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Research Article

Effect of *Spirulina platensis* and *Azolla nilotica* as feed additives on growth performance, antioxidant enzymes and fecundity of *Oreochromis niloticus*

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Abstract

This study was conducted in two experiments. Experiment 1 aimed to investigate the growth promoting and anti-oxidative effects of *Azolla nilotica* (AZN) and *Spirulina platensis* (SP) in *Oreochromis niloticus*. Seven fish groups (G1-G7), each in three replicates, were fed a basal diet (control), AZN 5%, AZN 10%, SP 0.5%, SP 1%, a mixture of *Azolla nilotica* 5% and *Spirulina platensis* 1% (AZN 5 %-SP 1% mix), and a mixture of *Azolla nilotica* 10% and *Spirulina platensis* 1% (AZN 10% - SP 1% mix), respectively, for 3 months. The results showed a significant increase in growth indices (weight gain, specific growth rate, average length gain, feed efficiency ratio), and a decrease in the feed conversion ratio in all supplemented groups compared to control. Hepato-somatic index in G4 and G5 groups, and intestinal-somatic index in G4 - G6, and in G5 and G6 were obviously higher than control. Spleno-somatic index and antioxidant enzymes (GSH-px, SOD and CAT) markedly increased in G5 and G6 compared to control. Significant increase (p<0.05) in white blood cells was recorded in G2 and G4. Experiment 2 verified the effect of one-month supplementation of SP 1% on males and females *O. niloticus* fecundity. Males fed with SP 1% had an enhanced fertility as indicated by an increase in sperm density, spermatozoa motility and livability rates compared with the control. There was a significant increase in eggs number and the fecundity index in unripe females and a tendency of egg size to increase in ripen fish. It could be concluded that SP 1% and AZN 5% supplementation is advantageous to improve *O. niloticus* growth and fecundity.

Keywords: Antioxidant enzymes, *Azolla nilotica*, Growth parameters, Fecundity, Nile tilapia, *Spirulina platensis*

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Introduction

Aquaculture is one of the fastest growing food production sectors in the world (FAO, 2018). Along with the increase in fish production, it has become a great need to offer the good quality and of high nutritive value food (Dawood, 2017). *Spirulina platensis* (SP) is a cyanobacterium that is utilized for more than 20 years in fish farming (Chen and Zhange, 1997). Early reports demonstrated that SP has antioxidant (Cao *et al*., 2018) and immune-stimulant (Bhowmik *et al.*, 2009) activities. *Spirulina* has high protein content (55–70%) (Bhowmik *et al.*, 2009); being rich in all essential amino acids, with higher beta-carotene than any other feed. SP is enriched with poly unsaturated fatty acids (PUFA) especially gamma linolenic acid (Malak and Sangak, 2017). The antioxidant effect of SP is due to its ability to combat with the reactive oxygen species (ROS) damaging the DNA (Almbro *et al.*, 2011).

Azolla is free-floating fresh water ferns which is rich in essential amino acids (25-35%), vitamins (A, B12 and Beta Carotene) and minerals (10-15%) e.g. calcium, phosphorous, potassium, iron, copper and magnesium, and low content of carbohydrate and oil. *Azolla* has growth promoting activities and contains a combination of amino acids, bio-active substances and biopolymers (7-10%). Azolla can be easily digested by livestock e.g. sheep, goat, pig and rabbit, owing to its high protein and low lignin contents (Kathirvelan *et al.*, 2015).

The present study aimed to investigate the effect of dietary administration of SP and *Azolla nilotica* (AZN) on growth and somatic indices, blood parameters (RBCs, WBCs and PCV), antioxidant enzymes (GSH-px, SOD and CAT), and fecundity in *O. niloticus*

Materials and methods

All the procedures were accomplished according to the Ethics for Humane Treatment of Animal Use inResearch Guidelines and complied with the relevant legislation of Faculty of Veterinary Medicine, Benha University, Egypt (Ref. No. 000R0119-2018). The present study was designed to include two experiments.

Experiment (I): Effect of SP and AZN on growth performance and antioxidant enzymes

Experimental fish

Nile tilapia (*Oreochromis niloticus*) with an average weight and body length of 18.00 ± 2.00 g and 8.00 ± 2.00 cm, respectively, and free from clinical signs were obtained from Central Lab for Aquaculture Research (CLARM), Abbassa, Sharkia Governorate, Egypt. Fish were placed in well prepared fiberglass tanks (750 L) filled with de-chlorinated water in the wet lab at the Faculty of Veterinary Medicine, Benha University. The water temperature was adjusted to 26 ± 2 °C and the oxygen level was maintained at an optimal level (6 mg/L) using aerators. Uneaten food and feces were siphoned, and about one third of water volume was exchanged daily.

