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Dietary DL-α-tocopheryl acetate enhances growth, survival, and resistance against an abrupt shift to higher salinity of the Asian catfish (*Clarias macrocephalus*) fry

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Abstract

The effect of dietary supplementation of DL-α-tocopheryl acetate (α-TA) on growth performance, feed efficiency, survival, and salinity resistance of Asian catfish (*Clarias macrocephalus*) fry was evaluated. A total of 750 catfish fry were divided into five treatments with three replicates and fed experimental diets containing varying levels of α-TA: 0, 100, 200, 300, and 400 mg·kg^{-1} diet for 45 days. Results show that catfish fry-fed diets supplemented with α-TA exhibited significantly higher weight gain (WG) and final average body weight (FABW), better specific growth rates (SGR) and feed conversion ratio (FCR), and significantly higher survival rates compared to those of the control group fed the basal diet ($P < 0.05$). Better salinity resistance was observed in the Asian catfish fed the α-TA-supplemented diets when abruptly exposed to higher salinity (10 ppt) for up to 48 h. In addition, the diet attractability test revealed that α-TA-supplemented diets attracted significantly more catfish fry than the basal diet lacking vitamin E ($P < 0.05$). In conclusion, the results demonstrated that dietary supplementation of α-TA (vitamin E) improved growth, feed efficiency, feed attractability, survival, and resistance to abrupt higher salinity exposure of *Clarias macrocephalus* fry.

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Introduction

Vitamin E is a general term for lipid-soluble antioxidants, tocopherols, and tocotrienols, where α - tocopherol has the highest biological activity (NRC, 2011). Vitamin E plays a vital role in animals' growth, reproduction, endocrine system function, oxidation resistance, and immunity (Yin et al., 2008; NRC, 2011; Zhao et al., 2018). The appropriate dietary vitamin E concentrations in fish could also enhance flesh quality (Zanon et al., 2018), organic fatty acid composition (Chen et al., 2018), growth, and reproductive performance (Erdogan and Arslan, 2019). Vitamin E performs many functions in farmed fish, including prevention of peroxidation of cellular and subcellular membrane phospholipids and leading to maintaining the structural and functional integrity of animal cells, improvement of the immune response, enhancing growth performance and feed efficiency, preventing muscle degeneration, reducing the risk of atherosclerosis, interaction with several micronutrients especially vitamin C and Se, improving reproductive efficiency and larval performance, and improving meat quality and shelf life of seafood (El-Sayed & Izquierdo, 2021).

Previous authors have demonstrated that vitamin E deficiency impairs growth performance in some aquaculture fish species such as the Atlantic salmon (Hamre and Lie, 1995), channel catfish fingerlings (Wilson et al., 1984), rainbow trout (Cowey et al., 1984), black sea bream (Peng et al., 2009), sea bream (Tocher et al., 2002) and spotted murrel juveniles (Abdel-Hameid et al., 2012). In contrast, some studies also demonstrated that the influence of vitamin E on growth was not observed (Cowey et al., 1983; Cowey et al., 1981; Wilson et al., 1984; Baker and Davies, 1996; Bell et al., 2000; Gaylord et al., 1998; Gatlin et al., 1992). The effect of vitamin E on growth performance is still unclear, and its importance needs to be assessed, particularly in Asian catfish. On the other hand, excess levels of vitamin E could induce lipid peroxidation by acting as a prooxidant in the generation of hydrogen peroxide (Tokuda & Takeuchi, 1999; Li et al., 2014; Abdel-Hameid et al., 2012). Thus, an optimum level of vitamin E should be provided to fulfill the fish's requirements, especially under cultural conditions.

