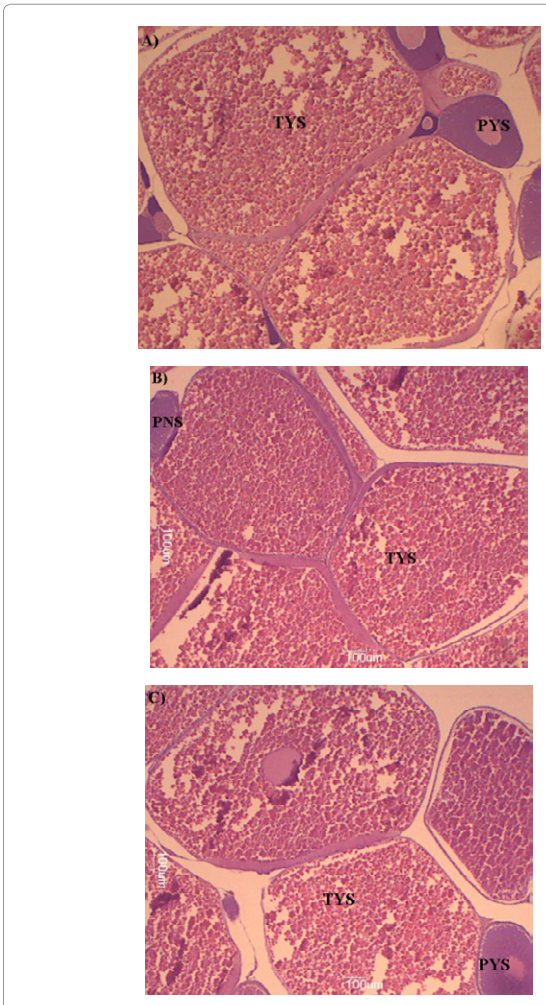

Figure 2: Effect of ZnAA treatment on ovarian histology of the *C. macrocephalus*. Cross section of an ovary of fish treated with control (A), ZnAA1 (B) and ZnAA2 (C). PNS; peri-nucleolus stage, PYS; primary yolk stage, TYS; tertiary yolk stage. Scale bar=100 µm.

Treatment	Control	ZnAA1	ZnAA2	*P* value
Weight gain (%)	10.3 ± 13	12.8 ± 10	14.2 ± 8	0.6
Estradiol (pg/ml)	486.7 ± 151	634.7 ± 191.5	1812 ± 2215	0.4
Histology tertiary yolk stage (TYS)	15.5%	50.5%	60.3%	-
Histology peri-nucleolus stage (PNS)	51.5%	28.7%	20.8%	-
Gonadosomatic index (%)	5.61^{b} ± 0.44	9.03^{a} ± 2.14	10.22^{a} ± 2.46	0.002
Fecundity (egg/g)	3034.1^{b} ± 329	4955.3^{a} ± 2028	5756.5^{a} ± 1766	0.025
Egg diameter (μm)	1448.4^{b} ± 79	1520.3^{a} ± 132.8	1530.5^{a} ± 81.0	0.001

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

Table 4: Maturation analysis of female *C. macrocephalus* with different ZnAA levels (mean ± SE).

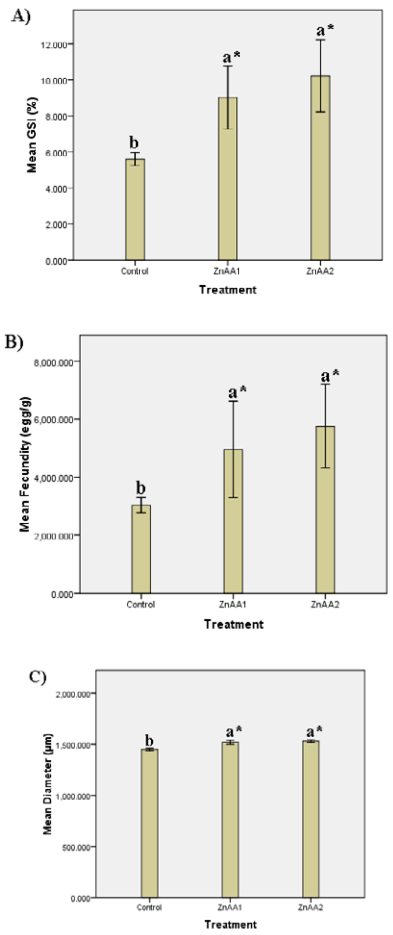

Figure 3: Mean gonadosomatic index (A), fecundity (B) and egg diameter (C) of female *C. macrocephalus* after eight weeks of ZnAA treatment. Values are expressed as mean ± SEM (GSI N: 6 animals/replicate, fecundity N: 6 animals/ replicate, egg diameter N: 180 eggs/replicate). p<0.05.