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Preparation of experimental diet

Azolla nilotica (dry powder) was obtained from Om Elqora for agricultural development and investment company, Cairo, Egypt and its chemical composition are presented in Table 1.

Table 1: The chemical composition (on DM basis) of *Azolla* meal (Pullin and Almazon, 1983)

Item	%
Moisture content (Fresh Azolla)	94
Crude protein	24-30
Crude lipid	3.0-3.3
Ash	10.5
Soluble sugar	3.5
Nitrogen	4.0-5.0
Phosphorus	2.0-4.5
Calcium	0.4-1.0
Magnesium	0.5-0.6
Manganese	0.11-0.16
Iron	0.06-0.26
Chlorophyll	0.34-0.55

Spirulina platensis (dry powder) was obtained from Algal Biotechnology Unit, National Research Centre, Dokki, Giza, Egypt with its chemical composition which are listed in Table 2. The experimental diet was prepared by incorporating formulated fish feed (Table 3) with 30 % protein by different concentration of AZN and SP. The basal diet was divided into seven portions: The first portion was kept free from any additives (control). Other portions were incorporated with AZN 5%, AZN 10%, SP 5%, SP 1%, AZN 5% - SP 1% mix and AZN 10%- SP 1% mix, respectively. A suitable amount of water was added to form moist dough then pelleted. Pellets were allowed to dry at room temperature prior to packaging and storage at 4°C till use.

Table 2: The chemical composition (on DM basis) of *Spirulina* meal (Kovács *et al.*, 2016).

Item	g/kg
Dry matter	944.9
Crude protein	658.1
Crude fat	8.6
Ash	65.1
Starch	35.6
Calcium	2.2
Phosphorus	9.2

Table 3: Formulation of basal diet from local ingredient according to NRC.

Components	%
Fish meal (60%C.P)	30.0
Soya bean meal	25.0
Yellow corn meal	37.9
Molasses	2.5
Vitamins premix	1.0
Mineral premix	2.1
Oil	1.5

Experimental design

Fish were divided into seven groups in three replicates (n=20 fish/group) as follow: group 1 (G1) was kept as control and received basal diet, groups

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2 (G2) received diet containing AZN 5% and group 3 (G3) received diet containing AZN 10%, group 4 (G4) received diet containing SP 0.5%, group 5 (G5) received diet containing SP 1%, group 6 (G6) received diet containing mixture of SP 1% and AZN 5% and group 7 (G7) received diet containing mixture of SP 1% and AZN 10%. Control and treated groups received experimental diets for three months at the rate of 4% of BW three times daily for the first two months then fish were fed at the rate of 3 % at the rest of the experimental period. Fish were weighted biweekly for adjustment the feeding rate.

Determination of growth parameters

At the end of the feeding trial, growth indices were evaluated including final weight, weight gain rate (WGR), length gain rate (LGR), feed conversion ratio (FCR) and feed efficiency ratio (FER) according to Liu *et al.* (2010).Furthermore, specific growth rate (SGR) was calculated as described by Laird and Needham (1988). Total body weight gain was estimated according to Jauncey and Ross (1982).

Final weight (w) was measured to the nearest 0.1g using portable electric balance:

Weight gain rate (%)= 100 × (Average final body weight −Average initial body weight / Average initial body weight

$$\text{Specific growth rate (SGR)} = \frac{\text{Ln (Final body weight (g)} - \text{Ln (Initial body weight (g)}}{\text{Time interval (days)}} \times 100$$

Where: Ln is the natural log.

Total weight gain (g/fish)= $W_t - W_0$

Where: W_0: is the initial fish weight (g) at start of the experiment, and W_t: is the final weight (g) at end of experiment.

Feed conversion ratio=feed consumption/weight gain rate.

Feed efficiency ratio= weight gain rate/ feed consumption.

Total fish length (L) from tip of mouth to tip of caudal fin was measured using graduated ruler:

Length gain rate (LGR) (%)= 100× (Average final body length − Average initial body length)/Average initial body length .

Determination of organo-somatic indices

Samples of ten fish were taken from each group at the end of the experiment for calculation of organo-somatic indices according to the following formulas:

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Hepato-somatic index= weight of liver /fish body weight × 100
Spleno-somatic index= weight of spleen / fish body weight × 100
Intestinal-somatic index= weight of intestine / fish body weight × 100

Determination of hematological parameters

Blood samples were taken with anticoagulant (EDTA 10%, Nile co. for pharmaceutical Cairo, Egypt) for counting RBCs and WBCs and PCV. Number of blood cells (RBCs and WBCs) and packed cell volume (PCV %) was evaluated as described by Dacie and Lewis (1991).