There is much evidence that nutritional vitamin imbalances affect the immune response. However, there are only a few studies on the relationships between dietary vitamins and stress responses in fish (Davis et al., 1998), most of them focusing on the role of vitamin C in fish stress responses (Henrique et al., 1998), and few of them concentrate on the role of vitamin E (Montero et al., 1999). Several unfavorable aquaculture conditions induce a stress response characterized by the release of hormones (e.g., cortisol and catecholamines) with subsequent physiological responses such as suppression of the immune system and increased mortality (Wedemeyer, 1997)

DL-α- tocopherol acetate (α-TA) is the form of vitamin E commonly supplied in aquafeeds because of its higher stability and oxidation resistance during feed processing and storage (Hamre and Lie, 1995; Peng et al., 2008). It can donate its phenolic hydrogen atoms to lipid-free radicals (Mourente et al., 2007), acting as quenchers of singlet oxygen free radicals, which renders these substances the capacity to protect tissues from damage, especially the unsaturated fatty acids (PUFA) of the cellular membrane that are more susceptible to oxidation. Previous studies have demonstrated that by supplementing diets with vitamin levels well above the requirement, approximately 50 $mg \cdot kg^{-1}$ for most fish species (NRC, 2011), fillet quality was improved by increasing oxidative stability and shelf-life (Peng & Gatlin III, 2009).

The clariid population of the Philippines and Southeast Asia, specifically *Clarias macrocephalus* and *C. batrachus,* is ever declining because of increased anthropogenic activities (Petkam & Moodie, 2001). In 2013, *C. macrocephalus* was listed as near threatened by the International Union for Conservation of Nature (IUCN). If *in situ* conservation measures for the Asian catfish, such as reintroduction, is desired, it is a requirement to transplant fingerlings whose growth and immune system have been

enhanced before release. The pure *C. macrocephalus* grows slowly and is susceptible to diseases (Diana et al., 1985). It is then necessary to explore dietary additives that would enhance both these factors before any *in situ* protocol is conducted. Hence, this study was performed to determine the potential growth-promoting effects of vitamin E in the form of DL-α- tocopherol acetate supplemented in the diet of the Asian catfish (*Clarias macrocephalus*) fry.

Materials and Methods

Induced spawning and artificial fertilization

Female breeders were induced to spawn, and the eggs were artificially fertilized with the suspension of extracted testis from the male Asian catfish as described previously (Bautista et al., 2022). Briefly, females were injected intramuscularly with gonadotropin and dopamine inhibitor (0.5 $ml \cdot kg^{-1}$; Ovaprim, Syndel Lab. Ltd., USA) into the dorsal muscle. Stripping was done the following morning, and the dry method of artificial fertilization was carried out. Male Asian catfish were sacrificed, testis excised, cut into small pieces, squeezed onto the stripped eggs in a small bowl, and gently stirred with chicken feathers for about 5 min, and the fertilized eggs were incubated.

Experimental fish and set up

A total of 750 (50 $fry \cdot tank^{-1}$) Asian catfish (*Clarias macrocephalus*) fry (24 days after hatching, DAH) were randomly distributed and stocked into fifteen experimental circular tanks (70 cm diameter x 25 cm height) containing 50 liters of freshwater (salinity of 0 parts per thousand, °/oo). Experimental fish were acclimatized to a basal (i.e. no α-TA) diet for one week before the conduct of the experiment. The setup was located at Salem Aquafarm in Puerto Princesa City, Palawan, Philippines.

Experimental diets

Five experimental diets were used: basal diet with no α-TA, 100 mg α-TA, 200 mg, 300 mg, and 400 mg α-TA. All diets contained ~44.0% crude protein and ~2.6% crude fat. Feed composition is shown in **Table 1**.

Feeding trial experiment

The experimental diets were fed to three replicate groups of Asian catfish fry at four times a day (0800h, 1200h, 1600h, 2000h) for a total of 45 days culture period. Feeding was *ad libitum* wherein the amount of feed was measured every first day of the week and was used as basis for the feeding rate for the whole week. This feeding method was repeated every week until the end of the experiment. Actual initial and final weights were obtained on the first and last day of the experiment. Siphoning of fish wastes and uneaten feeds were done regularly every morning and the water was replenished thereafter. Water change was done at 50% of the water volume twice a week. Water quality parameters such as DO, temperature, and pH were monitored three times a week while nitrite, ammonia and nitrate were measured once a week.