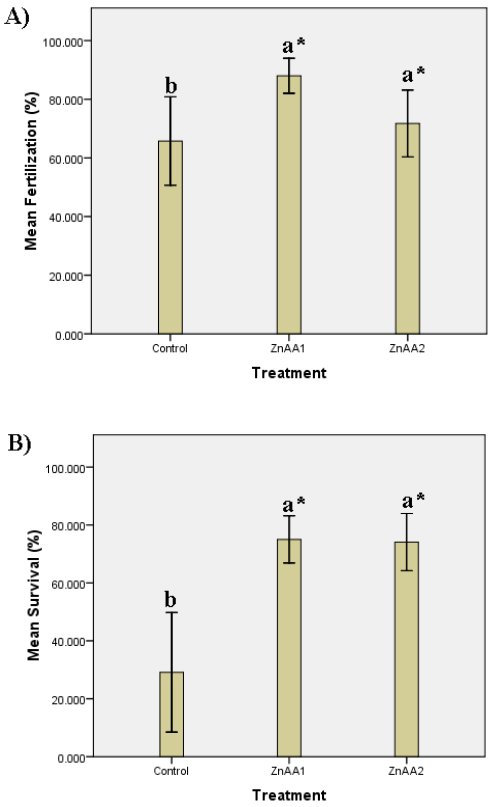

Figure 4: Mean spawning fertilization rate (A) and survival rate (B) after eight weeks of ZnAA treatment. Values are expressed as mean ± SEM p<0.05.

analysis indicated that a higher number of vitellogenic and matured follicles in ovaries were found in ZnAA treated treatment (ZnAA1 and ZnAA2). After eight weeks of ZnAA experiment trial, the female broodstock were artificially fertilized with semen from control males to evaluate the reproductive performance. The fertilization rate of the ZnAA treated fertilized female ZnAA1 and ZnAA2 were significantly higher (p=0.045) compared to the control groups (Figure 4A and Table 5). There was higher number of larval survival rate observed in females broodstock exposed in ZnAA treatment for *C. macrocephalus* with a P value at 0.001 (Figure 4B and Table 5). However, the ZnAA treatment has no significant different in the fecundity and hatching rate with the P value at 0.2, and 0.7, respectively. After the first breeding session, the fertilized female broodstock continued to be fed with trial feed for another four weeks and were artificially fertilized with semen from control males to evaluate the recovery reproductive performance. There was higher percentage of female that ready to breed observed in ZnAA exposed treatment with the percentage at 50% (Control), 71.4% (ZnAA1) and 87.5% (ZnAA2) (Table 6). The fecundity and hatching rate of the all ZnAA treatments were significantly higher with the P value at 0.001, and 0.025, compared to the control groups (Figure 5 and Table 6). However, the ZnAA treatment has no significant effects in the larval survival rate (p=0.3) (Table 6).

Discussion

In this study, ZnAA accumulations were found to be significantly

Treatment	Control	ZnAA1	ZnAA2	*P* value
Fertilization Rate (%)	65.75^{b} ± 21	88^{a} ± 7.9	72^{a} ± 16	0.045
Survival rate (%)	29.1^{b} ± 17.9	75^{a} ± 11	74^{a} ± 13	0.001
Hatching rate (%)	10.5 ± 8	14.3 ± 14	16 ± 13	0.7
Egg production (egg/kg)	2105 ± 1045	2715 ± 973	3371 ± 1997	0.2

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

Table 5: Breeding performance of female *C. macrocephalus* with different ZnAA levels (Mean ± SE).

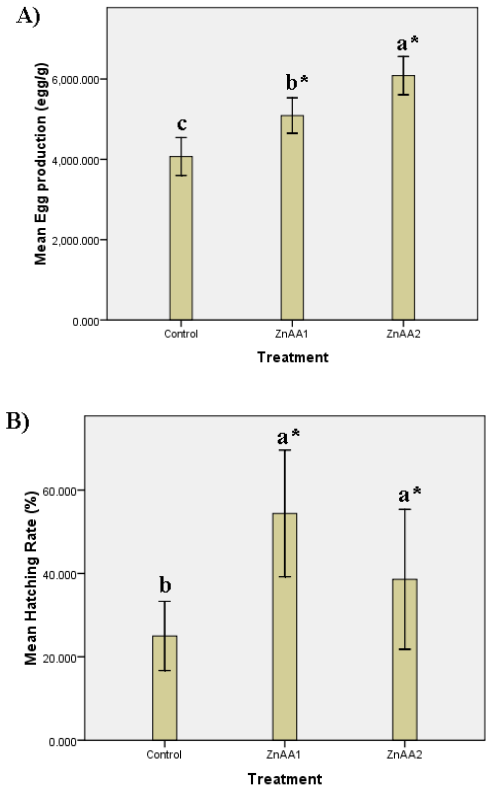

Figure 5: Mean egg production (A) and hatching rate (B) from the recovery breeding session. Values are expressed as mean ± SEM p<0.05.