Determination of antioxidant enzymes

Liver samples from both control and treated groups were preserved at -20°C until measurement. Glutathione peroxidase activity (GSH-Px) was determined in tissue by colorimetric method and its absorbance was measured at 405 nm according to Satoh (1978). Superoxide dismutase activity (SOD) was determined according to Fossati *et al.* (1980) at wave length of 560 nm. Catalase activity was measured by assay of hydrogen peroxide based on formation of its stable complex with ammonium molybdate with wave length at 405 nm (Fossati *et al.*, 1980).

Experiment (II): Effect of Spirulina platensis on fecundity

Experimental design

Mature males (n=5) and females (n=5) of *O. niloticus* with an average bodyweight and length of 47±2 g and 13±2 cm, respectively, were distributed into two groups: control and treated groups per each gender in two replicates. G1 and G2 were kept as controls for males and femalesrespectively and received basal diet, while G3 (males) and G4 (females) received SP 1% in diet for four weeks at the rate of 3% of BW three times daily. Water temperature was adjusted at 28±2°C and photoperiod at 13 hrs light : 11 hrs dark.

Determination of gonadosomatic index (GSI)

At end of the 4th week, fish were collected, weighted to the nearest 0.1 g and their gonads were excised and weighted for determination of GSI according to Kolding *et al*. (2008).

$$\text{GSI}= \frac{Gonadal\ weight(g)}{Body\ weight(g)} \times 100$$

Soon after dissection, the egg mass was carefully removed with a spatula from ovaries, and individual eggs were teased apart and counted. About 10-20 eggs were evaluated under a calibrated binocular stereomicroscope to measure the egg diameter (Coward and Bromage, 1999). Since eggs were ellipsoid shaped, both axes (long and short) were measured in order to calculate the mean egg size.

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Fecundity (%) = (Number of eggs/weight of fish (g)) × 100 according to Yamamoto (1958)

Reproductive effort (RE) = fecundity×egg size (mm) according to Celik *et al.* (2011)

Evaluation of semen characteristics

Semen was collected by gentle massage of the abdomen and the free released semen was assessed by one observer as described previously for hydrogen ion concentration (pH), individual sperm motility (Morita *et al.,* 2003), sperm viability and abnormalities in stained film with eosin-nigrosin stain (Musa, 2010).

The percentage of abnormal spermatozoa was counted according to Musa (2010) and sperm cell density was measured using hemocytometer (Tvedt *et al.,* 2001) according to below formula:

Sperm density= n ×r × 10,000

Where: n= average cells count and r=dilution rate.

Statistical analysis

The results were presented as mean ± SE (standard error) for three replicates. Statistical analysis was carried out (IBM SPSS Ver. 21) using one-way analysis of variance (ANOVA), Duncan in multiple range tests in experiment 1, and independent student *t*-test in experiment 2.

Results

Effect of Spirulina and Azolla on growth performance

Feed supplementation with AZN and/or SP enhanced ($p < 0.05$) *O. niloticus* growth performance compared with control as indicated by an augmentation of all growth parameters (Table 4).

Table 4: Effect of dietary supplementation of *Spirulina* (SP) and *Azolla nilotica* (AZN) on growth parameters of *O.niloticus.*

Fish groups	IW	FW	WG	WG%	SGR	FCR	FER	ALG
Control	17.20±0.20^{a}	22.10±0.40^{e}	5.30±0.20^{e}	0.30±0.02^{e}	0.30±0.01^{e}	3.20±0.10^{a}	0.34^{e}±0.01	18.40±1.20^{d}
AZN 5%	17.20±0.20^{a}	29.60±0.60^{c}	12.30±0.50^{c}	0.72±0.00^{b}	0.60±0.02^{c}	1.70±0.05^{c}	0.60^{c}±0.02	28.50±1.60^{c}
AZN 10%	17.28±0.23^{a}	25.19±0.50^{d}	7.90±0.50^{d}	0.46±0.03^{d}	0.41±0.03^{d}	2.48±0.17^{b}	0.45^{d}±0.03	30.10±2.40^{c}
SP 0.5%	17.28±0.23^{a}	34.56±0.49^{b}	17.20±0.50^{b}	1.01±0.04^{b}	0.70±0.02^{b}	1.42±0.05^{d}	0.73^{b}±0.02	29.70±1.90^{c}
SP 1%	17.28±0.23^{a}	43.24±0.57^{a}	25.90±0.40^{a}	1.51±0.02^{a}	1.02±0.01^{a}	1.15±0.02^{d}	0.87^{a}±0.01	48.20±1.50^{a}
AZN 5%-SP 1% mix	17.20±0.20^{a}	35.18±0.62^{b}	17.90±0.40^{b}	1.04±0.02^{b}	0.79±0.01^{b}	1.37±0.02^{d}	0.74^{b}±0.01	34.90±1.03^{b}
AZN 10%-SP 1% mix	17.28±0.23^{a}	29.70±0.59^{c}	12.40±0.50^{c}	0.72±0.04^{c}	0.60±0.02^{c}	1.74±0.08^{c}	0.60±0.02^{c}	25.90±1.50^{c}