Table 1 Composition of experimental diets.

Ingredients	α-TA (mg)				
	0	100	200	300	400
Fish meal (sardines)	496.00	496.00	496.00	496.00	496.00
Soybean meal	267.00	267.00	267.00	267.00	267.00
Corn starch	125.00	125.00	125.00	125.00	125.00
Cod liver oil	22.00	22.00	22.00	22.00	22.00
Vitamin mix	25.00	25.00	25.00	25.00	25.00
Mineral mix	25.00	25.00	25.00	25.00	25.00
CMC	40.00	39.90	39.80	39.70	39.60
DL-α-tocopheryl acetate	0.00	0.10	0.20	0.30	0.40
Total	1000.00	1000.00	1000.00	1000.00	1000.00
Proximate analysis (% dry weight)					
Moisture	7.70				
Crude protein	43.68				
Crude fat	2.62				
Crude fiber	1.61				
NFE	23.41				
Ash	2.07				

[a]Vitamin mix (estimated amount in 25 g mix per kg diet): Vitamin A, 9 mg; Vitamin D_3, 1.25 mg; Vitamin B_1, 200 mg; Vitamin B_2, 200 mg; Vitamin B_6, 125 mg; Vitamin B_{12} 50 mcg/kg; Niacin, 1000 mg; Calcium Pantothenate, 500 mg; Biotin, 1 mg; Folic Acid, 45 mg; Ethoxyquin, 12.5 mg; α-TA at 0, 100, 200, 300, 400 mg·kg^{-1} diet.
[b]Mineral mix (estimated amount in 25 per kg diet): Fe, 1000 mg; Mn, 250 mg; Zn, 1000 mg; Cu, 100 mg; Cu, 100 mg; I, 45 mg, Co, 0.5 mg; Se, 1 mg.

Growth response parameters

The growth parameters were computed using the following formula:

Weight Gain (g) = Final Average Body Weight - Initial Average Body Weight

SGR (% day^{-1}) = [Ln (Final ABW in g) – Ln (Initial ABW in g)] / (No. of days) x 100

Feed Intake (g) = Sum of daily feed given

Feed Conversion Ratio = Total Feed Intake / Weight Gain

Survival Rate (%) = Final count of catfish fry / Initial count of catfish fry x 100

Abrupt shift to higher salinity challenge

At the end of the feeding experiment, a total of 30 Asian catfish fry as representative of each treatment (10 catfish fry per replicate basin) were subjected to salinity challenge test for 2 days at 10 parts per thousand (°/oo). Two trials of salinity challenge tests were conducted. Saline water used in the experiment was prepared by dissolving commercially available salt in freshwater and verified using refractometer to achieve the salinity of 10 ppt. The saline water was then distributed into 15 square-shaped plastic containers (16 cm x 16 cm x 10 cm) containing 1.5 liters each. Mortality of catfish fry was monitored every 12 h until the end of the challenge test. Catfish fry that are immobile and showed no reaction when poked using a stick is considered dead. Mortality rate of fish was measured using the following formula:

Mortality Rate (%) = Dead fry count / Initial fry count * 100

Diet attractability

A diet attractability test was conducted using a square-shaped wooden tank (122 cm x 122 cm x 31 cm) with multiple chambers following the procedure of Suresh et al. (2011). The tank consisted of acclimatization, middle, and feeding chambers separated by a liftable shutter. The feeding chamber consisted of five sub-chambers designated for each experimental diet. The tank was filled with 150 L of freshwater (0 ppt). Three runs of diet attractability tests were conducted for this experiment.

