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Original Research Article

Interactive effect of dietary vitamin E and inorganic mercury on growth performance and bioaccumulation of mercury in juvenile olive flounder, *Paralichthys olivaceus* treated with mercuric chloride

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ABSTRACT

A 6-week feeding trial was carried out to evaluate the effects of dietary vitamin E (DL-α-tocopheryl acetate, TA) on growth and mercury (Hg) accumulation in juvenile olive flounder (*Paralichthys olivaceus*) treated with mercuric chloride ($HgCl_2$). Vitamin E and $HgCl_2$ were added to the semi-purified basal diet. Six semi-purified diets in a 2 × 3 factorial design were formulated to contain 2 levels of Hg (0 or 20 mg $HgCl_2$/kg diet) and 3 levels of vitamin E (0, 100, or 200 mg TA/kg diet). Experimental fish (n = 360, 9.99 ± 0.15 g) were randomly allocated into 30-L tanks at a density of 20 fish per tank with 3 replicates in each treatment and were fed twice a day. At the end of the feeding trial, dietary Hg depressed the growth performances in terms of weight gain (WG), specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) in fish, while fish fed the diets supplemented with vitamin E showed significant growth improvement in both presence and absence of $HgCl_2$ in the diets ($P < 0.05$). Survival rate was not affected in fish fed the experimental diets. Whole body compositions of fish such as lipid and moisture contents were influenced by dietary vitamin E supplementation. Total Hg contents of muscle, liver and kidney tissues were significantly reduced in fish fed diets supplemented with vitamin E ($P < 0.05$), while the two-way ANOVA showed that increasing Hg concentration has resulted in a reduction in vitamin E. Whole body fatty acids of fish like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents were decreased by dietary Hg. However, supplementation of dietary vitamin E improved the α-linolenic acid (ALA) and EPA contents in fish. Our results suggest that dietary supplementation of vitamin E has potential effects on growth improvement and ameliorating inorganic Hg bioaccumulation in juvenile olive flounder.

1. Introduction

Fish is considered as a well-balanced diet because of its nutritional benefits such as high-quality protein, vitamin, high energy and minerals (Pieniak et al., 2010). Marine fish are a rich source of omega-3 polyunsaturated fatty acids (PUFAs) especially α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and they contain a very low level of cholesterol (Carvalho et al., 2005; Castro-González and Méndez-Armenta, 2008; Storelli, 2008; Groth, 2010; Vieira et al., 2011). They help in the neurodevelopment of children and have an active role in coronary heart disease (Mozaffarian and Wu, 2011; Swanson et al., 2012). In contrast, marine fish can also be a potential source of toxic metals at a higher trophic level through their consumption, such as Minamata disease in Japan in the 1950s that was caused by methylmercury (MeHg) poisoning (Wen-Xiong et al., 2012). Although fish intakes have potential health benefits, the presence of mercury

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(Hg) in seafood has raised public health concerns (Olmedo et al., 2013) due to their toxicity, persistence, bioaccumulation and biomagnification into the food chain (Castro-González and Méndez-Armenta, 2008; Storelli, 2008; Jaeger et al., 2009; Groth, 2010; Mendil et al., 2010; Carrasco et al., 2011; Gewurtz et al., 2011). Therefore, fish exposure to Hg presents unique problems from both toxicological and nutritional perspectives (Clarkson, 1998).

Mercury is considered as one of the most dangerous toxic metals especially in the aquatic environment (Asefi and Zamani-Ahmadmahmoodi, 2015). It can be found in 3 major forms, namely elemental Hg (Hg^0), inorganic Hg (Hg^{+2}), and organic Hg (CH_3Hg^+) (Looi et al., 2016). Mercuric chloride ($HgCl_2$) is a cumulative poison and is considered as a direct-acting toxicant (Sharma and Bhattacharya, 2010). The natural and anthropogenic emissions of Hg exist as inorganic forms (Wiener, 2013). Increases in the deposition of Hg in marine environments result from increased anthropogenic activities that could enhance food chain bioaccumulation and higher concentrations of Hg in marine fish (Jones et al., 2013). The typical concentration of Hg in edible tissues of various species of fish ranges from 50 to 1,400 μg/kg fresh weight; however, fish from contaminated aquatic environments can have 10 mg/kg (IPCS, 2003). Niimi and Kissoon (1994) reported that the level could be reached to 20 mg/kg which may cause toxicity in fish. Kruzikova et al. (2008) confirmed that Hg can be predominantly deposited as MeHg in fish tissue. However, Ikingura and Akagi (2003) postulated that a substantive level of Hg (0 to 44%) can also be present as inorganic Hg in fish especially in seafood (Moon et al., 2011). Mercury exposure in fish at high levels in inorganic or organic form can permanently damage the brain, kidneys, and fetus (ATSDR, 2003). However, most researchers do not give enough attention to nutritional factors that might influence organism in response to heavy metal intoxication (Chapman and Chan, 2000). Therefore, in addition to organic Hg, it is imperative to assess the inorganic Hg toxicity and its interaction with nutritional factors in fish on the dietary basis.