IW, FW, WG, WG%, SGR, FCR, FER and ALG refereed to initial weight, final weight, weight gain, specific growth rate, feed conversion rate, feed efficacy rate and Length gain rate, respectively. Values (Mean±SEM) with different superscript letters in the same column are significantly different (p<0.05).

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However, the maximal values of growth indices were obtained in G5 (SP 1 %) followed by G6 (AZN 5%-SP 1% mix) groups. In the meantime, feed conversion ratio showed a significant (p<0.05) decrease in all supplemented groups compared to the control, with the highest and lowest values were recorded in G3 (AZN 10%) and G5 (SP 1%), respectively.

Hepato-somatic index in G4 (SP 0.5 %) and G5 (SP 1%) groups, ISI in G4 (SP 0.5 %) and G5 (SP 1%) and G6 (AZN 5%-SP 1% mix), and SSI index in G5 (SP 1%) and G6 (AZN 5%-SP 1% mix) groups were substantially (p<0.05) higher than the control (Table 5).

Table 5: Effect of dietary supplementation of *Spirulina* (SP) and *Azolla nilotica* (AZN) on Bio-somatic indices of *O.niloticus.*

Fish groups	HSI	SSI	ISI
Control	1.30±0.14 ^{c}	0.16±0.03 ^{a}	3.21 ±0.22 ^{d}
AZN 5%	1.57±0.12^{bc}	0.17±0.02 ^{a}	3.25 ±0.14 ^{d}
AZN 10%	1.65±0.13^{abc}	0.14±0.02 ^{a}	3.35 ±0.14 ^{d}
SP 0.5%	2.02 ±0.15 ^{a}	0.16±0.02 ^{a}	4.29 ±0.20 ^{b}
SP 1%	1.92±0.03^{ab}	0.58±0.47 ^{a}	6.21±0.20 ^{a}
AZN 5%-SP 1% mix	1.55±0.17^{bc}	0.50±0.28 ^{a}	3.86±0.15^{bc}
AZN 10%-SP 1% mix	1.52±0.15^{bc}	0.13±0.02 ^{a}	3.55±0.14 ^{cd}

HSI, SSI and ISI referred to hepato-somatic, Spleno-somatic and intestinal somatic indices, respectively. Values (means±SE) with different letters in the same column are significantly different (p<0.05).

Effect of Spirulina and Azolla on hepatic antioxidant enzymes and hematological parameters

Data presented in Figure 1 verified a synergism between SP and AZN in increasing the hepatic antioxidant enzymes. The levels of GSH-px, CAT and SOD exhibited the highest significant (P < 0.05) values in fish fed with a mixture of AZN 10% and SP1% (G7 group) followed by AZN 5% - SP1% mix (G6 group).

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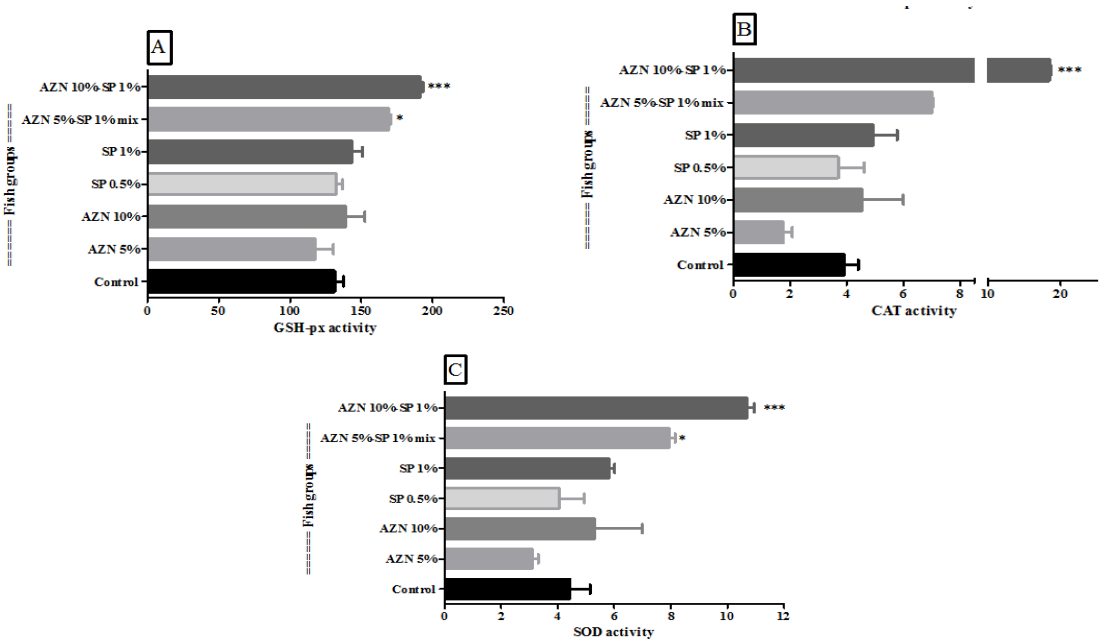