A total of 30 catfish fry (ABW = ~0.5 g) were placed in the acclimatization chamber and then acclimatized for 1 h. Before commencing the test, 2 g of each experimental diet were placed into the feeding sub-chambers. Three min after the placement of feed, the shutter was lifted to allow the catfish fry to access the test diets. The number of catfish fry that entered the feeding chamber and fed on each test diet was recorded at 1, 5, and 10 min. The percentage of catfish fry attracted to a particular test diet was averaged for the 3 different time intervals.

Statistical analysis

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) software. Data on final average body weight, weight gain, specific growth rate, survival, and feed utilization parameters were presented as mean (± SEM i.e., standard error of the mean). One-way analysis of variance ANOVA ($p<0.05$) was performed after passing the tests for both normalities of data distribution and homogenous variance, followed by Post hoc analyses using Duncan's Multiple Range Test (DMRT) to determine significant differences among the means.

Results

Growth Performance, Feed Efficiency, and Survival

The growth performance, feed efficiency, and survival of Asian catfish fry-fed the experimental diets are presented in **Table 2**. At the end of the experiment, results showed that catfish fry fed with experimental diets supplemented with vitamin E (i.e., α-TA) exhibited significantly higher weight gain (WG) and final average body weight (FABW) compared with those of the control group fed the basal diet ($P < 0.05$). Dietary supplementation of α-TA also revealed significantly higher values for specific growth rate (SGR) and feed conversion ratio (FCR) than those in the control group. The lowest feed intake was observed in the control group ($P< 0.05$). Survival of Asian catfish fry fed α-TA-supplemented diets was significantly higher than that of the control group fed the basal diet ($P< 0.05$). The optimum level of dietary α-TA was estimated using the WG vs α-TA relationship using both broken-line and second-order polynomial regression analyses to be in the range 170.23 - 233.3 mg·kg^{-1} α-TA (Fig. 1). The optimum level to elicit the highest survival after 48 h abrupt exposure from 0 ppt to 10 ppt of salinity was in the range 200 – 276.75 mg·kg^{-1} α-TA.

Table 2 Growth performance, feed efficiency, and survival of Asian catfish fry-fed experimental diets containing varying levels of α-TA for 45 days.

α-TA *(mg)*	IABW (g)	FABW (g)	WG (g)	FI (g)	SGR (% day^{-1})	FCR	Survival (%)
0	0.05	0.46±0.02^{a}	0.40±0.02^{a}	1.02±0.02^{a}	4.83±0.15^{a}	2.53±0.11^{b}	72.67±1.76^{a}
100	0.05	0.59±0.01^{b}	0.53±0.01^{b}	1.05±0.01^{ab}	5.36±0.05^{b}	1.98±0.04^{a}	83.33±2.4^{b}
200	0.05	0.65±0.00^{b}	0.59±0.00^{b}	1.09±0.02^{b}	5.56±0.07^{b}	1.83±0.05^{a}	94.00±1.15^{c}
300	0.05	0.61±0.03^{b}	0.56±0.03^{b}	1.07±0.02^{b}	5.46±0.09^{b}	1.92±0.09^{a}	91.33±2.4^{c}
400	0.05	0.59±0.00^{b}	0.54±0.01^{b}	1.05±0.00^{ab}	5.36±0.05^{b}	1.95±0.02^{a}	90.67±1.33^{c}