Vitamin E (α-tocopherol) as a lipid-soluble vitamin can act as antioxidants by scavenging reactive oxygen species from different tissues of organisms (Agarwal et al., 2010). It has several naturally occurring forms with d-α-tocopherol having the highest biopotency (NRC, 2011). It is well documented that the toxic effects of divalent Hg can be prevented by chelating or enhancing antioxidant defense systems (Pillai and Gupta, 2005). Vitamin E can inhibit oxidative damage in the liver and other tissues caused by mercury and cadmium intoxication in animal (Rana et al., 1996; Rao and Sharma, 2001). In juvenile olive flounder, the dietary vitamin E requirement is 22 mg/kg diet (Korea-US Aquaculture, 2017).

Fish consumption is considered as the main exposure route for Hg contamination in human (Cerveny et al., 2016). Thus, different tissues of fish can be of interest to researchers for assessing total Hg content in the fish (Oost et al., 2003). This approach can be appropriate in the case of commercial fish species, such as olive flounder, *Paralichthys olivaceus*, in Korea, which is representing the highest aquaculture production and the highest consumer demand (Okorie et al., 2013). So far, very limited information is available about the dietary impact of inorganic Hg and its interaction with different antioxidants *in vivo* (Agarwal et al., 2010; Lee et al., 2016).

In our previous experiments, we reported that vitamin C, E and selenomethionine had protective effects on dietary-induced organic Hg toxicity in juvenile olive flounder (Moniruzzaman et al., 2017; Park et al., 2016). In another study, Lee et al. (2016) reported that dietary vitamin C had reducing effects on tissue Hg accumulation in the same species of fish when exposed with inorganic Hg. Based on our previous findings and importance of inorganic Hg, we aimed to evaluate the effects of dietary vitamin E on inorganic Hg in terms of dietary $HgCl_2$ accumulation in different tissues as well as growth performance of juvenile olive flounder in this study.

2. Materials and methods

2.1. Test diets

In the present study, we used a semi-purified basal diet as a control without supplementing vitamin E and/or Hg (Table 1). Total 6 experimental diets with a 2 × 3 factorial design were formulated to contain 2 dietary Hg levels at 0 and 20 mg Hg/kg in the form of $HgCl_2$ and 3 vitamin E (DL-α-tocopheryl acetate, TA) levels at 0, 100 and 200 mg TA/kg (Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg Hg with 0, 100 and 200 mg TA, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg Hg with 0, 100 and 200 mg TA, respectively) in triplicates. However, the actual analyzed Hg and TA levels are shown in Table 2. In basal diets when supplemented with $HgCl_2$ (Sigma-Aldrich, MO, USA) and TA (Sigma-Aldrich, MO, USA) sources, an equivalent amount of cellulose was removed. The formulated experimental diets (Hg_0E_0, Hg_0E_{100}, Hg_0E_{200}, $Hg_{20}E_0$, $Hg_{20}E_{100}$ and

Table 1
Composition of the experimental basal diet (DM basis).

Item	Content
Ingredients, g/kg	
Casein (vitamin-free)[1]	432
Defatted fishmeal[2]	100
Wheat flour[3]	60
Dextrin[1]	188
Corn starch[3]	50
Fish oil (DHA + EPA enriched)[4]	90
Vitamin premix (vitamin E-free)[5]	10
Mineral premix[6]	10
Cellulose[1]	60
Proximate analysis, %	
Moisture	8.29 ± 0.67
Crude protein	50.37 ± 0.20
Crude lipid	8.71 ± 0.29
Crude ash	4.03 ± 0.25

DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid.
[1] United States Biochemical (Cleveland, OH) 44122.
[2] Suhyup Feed Co. Ltd., Busan, Republic of Korea.
[3] Young Nam Flour Mills Co., Busan, Republic of Korea.
[4] E-Wha oil Co., Ltd., Busan, Republic of Korea.
[5] Vitamin premix contains (as mg/kg diet): DL-calcium pantothenate, 150; choline bitartrate, 3,000; inositol, 150; menadione, 6; niacin, 150; pyridoxine·HCl, 15; riboflavin, 30; thiamine mononitrate, 15; retinyl acetate, 6; biotin, 1.5; folic acid, 5.4; vitamin-B_{12}, 0.06; cholecalciferol, 2.4.
[6] Mineral premix contains (as mg/kg diet): Al, 1.2; Ca, 5,000; Cl, 100; Cu, 5.1; Co, 9.9; Na, 1,280; Mg, 520; P, 5,000; K, 4,300; Zn, 27; Fe, 40; I, 4.6; Se, 0.2; Mn, 9.1.