Figure 1: Effect of *Spirulina* (SP) and *Azolla nilotica* (AZN) on hepatic antioxidant enzymes in *O. niloticus*. * and * referred significant differences at $p<0.05$ and 0.001, respectively as compared with control.**

White blood cells significantly increased ($p < 0.05$) in G2 (AZN 5%), G4 (SP 0.5%) and decreased in G7 (AZN 10%-SP 1% mix) when compared to the control. Nevertheless, other blood parameters (RBCs and PCV) were not significantly varied between treated and control groups (Fig. 2).

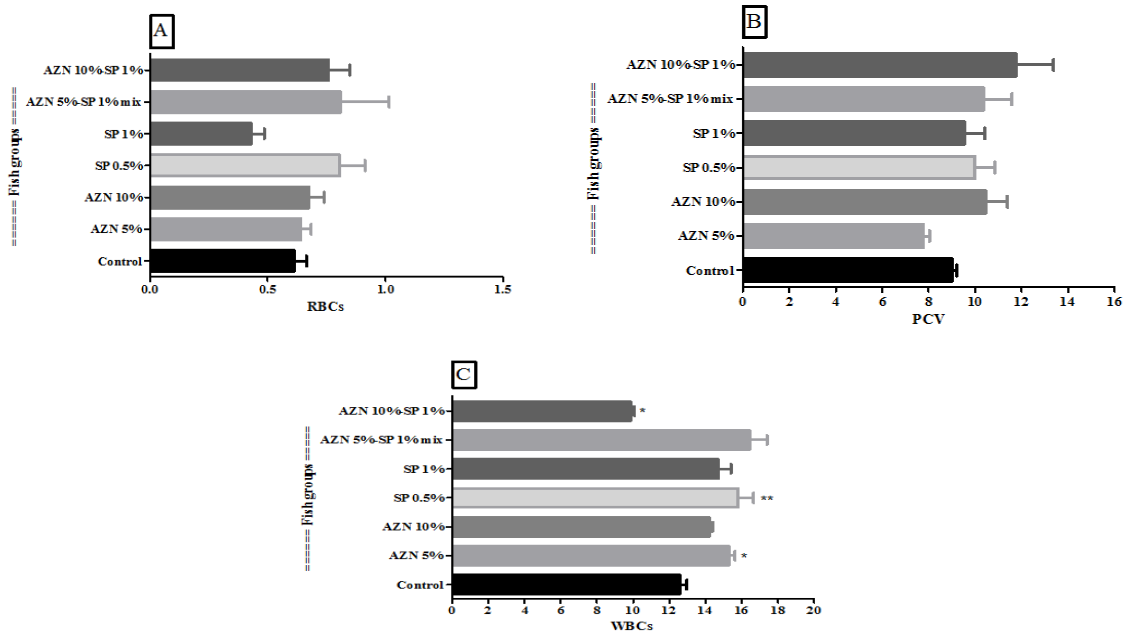


Figure 2: Effect of dietary supplementation of *Spirulina* (SP) and azolla nilotica (AZN) on hematological parameters of *O. niloticus*. * and ** referred significant differences at $p<0.05$ and 0.01, respectively as compared with control.

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The effect of Spirulina platensis incorporated diet on male and female fecundity

In male *O. niloticus*, the motility rate, duration, livability (p<0.05) and semen density (p<0.01) greatly improved after feeding with SP 1% compared to the control (Fig. 3). In unripe female *O. niloticus*, while the egg population (p<0.01) and fecundity index (p<0.05) increased, the gonadosomatic index, gonad index, ovarian weight, (p<0.05) and size of eggs (p<0.01) decreased in SP 1% fed females compared to the control groups. In ripen fish, the size of eggs tended (p = 0.09) to increase in SP 1% fed group compared to the control (Fig. 4).