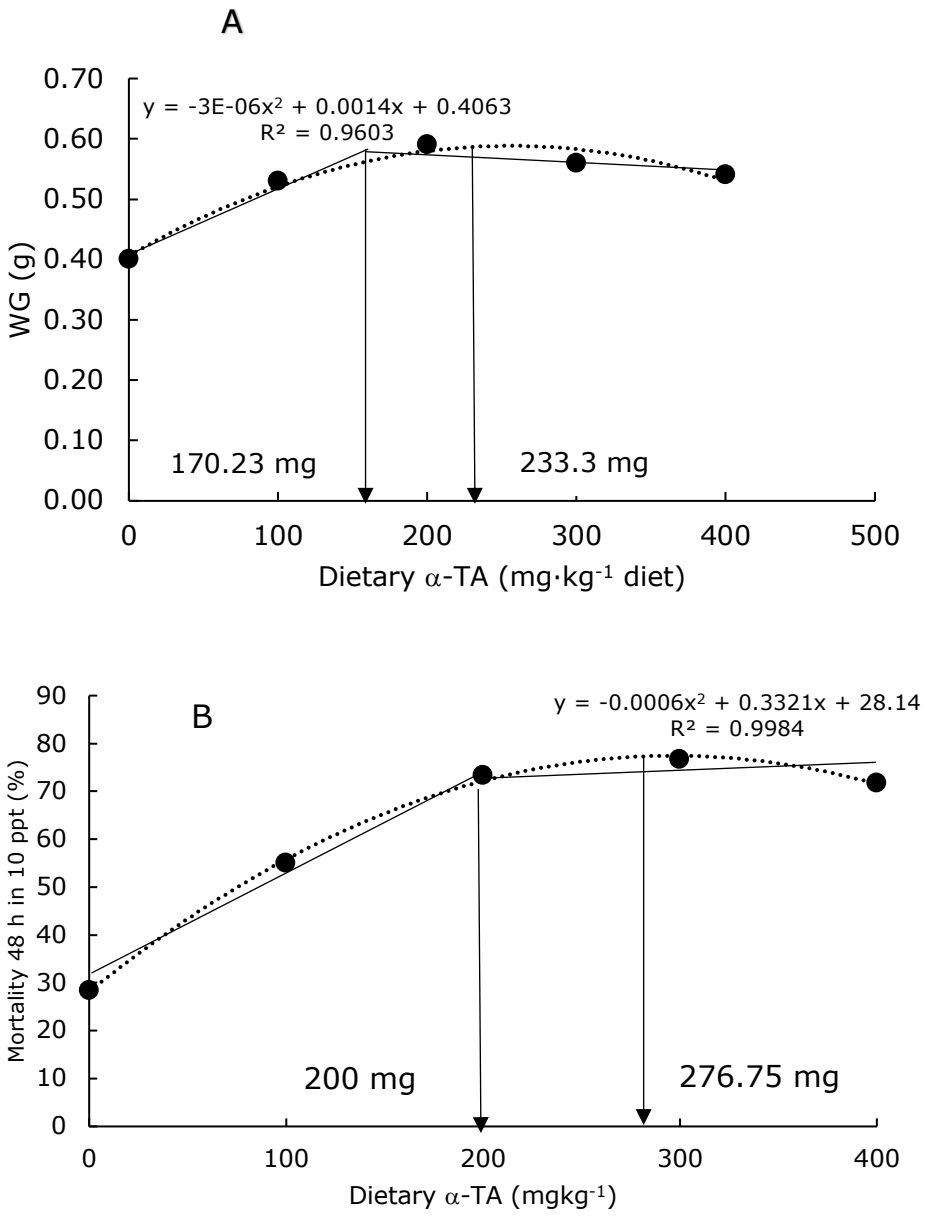

Figure 1 Broken-line and second-order polynomial regression analyses for the relationship between (**A**) WG and (**B**) % cumulative mortality at 48 h abrupt exposure from 0 to 10 ppt salinity, against varying levels of dietary α-TA in the Asian catfish fry.

Diet Attractability

Evaluation of diet attractability is presented in **Table 3**. Results showed that the α-TA-supplemented diets significantly attracted more Asian catfish fry than the basal diet without vitamin E ($P< 0.05$).

Table 3 Percent of Asian catfish fry attracted to the experimental diets containing varying levels of α-TA.