Table 2
Supplemented and analyzed concentration of mercury and vitamin E (as Hg and TA mg/kg, respectively) from the experimental diets.

Item	Diets[1]					
	H_0E_0	H_0E_{100}	H_0E_{200}	$H_{20}E_0$	$H_{20}E_{100}$	$H_{20}E_{200}$
Supplemented Hg[2]	0	0	0	20	20	20
Analyzed Hg	0.07	0.07	0.07	17.6	16.7	19.1
Supplemented vitamin E[3]	0	100	200	0	100	200
Analyzed vitamin E[4]	0.08	73.4	189	0.08	82.7	186

[1] Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg/kg Hg with 0, 100 and 200 mg/kg TA, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg/kg Hg with 0, 100 and 200 mg/kg TA, respectively.
[2] As mercuric chloride (H_gCl_2) at 1,000 mg $HgCl_2$/kg.
[3] As DL-α-tocopheryl acetate (TA).
[4] As α-tocopherol.

$Hg_{20}E_{200}$) were isonitrogenous (50% crude protein) and isocaloric (16.7 kJ/g gross energy) based on the physiological fuel value of 16.7, 16.7 and 37.7 kJ/g of protein, carbohydrates and lipid, respectively, described by Halver and Hardy (2002). Casein without vitamin and defatted fishmeal were used as the main protein sources. Fishmeal was defatted thrice by mixture of chloroform and methanol (2:1, vol/vol) to avoid the influence of lipid in fishmeal which may contain fat soluble vitamins especially vitamin E. All the ingredients were mixed completely and then pelleted by using 1- and 2-mm diameter dies according to Lee and Bai (1998). Diets were dried at room temperature for 72 h and packed into airtight small bags according to diet numbers, and then kept at −20 °C until use.

2.2. Fish and feeding trial

The experiment was conducted under the guidelines of Animal Ethics Committee Regulations, No. 554 issued by the Pukyong National University, Busan, Republic of Korea. Juvenile olive flounder were obtained from a local hatchery (Hampyeong, Cheonnam, Republic of Korea) and transported to the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan, Republic of Korea. Fish were fed the basal diet (Table 1) without Hg and TA for 2 weeks before the start of the experiment to acclimate the fish to the diets and to the experimental conditions. Experimental fish (n = 360, 9.99 ± 0.15 g) were then randomly allocated into 30-L tanks at a density of 20 fish per tank with continuous air supply to maintain dissolved oxygen near saturation, with 3 replicates in each treatment. The experiment was conducted in a semi-recirculation system. Water temperature and pH were maintained at 20 ± 1 °C and 7.7 ± 0.1, respectively. Dissolved oxygen was maintained near saturation by continuous aeration with electric aerators. Fish were fed at an amount of 1.5% to 2% of wet body weight up to satiation twice a day. Feeding was done by hand very carefully and slowly to ensure ingestion of all feed. Uneaten feed was removed regularly by siphoning just after feeding to avoid possible leaching of Hg. Each tank inside was scrubbed once a week to minimize algal and fungal growth. A photoperiod of 12 h light: 12 h dark was maintained during the experimental period. Total fish weight per tank was determined after 3 weeks as midterm sampling and the feeding rates were also adjusted accordingly.

2.3. Sample collection and analyses

At the end of the feeding trial, fish were starved for 24 h, and then every fish was counted and weighed individually, to calculate weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival rate. Growth parameters were calculated as follows: WG (%) = 100 × (Final weight, g − Initial weight, g)/Initial weight (g); SGR (%/day) = 100 × (ln Final weight, g − ln Initial weight, g)/Days; FE (%) = 100 × Wet weight gain (g)/Dry feed intake (g); PER = Wet weight gain/Protein intake.

Three fish from each aquarium were collected and euthanized with tricaine methanesulfonate solution (MS 222, 0.5 g/L; Argent Chemical Laboratories, Redmount, WA, USA) to analyze whole-body proximate composition. Five additional fish per tank were randomly captured, euthanized (MS 222) and dissected to collect liver, kidney and muscle samples for the analyses of vitamin E, Hg concentration and fatty acid composition. All the analyses were performed at the Feeds and Foods Nutrition Research Center of Pukyong National University, Busan, Republic of Korea.