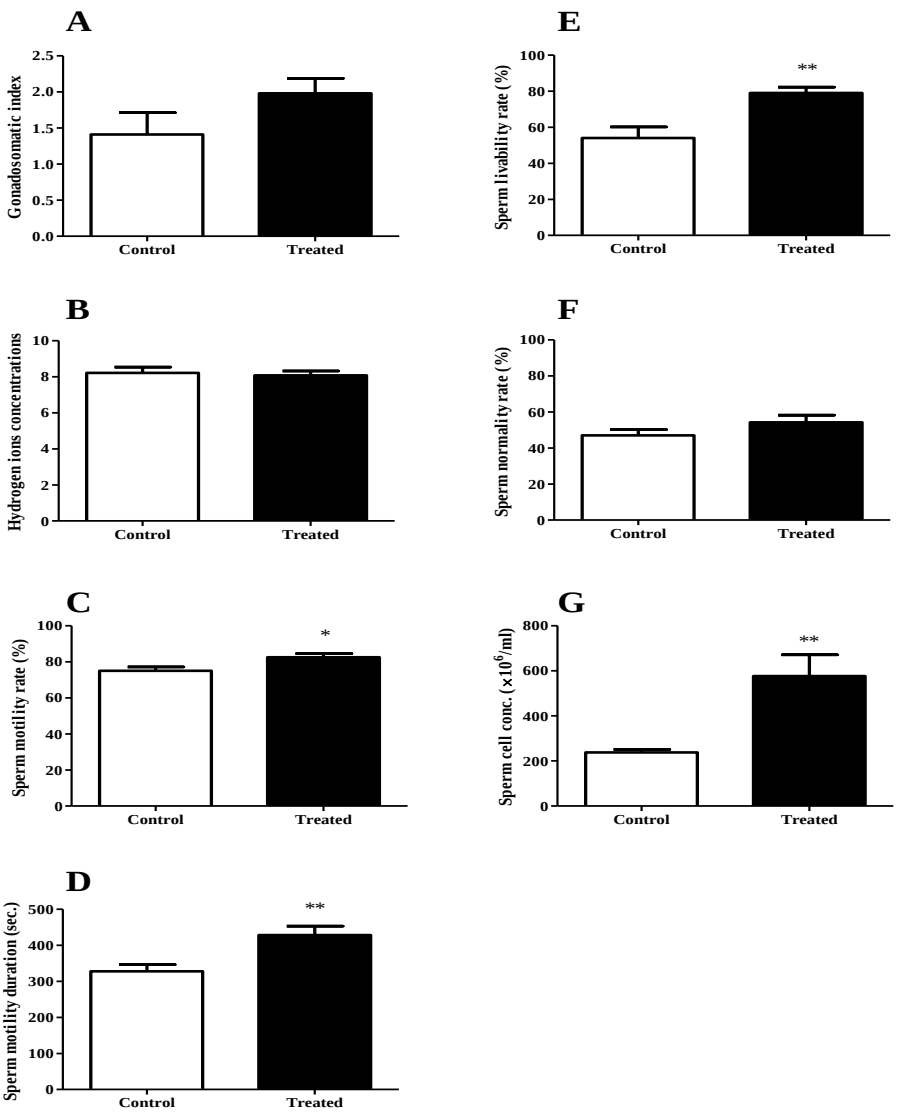


Figure 3: Effect of dietary supplementation of *Spirulina* on male fecundity of *O. niloticus*. * and ** referred significant differences at p<0.05 and 0.01, respectively as compared with control.