α-TA *(mg)*	*Ave.no. of fry attracted (%)*
0	7.78±0.64^{a}
100	12.58±1.61^{b}
200	14.82±1.49^{bc}
300	17.78±0.64^{c}
400	13.70±1.50^{bc}

Abrupt shift to higher salinity

Better resistance to abrupt shift to higher salinity was observed in fry-fed diets containing DL-α-tocopheryl acetate (**Table 4)**. After 12 h of abrupt exposure to higher salinity water, cumulative mortality rates ranged from 3.33 to 8.33% in groups fed diets containing α-TA, which were significantly lower than the 21.67% mortality rate in the control group ($P < 0.05$). After 24 and 36 h exposure to higher salinity, mortality rates of catfish fry in α-TA-treated groups remained significantly lower than those in the control group ($P<0.05$). At 48 h, the control group exhibited significantly the highest mortality rate with 71.67%, compared with those in the vitamin E-treated groups ($P < 0.05$).

Table 4 Mean cumulative mortality of Asian catfish fry after abrupt exposure to 10ppt salinity (± SEM).

α-TA (mg)	*Mortality of fry (%)*			
	12 h	*24 h*	*36 h*	*48 h*
0	21.67±4.41[a]	40.00±2.89[a]	58.33±3.33[a]	71.67±4.40[a]
100	8.33±1.67[b]	20.00±2.89[a]	36.67±4.41[b]	45.00±2.89[b]
200	3.33±1.67[b]	10.00±0.00[c]	20.00±0.00[c]	26.67±4.40[c]
300	5.00±0.00[b]	13.33±3.33[bc]	20.00±5.77[c]	23.33±4.40[c]
400	5.00±2.89[b]	13.33±3.33[bc]	21.67±±1.67[c]	28.33±3.33[c]

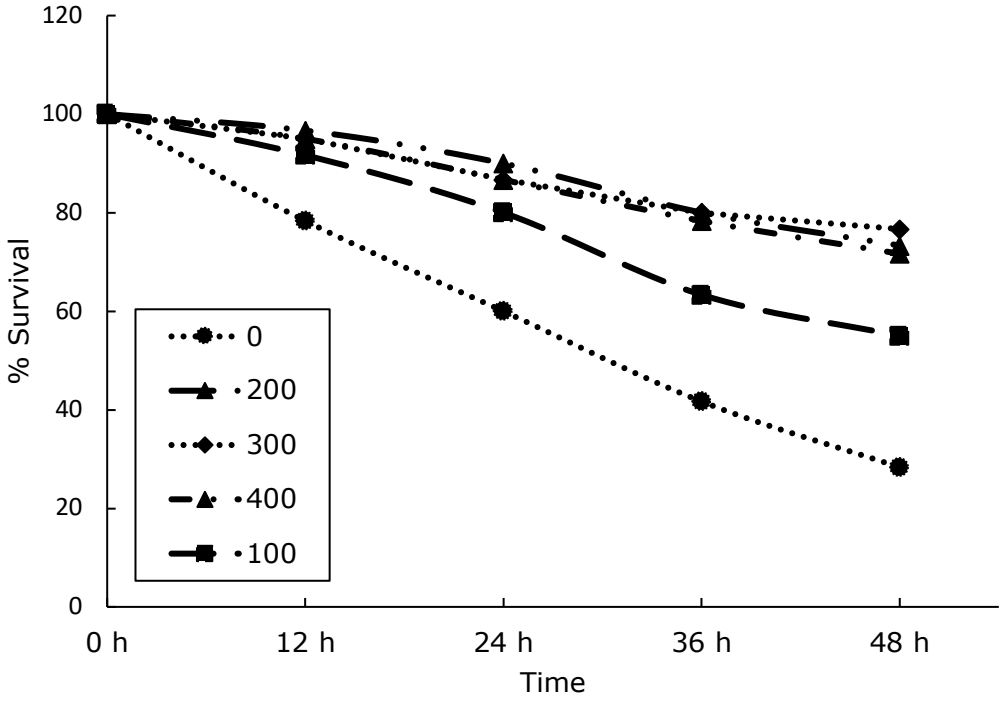

Figure 2 Periodic cumulative mortality of Asian catfish fry *Clarias macrocephalus* subjected to abrupt shift to higher salinity 10 ppt).