Treatment	Control	ZnAA1	ZnAA2	*P* value
Breeding broodstock (%)	50%	71.4%	87.5%	-
Egg production(egg/kg)	4069.5^{c} ± 472	5644.8^{b} ± 493	6499^{a} ± 630	0.001
Hatching rate (%)	25^{b} ± 36	54^{a} ± 29	39^{a} ± 30	0.025
Survival Rate (%)	40.7 ± 9	54.3 ± 27	46.5 ± 25	0.6

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

Table 6: Recovery breeding performance of female *C. macrocephalus* with different ZnAA levels (Mean ± SE).

different in serum, meat and ovary. According to Thompson et al. [21], exogenous ZnAA was then absorbed by intestine to the liver and was passed on to the ovary during reproductive development. This mechanism is regulated with metal-binding protein metallo thionein in the liver where the primary function of metallo thionein is to control a pool of loosely ligated ZnAA within the cell. In other study, Thompson et al. [21] stated that ZnAA is accumulated in the liver and transported by the bloodstream to other organs. Thus, it explains the significant increase of ZnAA in ovary in the current study. The ZnAA accumulation in other parts of the treated female such as bone suggested that zinc stimulates bone formation [22]. Zinc also helps bone mineralization by acting as a cofactor for alkaline phosphatase [23]. In this study, the estradiol profile in the *C. macrocephalus* female broodstock was not significant with ZnAA treatment compared to the control group. Usually, estradiol is secreted into the bloodstream to the liver and stimulates the synthesis of vitellogenin production by hepatocytes [24]. In teleost, estradiol concentration gradually elevates during vitellogenesis and declines in response of luteinizing hormone (GTH-II) as oocytes begin their maturation [9,25]. Thus, it explains the insignificant difference of estradiol profile in this study. The level of GSI, fecundity, egg diameter and mature cells in histology analysis from the ZnAA treated group demonstrated a significant difference compared to the control group. Previous studies demonstrated that the estradiol induced vitellogenesis process in the liver [24]. Vitellogenesis is a process where vitellogenin is transported from the liver via the bloodstream to the ovary. In ovary, vitellogenin is incorporated by a receptor-mediated process into subsequently nourishing the developing embryo [21,26,27]. Vitellogenin is also known as zinc bounded protein [27]. The role of ZnAA protein is essential as cofactor for enzymes which are involved in DNA, RNA and protein synthesis as well as a requirement for membrane and polyribosome stability [8]. During vitellogenesis, oocytes increase in size, thus it explains the significant increase in GSI, fecundity, egg diameter and prominent mature cells in histology analysis. ZnAA metallo thionein influences the reproductive cycle by being involve in the hepatic synthesis of a yolk precursor (vitellogenin) and induces oocytes growth prior to fertilization. According to Banks et al. [8], vitellogenin are transported via the blood to the growing oocytes and processed, for the developing embryo and larvae after fertilization. In the present study, the fertilization rate and survival rate of the ZnAA treated fertilized female were significantly higher compare to the control group. According to Riggio et al. [28], the role for zinc during embryonic development had to act as a regulator of cell division and morphogenesis. While zinc application enhances the cell proliferation, zinc deprivation reduces cell division and stimulates congenital abnormalities of foetal organs derived from ectodermal, mesodermal and endodermal germ lines [5,28]. In recovery reproductive performance, the ZnAA treatment groups demonstrated a higher percentage of female that prompt to spawn. The other parameter such as fecundity and hatching rate showed a significant difference from the control group. The female broodstock in recovery experiment were fed with ZnAA treatment have longer duration. This condition favours more ZnAA accumulation in the liver thus, increased the levels of ZnAA metallothionein for vitellogenesis. The vitellogenesis produces vitellogenin in the liver and transports it to the ovary and nourishing the developing embryo [21].

Conclusion

The recent study indicated that the optimum zinc amino acid treatment in enhancing the *Clarias macrocephalus* female broodstock first sexual maturation and improving the reproductive performance is ZnAA1 (100 ppm ZnAA).

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