2.4. Proximate composition analysis

Analyses of proximate composition of experimental diets and whole fish body were performed by the standard methods of AOAC (1995). Moisture contents (1 g for each sample) in the diets and fish were determined by drying at 135 °C for 2 h. Ash content (1 g for each sample) was determined by using a muffle furnace at 550 °C for 4 h. Crude lipid (1 g for each sample) was determined by soxhlet extraction unit (Soxtec system 1046; Foss, Hoganas, Sweden) and crude protein content (0.1 g for each sample) by Kjeldahl method (N × 6.25) after acid digestion, distillation and titration of the samples.

2.5. Vitamin E analysis

Vitamin E concentration in experimental diets (0.1 g for each sample) supplemented with TA and tissues (0.1 g for each sample) such as liver, kidney and muscle of pooled fish was determined by High-Performance Liquid Chromatography (HPLC; Dionex Softron, Sunnyvale, CA, USA) with an ultraviolet (UV) detector at 254 nm (Moniruzzaman et al., 2017). The mobile phase was 0.05 mol/L KH_2PO_4 at pH 2.8, and the flow rate was 1.0 mL/min. Weighed samples were homogenized in 10% cold metaphosphoric acid. Homogenates were centrifuged at 3,000 × g for 20 min, and supernatants were analyzed by HPLC after being filtered through a 0.45 μm pore size syringe filter (Sartorius, Gottingen, Germany).

2.6. Mercury analysis

Diet and tissue Hg concentrations were determined by AOAC (2000) method as described by Lee et al. (2016). The Hg contents in each sample (1 g per sample) were determined by using Inductively Coupled Argon Plasma Mass Spectrometer (ICP-MS; Perkin-Elmer 3300, Waltham, MA, USA). The Hg detection limit was 0.001 μg/L. Trueness and precision of the analyses with each set of samples were checked by analyses of standard reference material dogfish muscle tissue, DORM-1 (Dogfish muscle Reference Material, National Research Council, Canada). The methods were found satisfactory and are within the 95% confidence limits of the certified values.

2.7. Fatty acids analysis

The whole body lipid was extracted according to Folch et al. (1957). Fatty acid methyl esters (FAMEs) of whole body lipid were prepared using acid-catalyzed transesterification method. The composition of FAMEs was determined by Gas Liquid Chromatography (Trace GC, Thermo Finnigan, San Jose, CA, USA) with flame ionization detector, equipped with a Carbowax 007 capillary column (30 m × 0.25 mm inside diameter, film thickness 0.25 μm, QUADREX, Bethany, CT, USA). Injector and detector temperatures were 250 °C. The column temperature was programmed from 100 to 220 °C at a rate of 5 °C/min and 220 to 240 °C at a rate of 3 °C/min. Helium was used as the carrier gas. Fatty acid methyl esters were identified by comparison with known standards of FAME mix-37 (Supelco, Bellefonte, PA, USA).

2.8. Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) to determine if there was any interaction between dietary Hg and vitamin E. When we found interaction, all data were analyzed by one-way ANOVA to test for the effects of the dietary treatments. When a significant treatment effect was observed, Fisher's protected least significant difference (LSD) test was used to compare means amongst treatments with significant effects. Treatment effects were considered with the significant level at $P < 0.05$. All

statistical analyses were tested using SAS version 9.1 analytical software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Growth and survival

Weight gain, SGR, FE, feed intake (FI), PER and survival rate of juvenile olive flounder fed the experimental diets for 6 weeks were shown in Table 3. Dietary Hg and vitamin E were significantly depressed or enhanced the WG, SGR, FE and PER in fish fed the diets ($P < 0.05$). Significant interaction effect was observed between Hg and vitamin E in those parameters ($P < 0.05$) except for WG ($P > 0.05$). Feed intake and survival rate were not significantly affected by dietary Hg and vitamin E ($P > 0.05$). The WG, SGR, FE and PER in fish fed $Hg_{20}E_0$ diet were significantly lower than those in fish fed Hg_0E_0 diet ($P < 0.05$), and there were no significant differences in FI and survival rate from fish fed Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} diets ($P > 0.05$). Fish fed the Hg_0E_{100} and $Hg_{20}E_{100}$ diets showed no significant differences in terms of WG, SGR, FE, PER and FI in fish. Moreover, fish fed the Hg_0E_{200} and $Hg_{20}E_{200}$ diets did not show significant differences in WG, FE and FI ($P > 0.05$). There were no significant differences in WG, SGR, FE, FI and survival rate for fish fed $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ diets ($P > 0.05$). Results demonstrated that both Hg and vitamin E had significant dietary decreasing or increasing effects on the WG, SGR, FE and PER of fish.