Discussion

In the evaluation of incorporating a dietary additive in aquafeeds, it is important that the test diet is sufficiently attractive and palatable to the test fish, which is a key concern when developing artificial aquaculture diets (Dworjanyn et al., 2007). This is because palatability indirectly affects consumption and digestibility by making the food more or less attractive to the grazer (Bowker, 2013). If test diets are unpalatable, other observed parameters would be less meaningful in the point of view of animal nutritionists. Also, the unpalatable feed will result in a large amount of waste from uneaten/undigested food and

poor animal performance (growth and quality). Unpalatable diets cause the fish to become satiated more quickly and therefore eat less food, decreasing their growth in the allotted time frame, making the evaluation of the feed additive at best doubtful. In the present study, the formulated test diets which contained α-TA elicited attractability in the Asian catfish fry. Thus, the growth and efficiency parameters observed in the present study were considered to be highly reliable.

Successful fish farming of a particular species requires using complete, efficient, and optimal feed to provide all nutritional requirements for fast and healthy fish growth. Results of the present study demonstrated that supplementation has significantly improved the growth, feeding utilization, and survival of Asian catfish *(Clarias macrocephalus)* fry. This agrees with several studies that reported the advantageous effects of dietary supplementation of vitamin E fed to several fish species. The study of Wang et al. (2019) demonstrated that proper dietary vitamin E could improve growth in yellow drum (*Nibea albiflora*) juveniles; in juvenile olive flounder (*Paralichthys olivaceus*) (Moniruzzaman et al., 2017); and large yellow croaker (*Larimichthys crocea*) (Zhang et al., 2022). The highest mortality of fish fed the basal diet containing no α-TA in the present study also suggests that dietary α-TA improves the survival of *C. macrocephalus* fry. On the other hand, Montero et al. (2001) and Blazer (1982) observed no alterations in the growth of rainbow trout fed a non-vitamin E-supplemented diet for 4 months.

The optimum amounts of dietary α-TA to elicit the maximum WG in the present study are in the range of 170.23 - 233.3 mg·kg^{-1} and to produce the highest survival when exposed abruptly from 0 ppt to 10 ppt, 200.00 – 276.75 mg·kg^{-1}; these values were higher than those of other fish species studied so far. The optimum level for broodfish meagre (*Ompok pabda*) was 100 mg·kg-^{1} feed (Sarowar & Mollah, 2009). Optimum values for growth for other fish species were 99 mg·kg^{-1} for *Cirrhinus cirrhosis* (Paul et al., 2004), 100 mg·kg^{-1} (Watanabe et al., 1970), and 80-100 mg·kg^{-1} (Halver, 2002) for the common carp *Cyprinus carpio*, 120 mg·kg^{-1} for the Atlantic salmon, *Salmo salar* (Hamre & Lie, 1995), 24-33 mg·kg^{-1} diet (Hung et al., 1981) and 2-3 mg·kg^{-1} diet (Cowey et al., 1981) for rainbow trout (*Salmo gairdneri*), 79.44 mg·kg^{-1} for Caspian trout (*Salmo caspius*; Saheli et al., 2021), 78-111 mg·kg^{-1} for cobia *Rachycentron canadum* (Zhou et al., 2013), 61-115 for grouper *Epinephelus malabaricus* (Lin & Shau, 2005), 100-200 mg·kg^{-1} for grass carp *Ctenopharyngodon idella* (Li et al., 2014). In general, studies on vitamin E requirements of various species range from 2 to 200 mg·kg^{-1} diet α-TA. The high optimum level for growth of Asian catfish fry in the present study could be due to several factors such as the fish species, life stage, feeding regime, rearing environment, diet composition, and even method of sample analysis (Hamre, 2011; Lu et al., 2016). It has been reported that high dietary lipid content, especially in the form of unsaturated fatty acids or low levels of other antioxidants like vitamins C, selenium, and astaxanthin, can provoke the dietary vitamin E requirement to protect the cell against lipid peroxidation (Hamre, 2011; Puangkaew et al., 2004). On the other hand, although the WG values of the catfish fry to levels above the estimated optimum were not significantly different in the present study, the slight numerical decrease in WG above this level is most likely explained by the slight imbalance and accumulation of vitamin E radicals, which may act as prooxidants (Li et al., 2013). High levels of vitamin E depressed growth performance and impaired feed utilization in rainbow trout *Oncorhynchus mykiss* (Kiron et al., 2004), spotted snakehead *Channa punctatus* (Abdel-Hameid et al., 2012), and yellow catfish *Pelteobagrus fulvidraco* (Lu et al., 2016). With regard to the level of minimum requirement level for vitamin E, Liu et al. (2022) have reviewed recent studies, and current industry practices in connection with vitamin E requirement in fish and have suggested increasing the dietary levels of vitamin E above the NRC recommendation of 50-60 mg·kg^{-1} diet to values ranging between 200 and 600 mg·kg-^{1} diet to avoid deficiencies, improve performance, immune status and decrease nutrient loss.