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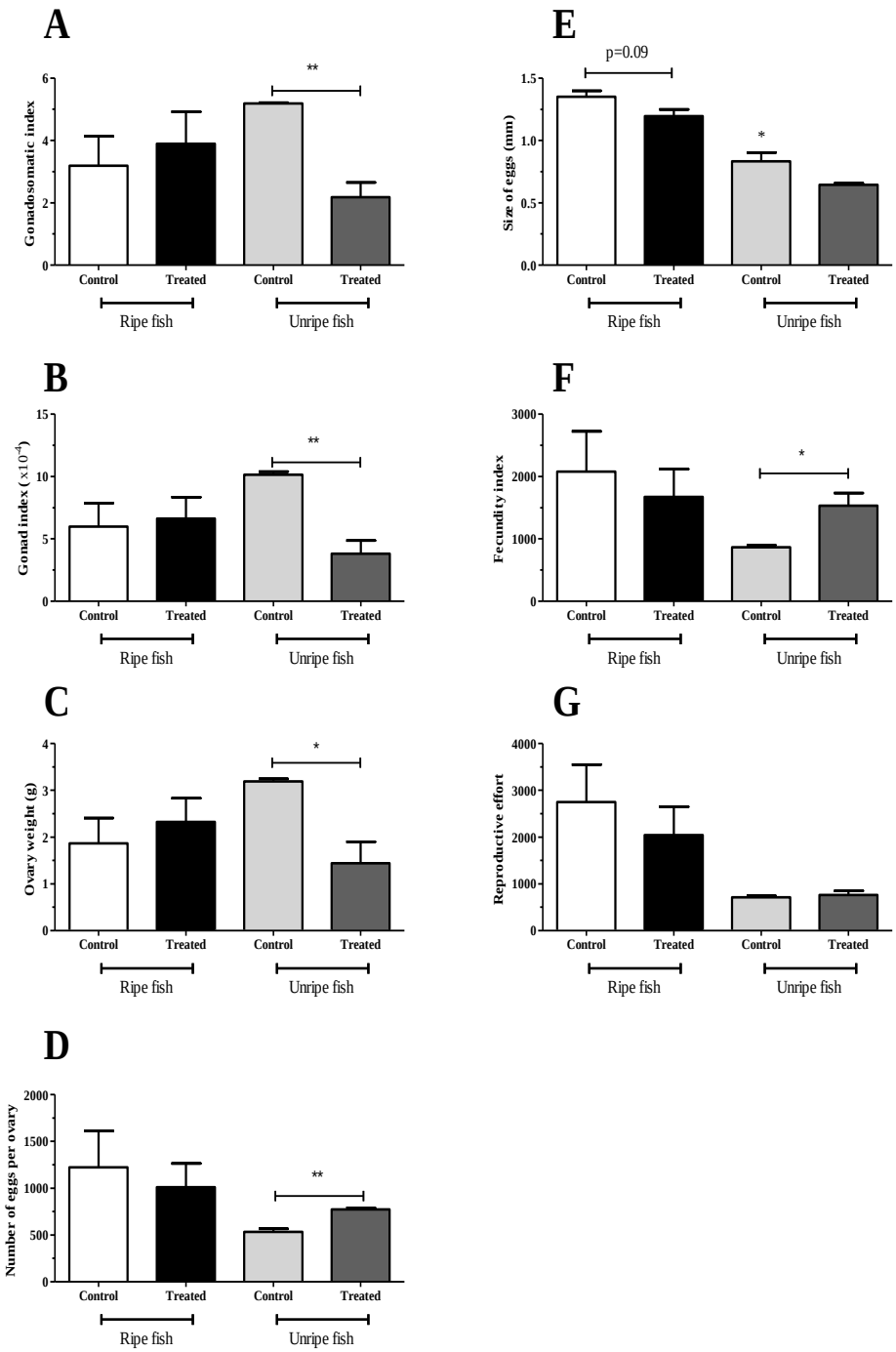


Figure 4: Effect of dietary supplementation of *Spirulina* on female fecundity of *O. niloticus*. * and ** referred significant differences at $p<0.05$ and 0.01, respectively as compared with control

Discussion

The recent trend in aquaculture directed toward the addition of protein rich substitutes to fish feed for improving the growth parameters (Dawood, 2017). The current study indicated that the supplementation of SP 1% greatly influenced the growth performance and

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fecundity of *O. niloticus*, and SP acted synergistically with AZN to enhance the hepatic antioxidative enzymes.

Regarding the growth promoting effect of AZN, SP and their mixture, the current study revealed that SP significantly increased growth performance indices with the superior effect obtained in SP-1%. For the meantime, incorporation of AZN at the rate of 5% was superior to 10 % and both were higher than the control group, but were lower than SP fed groups. The high growth performance in SP fed groups may be due to its high protein (60–70%), essential amino acids, vitamins, minerals and fatty acids contents (Hayashi *et al*, 1994). Moreover, the cellular structure of SP lacks cellulose and this reduces stress and enhances fish health against invasive pathogens (Nakono *et al.*, 2003). Although, AZN is a rich source in all essential amino acids, vitamin A, vitamin B complex, beta-carotene and minerals (Anitha *et al.*, 2016), poor growth response may be attributed due to its high neutral detergent fiber and possibly adenine that limit its usefulness as a food ingredient for simple-stomach animals (Buckingham *et al*., 1978). The findings related to SP effect here were harmonized with former studies (Abdel-Tawwab and Ahmed, 2009; Amer, 2016; Mahmoud *et al.*, 2018) who indicated an improvement in weight gain and specific growth rate of *O. niloticus* with SP. Moreover, the result related to AZN effect was supported by previous work in *O. niloticus* (Abou *et al.*, 2012) and grass carp (Nekoubin and Sudagar, 2013). The decrease in FCR in our study in all supplemented groups compared to the control agreed with Abdel-Tawwab (2008) in *O. niloticus* fed with AZN, and De Chavez and Bolivar (2018) in *Clarias gariepinus* fed SP.