3.2. Whole body proximate composition

Whole body proximate composition of olive flounder fed the experimental diets is shown in Table 4. There was no significant effect of Hg on whole body moisture, crude protein, lipid, or ash contents of olive flounder ($P > 0.05$). Dietary supplementation of vitamin E led to increased lipid level but decreased moisture content of whole body of fish. There was no significant interaction between Hg and vitamin E in terms of whole body protein and ash contents of juvenile olive flounder ($P > 0.05$).

Table 4
Whole-body proximate composition of juvenile olive flounder fed the experimental diets for 6 weeks (% dry matter basis).

Item[1]	Crude protein	Crude lipid	Crude ash	Moisture
Individual treatment means				
Hg_0E_0	70.3 ± 3.1	3.6 ± 1.1	19.9 ± 4.2	78.7 ± 0.8
Hg_0E_{100}	75.4 ± 0.6	3.4 ± 0.7	18.3 ± 0.5	78.1 ± 0.3
Hg_0E_{200}	72.1 ± 2.1	4.2 ± 1.2	19.4 ± 1.3	77.9 ± 0.2
$Hg_{20}E_0$	71.0 ± 2.8	3.4 ± 0.2	21.7 ± 2.3	79.2 ± 0.8
$Hg_{20}E_{100}$	71.6 ± 0.5	4.1 ± 0.6	20.5 ± 1.5	78.5 ± 0.1
$Hg_{20}E_{200}$	71.8 ± 2.9	5.3 ± 1.3	21.2 ± 2.6	77.7 ± 0.4
Means of main effect				
Hg_0	72.6	3.7	19.2	78.3
Hg_{20}	71.5	4.3	21.1	78.5
E_0	70.7	3.5^{b}	20.8	78.9^{a}
E_{100}	73.5	3.8^{ab}	20.3	78.3^{b}
E_{200}	71.9	4.7^{a}	19.4	77.8^{b}
Two-way ANOVA: *P*-value				
Hg effect	0.3123	0.2203	0.1221	0.4094
Vitamin E effect	0.1383	0.0414	0.6081	0.0088
Hg × Vitamin E	0.2410	0.1170	0.9890	0.4758

[a,b] Values are means ± SD from triplicate groups of fish ($n = 3$) where the values within a column without a common superscript differ ($P < 0.05$).
[1] Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively.

3.3. Total Hg concentrations in tissues

Total Hg accumulations in the liver, kidney and muscle of fish fed the experimental diets for 6 weeks are shown in Table 5. Higher Hg significantly increased the Hg contents in liver, kidney and muscle tissues of fish ($P < 0.05$). The fish fed $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ diets showed significantly higher total Hg concentrations in liver and kidney tissues than the fish fed Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} diets, respectively ($P < 0.05$), and there were no significant differences in total Hg concentrations in the liver, kidney and muscle tissues of fish fed Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} diets ($P > 0.05$). Total Hg concentrations in the liver and kidney tissues of fish fed $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ diets were similar but they were

Table 3
Growth performances and survival rate of juvenile olive flounder fed the experimental diets for 6 weeks.

Item[1]	WG,[2] %	SGR,[3] %/day	FE,[4] %	PER[5]	FI[6]	Survival, %
Individual treatment means						
Hg_0E_0	31.2 ± 8.0	0.99 ± 0.1^{b}	60.8 ± 0.7^{bc}	1.23 ± 0.1^{b}	3.5 ± 0.4	96.7 ± 2.9
Hg_0E_{100}	34.3 ± 2.6	0.94 ± 0.1^{b}	58.0 ± 0.9^{bc}	1.15 ± 0.1^{b}	3.7 ± 0.1	100 ± 0.0
Hg_0E_{200}	41.6 ± 5.6	1.12 ± 0.1^{a}	70.5 ± 8.5^{a}	1.44 ± 0.2^{a}	3.8 ± 0.1	98.3 ± 2.9
$Hg_{20}E_0$	19.6 ± 3.1	0.58 ± 0.1^{c}	35.7 ± 6.8^{d}	0.71 ± 0.1^{c}	3.7 ± 0.3	96.7 ± 2.9
$Hg_{20}E_{100}$	30.0 ± 4.4	0.92 ± 0.1^{b}	55.1 ± 3.1^{c}	1.10 ± 0.1^{b}	3.8 ± 0.1	96.7 ± 2.9
$Hg_{20}E_{200}$	36.0 ± 3.1	0.98 ± 0.1^{b}	64.4 ± 8.7^{ab}	1.20 ± 0.1^{b}	3.7 ± 0.1	98.3 ± 2.9
Means of main effect						
Hg_0	35.7^{a}	1.0^{a}	63.1^{a}	1.3^{a}	3.6	98.3
Hg_{20}	28.5^{b}	0.8^{b}	51.7^{b}	1.0^{b}	3.7	97.2
E_0	25.4^{c}	0.8^{c}	48.3^{c}	0.9^{c}	3.5	96.6
E_{100}	32.1^{b}	0.9^{b}	56.5^{b}	1.1^{b}	3.8	98.3
E_{200}	38.8^{a}	1.1^{a}	67.5^{a}	1.3^{a}	3.8	98.3
Two-way ANOVA: *P*-value						
Hg effect	0.0085	0.0001	0.0004	0.0002	0.3753	0.3887
Vitamin E effect	0.0017	0.0001	0.0001	0.0003	0.1519	0.4719
Hg × Vitamin E	0.4151	0.0006	0.0033	0.0068	0.4716	0.4719