In the abrupt shift to higher salinity challenge test, *C. macrocephalus* fed the α-TA-supplemented diets showed significantly higher survival rates than the control group fed with the basal diet. This result demonstrated that α-TA could have improved the immunity and resistance of Asian catfish fry against environmental stress, which is the abrupt shift to higher salinity change. Hamre et al. (2022) presented evidence that initially contributed proof to their hypothesis that increasing temperature in spring led to oxidative stress in Atlantic salmon (*Salmo salar*) with an accumulation of oxidation products in the tissues and increased utilization of antioxidants. Previous to this study was that of Nordgarden et al. (2003) found that Atlantic salmon fillet exhibited elevated thiobarbituric acid reactive substances (TBARS) and lowered fillet concentration of astaxanthin and α-tocopherol in spring. These studies have somewhat connected environmental stress to cellular oxidative stress in fish. The challenge of abrupt salinity change in the Asian catfish probably led to a condition of oxidative stress in the cells. Gilthead seabream (*Sparus aurata*) juveniles were fed either a diet containing 150 mg of α-tocopherol or with no additive for 15 weeks and subjected to overcrowding as a chronic stressor and repetitive chasing as an acute repetitive stressor; the results demonstrated that a fish-fed diet with no vitamin E exhibited lower stress resistance (Montero et al., 2001). Furthermore, increased fish mortality fed the reduced vitamin E diet after 8 days of repetitive stress, denoting a combined effect of low dietary levels and stress.

Vitamin E (i.e., α-tocopherol) plays an important role in detoxification and protection of the animal body, mainly in anti-oxidation, prevention of tissue and cell damage, enhancement of animal immunity, which can effectively protect the animal body and alleviate oxidative stress and other damages (Yan et al., 2022). Liu et al. (2022) also recommended that to ensure that fish growth or health is not compromised, it would be beneficial to identify the optimal dietary vitamin E inclusion level using practical formulation under stressful and sub-optimal farming conditions. In the present study, we determined the optimum α-TA supplementation to elicit the highest survival after abrupt exposure of the catfish fry from 0 ppt to 10 ppt in 48 h, the value of which was higher than that for growth: 200 mg·kg^{-1} α-TA to 276.75 mg·kg^{-1} α-TA.

In conclusion, α-TA supplementation in the diet of Asian catfish (*Clarias macrocephalus*) fry significantly improved its growth performance, feed utilization, and survival, as well as elevated its resistance to abrupt shift to higher salinity exposure. The minimum requirement ranges for dietary α-TA to elicit optimum growth and survival under salinity stress were 170.23 - 233.3 mg·kg^{-1} α-TA and 200 mg·kg^{-1} α-TA to 276.75 mg·kg^{-1} α-TA, respectively.

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