The bio-somatic indices are considered an indicator of fish response to environmental stress (Barton *et al.*, 2002). In the present study, SP was a strong inducer of increment in all bio-somatic indices where HSI and ISI significantly increased in SP groups, while ISI and SSI markedly increased in AZN 5%-SP 1% group. The increase in HSI may be attributed to hypertrophy and/or hyperplasia of liver cells (Ayoola, 2008). These results were in partial agreement with Mahmoud *et al.* (2018), who reported an increase in HSI of *O. niloticus* with Spirulina 2%. An increase in ISI may be recognized to the increase in the intestinal tract villi thickness (Wang *et al.*, 2008) which enabling more usage of aquatic plant food and enhances tilapia digestion. These results were matched with those obtained from feeding of *O. niloticus* with bee pollen incorporated diet (El Asely *et al.*, 2014).

Regarding the consequence of SP and AZN on liver antioxidant enzymes activity, high GSH-px, SOD and CAT values were found in the AZN 10%-SP 1% mix followed by AZN 5%-SP 1% mix compared to control. AZN contains high content of vitamins A, B12 and

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beta carotene. The algal carotenoid extract has significant antioxidant activity (Hu *et al*, 2008). SP is rich in phytopigments such as phycobilins, phycocyanin and allophycocyanin, and xanthophylls which has antioxidant activity (Bermejo *et al.*, 2008). Therefore, we suggest that AZN acts synergistically with SP in improving ISS which enhances digestion and absorption of proteinaceous feed staff that help in antioxidant enzymes biosynthesis. These antioxidants protect cells from damage by the excessive ROS from cells (Amin, 2014).

Regarding the effect of SP and AZN on blood parameters, our study revealed that leukocytes significantly increased in *O. niloticus* fed AZN 5% and SP 0.5 %. The increase in WBCs with SP demonstrates its immune-stimulating effect and anti-infection properties (Mahmoud *et al.*, 2018). Former studies by Abdel-Tawwab and Ahmed (2009) (used SP at the levels of 1.25-10.0 g/kg feed) and Ragap (2009) (used SP at the levels of 2.5, 5 and 10 %), which is considered high levels compared to the recent study, showed an increase in WBC. In the meantime, Amin (2014) fed *O. niloticus* with 1% Spirulina for shorter time (30 days) observed significant increase in WBCs.

Concerning to male fecundity, most spermatozoa parameters were improved by SP 1% feeding. No former study affirmed the influence of SP on male fecundity. The positive impact of SP on semen quality possibly due to its high content of beta-carotene and its antioxidative mechanism in ROS scavenge (Almbro *et al.*, 2011). Similarly, Tizkar *et al.* (2015) found an increase in sperm concentration and motility of goldfish *Carassius auratus* when fed carotenoid-based diet for 150 days. The effect of SP on female reproductive performance upon short term supplementation (one month) was observed in unripe fish characterized by an increase in egg population and fecundity index. In ripen fish, we noticed that the eggs size tended (p=0.09) to be larger than the control. Former study with supplementation of SP to the diet of *O. niloticus* for long period (19 weeks) showed an increase in the gonadosomatic index (Malak and Sangak, 2017). The improvement of either male or female reproductive performance may be accredited to high nutritional value of SP e.g. amino acids, vitamins, minerals, fatty acids and β-carotene (Vonshak, 1997) that provide energy and other essential requirements for spawning activities (Malak and Sangak, 2017). In addition, the incorporation of carotenoid alleviates the harmful effect of ROS, which reduce spermatozoa damage (Almbro *et al.*, 2011). Some of SP contains large quantities of eicosatetraenoic acids and arachidonic acids which influence the reproductive performance of fish (Kyle *et al.*, 1992).

In conclusion,aquatic plants either algae or ferns have positive impacts on growth performance, bio-somatic indices and antioxidant enzymes activity which enhance fish productivity

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and protect fish under stressful conditions. The effect of these substances (SP and AZN) not only confined to growth promoting effect, but also extended to *O. niloticus* (male and female) fecundity.

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