WG = weight gain; SGR = specific growth rate; FE = feed efficiency; PER = protein efficiency ratio; FI = feed intake.
[a,b,c] Values are means ± SD from triplicate groups of fish ($n = 3$) where the values within a column without a common superscript differ ($P < 0.05$).
[1] Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively.
[2] WG (%) = 100 × (Final weight−Initial weight)/Initial weight.
[3] SGR (%/day) = 100 × (ln Final weight−ln Initial weight)/Days.
[4] FE (%) = 100 × Wet weight gain (g)/Dry feed intake (g).
[5] PER = Wet weight gain/Protein intake.
[6] FI = 100 × Total feed fed (dry matter)/[(Initial weight + Final weight + Dead fish weight)/2 × Days].

Table 5
Tissue total mercury contents of juvenile olive flounder fed the experimental diets for 6 weeks (μg/g of wet matter basis).

Item[1]	Muscle	Kidney	Liver
Individual treatment means			
Hg_0E_0	0.08 ± 0.01	1.6 ± 0.7^{c}	1.2 ± 0.2^{c}
Hg_0E_{100}	0.07 ± 0.01	1.6 ± 0.4^{c}	1.6 ± 0.3^{c}
Hg_0E_{200}	0.08 ± 0.02	2.1 ± 1.4^{c}	1.7 ± 1.0^{c}
$Hg_{20}E_0$	0.4 ± 0.2	35.9 ± 11.9^{a}	35.1 ± 0.7^{a}
$Hg_{20}E_{100}$	0.3 ± 0.1	22.6 ± 0.6^{b}	18.7 ± 0.7^{b}
$Hg_{20}E_{200}$	0.2 ± 0.1	19.6 ± 6.3^{b}	22.1 ± 6.9^{b}
Means of main effect			
Hg_0	0.1^{b}	1.8^{b}	1.5^{b}
Hg_{20}	0.3^{a}	25.6^{a}	25.2^{a}
E_0	0.3^{a}	18.2^{a}	18.1^{a}
E_{100}	0.2^{ab}	12.1^{b}	11.9^{b}
E_{200}	0.1^{b}	10.8^{b}	10.2^{b}
Two-way ANOVA: *P*-value			
Hg effect	0.0002	0.0001	0.0001
Vitamin E effect	0.0527	0.0318	0.0059
Hg × Vitamin E	0.0618	0.0241	0.0458

[a,b,c] Values are means ± SD from triplicate groups of fish ($n = 3$) where the values within a column without a common superscript differ ($P < 0.05$).
[1] Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively.

significantly lower than those measured in the tissues of fish fed the $Hg_{20}E_0$ diet ($P < 0.05$). The two-way ANOVA showed the significant reducing effect of dietary vitamin E in the muscle ($E_0 > E_{200}$), liver ($E_0 > E_{100}$) and kidney ($E_0 > E_{100}$) tissues in terms of Hg bioaccumulation. However, significant interaction effect between Hg and vitamin E was found in liver and kidney tissues only ($P < 0.05$).

3.4. Total vitamin E concentrations in tissues

The vitamin E levels in the liver, kidney and muscle of fish fed the experimental diets for 6 weeks are shown in Table 6. There were significant effects of dietary Hg and vitamin E observed in liver, kidney and muscle tissues of juvenile olive flounder ($P < 0.05$). However, significant interaction between Hg and vitamin E was found in liver and kidney tissues only ($P < 0.05$). No significant interaction between Hg and vitamin E was found in muscle tissue ($P > 0.05$). The two-way ANOVA showed that vitamin E levels in the muscle, liver and kidney tissues increased significantly in a dose-dependent manner in fish fed diets supplemented with vitamin E. Fish fed the Hg_0E_{200} and $Hg_{20}E_{200}$ diets showed significantly higher vitamin E levels in the liver and kidney tissues than fish fed Hg_0E_0 and $Hg_{20}E_0$ diets, respectively, and vitamin E in the liver tissue of fish fed Hg_0E_{200} showed significantly higher contents than that of fish fed Hg_0E_{100} diet ($P < 0.05$). However, fish fed the $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ diets showed significantly lower vitamin E in the liver and kidney tissues than fish fed Hg_0E_{100} and Hg_0E_{200} diets, respectively ($P < 0.05$).

Table 6
Tissue total vitamin E contents of juvenile olive flounder fed the experimental diets for 6 weeks (μg/g of wet matter basis).

Item[1]	Muscle	Kidney	Liver
Individual treatment means			
Hg_0E_0	9.8 ± 0.6	33.3 ± 1.3^{b}	17.8 ± 2.9^{e}
Hg_0E_{100}	17.0 ± 0.7	61.12 ± 3.2^{a}	44.1 ± 3.7^{c}
Hg_0E_{200}	40.1 ± 1.6	61.4 ± 1.5^{a}	63.8 ± 0.8^{a}
$Hg_{20}E_0$	4.7 ± 0.6	26.6 ± 2.2^{c}	14.3 ± 0.3^{e}
$Hg_{20}E_{100}$	10.6 ± 0.6	28.4 ± 2.3^{c}	31.8 ± 0.8^{d}
$Hg_{20}E_{200}$	36.6 ± 1.6	34.9 ± 0.8^{b}	50.5 ± 0.7^{b}
Means of main effect			
Hg_0	22.3^{a}	51.9^{a}	41.9^{a}
Hg_{20}	17.3^{b}	30.0^{b}	32.2^{b}
E_0	7.3^{c}	30.0^{c}	16.1^{c}
E_{100}	13.8^{b}	44.7^{b}	37.9^{b}
E_{200}	38.3^{a}	48.2^{a}	57.1^{a}
Two-way ANOVA: *P*-value			
Hg effect	0.0001	0.0001	0.0001
Vitamin E effect	0.0001	0.0001	0.0001
Hg × Vitamin E	0.1057	0.0001	0.0023

[a,b,c,d,e] Values are means ± SD from triplicate groups of fish ($n = 3$) where the values within a column without a common superscript differ ($P < 0.05$).
[1] Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively.

3.5. Whole body n-3 fatty acids composition

Whole body n-3 fatty acid composition of fish fed the experimental diets for 6 weeks are shown in Table 7. There were significant effects of Hg on EPA and DHA ($P < 0.05$) except for α-linolenic acid (ALA) ($P > 0.05$). However, vitamin E showed significant effects on EPA and ALA ($P < 0.05$) except for DHA ($P > 0.05$). There was no significant interaction between Hg and vitamin E on EPA or DHA or ALA ($P > 0.05$). Dietary Hg level significantly decreased the levels of EPA and DHA, and dietary vitamin E increased the levels of EPA and ALA in the whole body of fish.

4. Discussion

In the present study, olive flounder fed diets containing 20 mg $HgCl_2$/kg and 0 mg TA/kg showed growth depression compared with fish fed other experimental diets. Zahir et al. (2005) reported that mercuric ion (Hg^{2+}) has a strong thiol-binding capacity which may cause oxidative stress in mice (Hussain et al., 1999) and tissue damage in rats (Reus et al., 2003). Moreover, the growth depression in fish in the present study may be due to the toxicity of Hg through the production of superoxide radicals and glutathione enzyme depletion (Miura et al., 1995; Agarwal et al., 2010). The growth depression of fish due to Hg toxicity may attributed to the use of body energy for repairing damaged cells which may lower the somatic and reproductive growth (Houck and Cech, 2004). The results

Table 7
Whole-body fatty acids composition in terms of α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) contents in juvenile olive flounder fed the experimental diets for 6 weeks (% dry matter basis).

Item[1]	ALA	EPA	DHA
Individual treatment means			
Hg_0E_0	0.29 ± 0.01	6.7 ± 2.1	4.4 ± 1.7
Hg_0E_{100}	0.37 ± 0.1	7.4 ± 2.7	4.6 ± 2.6
Hg_0E_{200}	0.47 ± 0.04	12.8 ± 0.1	6.2 ± 0.9
$Hg_{20}E_0$	0.31 ± 0.1	3.3 ± 1.8	2.7 ± 1.5
$Hg_{20}E_{100}$	0.28 ± 0.04	4.7 ± 0.5	2.2 ± 0.1
$Hg_{20}E_{200}$	0.35 ± 0.05	5.6 ± 0.4	3.4 ± 1.8
Means of main effect			
Hg_0	0.4^{a}	8.9^{a}	5.1^{a}
Hg_{20}	0.3^{a}	4.5^{b}	2.8^{b}
E_0	0.3^{b}	5.1^{b}	3.6^{a}
E_{100}	0.3^{b}	6.1^{b}	3.4^{a}
E_{200}	0.4^{a}	9.1^{a}	4.8^{a}
Two-way ANOVA: *P*-value			
Hg effect	0.0916	0.0020	0.0128
Vitamin E effect	0.0399	0.0242	0.3134
Hg × Vitamin E	0.1673	0.1029	0.8339

[a,b] Values are means ± SD from triplicate groups of fish ($n = 3$) where the values within a column without a common superscript differ ($P < 0.05$).
[1] Hg_0E_0, Hg_0E_{100} and Hg_0E_{200} designate 0 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively; $Hg_{20}E_0$, $Hg_{20}E_{100}$ and $Hg_{20}E_{200}$ designate 20 mg/kg Hg with 0, 100 and 200 mg/kg vitamin E, respectively.

of the present study are in agreement with that observed in juvenile walleye, Sacramento blackfish, green and white sturgeon (Friedmann et al., 1996; Houck and Cech, 2004; Lee et al., 2011). However, survivability of fish in the present study was not affected by dietary Hg supplementation which is in accordance with Wobeser (1975) and Lee et al. (2016), but in contrast to Abdel-Tawwab et al. (2004). Our results also showed that as the dietary vitamin E levels increased in the diets, feed utilization in terms of FE and PER also increased in the fish (Moniruzzaman et al., 2017).

Kim et al. (2012) reported that inorganic Hg may cause significant changes in hematological parameters and hepatic oxidative stress enzyme activity in olive flounder *P. olivaceus*. It is widely accepted that the high affinity of Hg for the thiol or sulfhydryl groups of proteins underlies the mechanisms of Hg toxicity (NRC, 2005). Binding of Hg with sulfhydryl groups results in decreased enzyme activities, altered structural functionality, and problems in transport processes (Zalups and Lash, 1994). The present experiment showed that the fish fed diets containing more than 100 mg TA/kg exhibits growth improvement in fish. Howard (1980) reported that vitamin E is as effective as selenium in overcoming the growth depression produced by metal in rats. Susan (1979) reported that there were greater growth and survival and fewer signs of Hg toxicity in the rat's given supplemental vitamin E at 500 mg/kg. Vitamin E was first reported to protect against Hg toxicity in fowl (Welsh, 1974), and it was shown that high level of vitamin E decreased mortality in Japanese quail fed 30 mg/kg MeHg (Welsh and Soares, 1976). Administration of vitamin E in drinking water protects male mice against heavy metals-induced renal and testicular oxidative stress and injuries (Atef, 2001). In another report, Agarwal et al. (2010) had found the ameliorating and protecting effect of vitamin E in tissue levels, such as kidney, liver and brain of rats, against acute Hg toxicity, where rats were subjected to intraperitoneal injection with vitamin E as post-treatment. Moreover, Agarwal et al. (2010) reported that vitamin E provides complete protection from Hg-induced hepatotoxicity both in pre- and post-treatments. Interestingly, in the present study, we observed that Hg contents in all tissues of fish, such as muscle, liver and kidney tissues, were significantly reduced by dietary supplementation of vitamin E (100 or 200 mg TA/kg diet) which may also support the results found by Agarwal et al. (2010). Inorganic Hg does not readily cross cell membranes (Lee et al., 2016). For this reason, following absorption of $HgCl_2$, the liver and kidneys have the highest Hg levels (Lee et al., 2016), whereas muscle tissue has substantially lower levels (NRC, 2005). Syversen and Parvinder (2012) reported that inorganic Hg accumulates primarily in the kidney, followed by its accumulation in the liver. Present experiment also showed higher Hg concentrations in the liver and kidney tissues than in muscle tissue. Urano and Matsuo (1976) reported the methyl radical, generated by addition of ferrous sulfate to dimethyl sulfoxide, reacts with tocopherol to form stable methylated products. In this study, olive flounder fed diets containing Hg showed lower vitamin E levels in liver, kidney and muscle tissues than fish fed diets without Hg. Fukino et al. (1984) reported inorganic Hg has been shown to alter the levels of nutrients such as vitamins C and E in the tissues because the vitamin C and E interacts with Hg. Interaction of vitamin E and Hg showed it alleviates MeHgCl and $HgCl_2$ toxicity and neuronal degeneration which ultimately prevents lipid peroxidation due to Hg toxicity (Kasuya, 1975). As novel findings, dietary Hg and vitamin E showed significant interaction effects on tissue Hg reduction in liver and kidney tissues of fish in this study. In our previous study (Moniruzzaman et al., 2017), we reported dietary vitamin E had a profound effect in reducing Hg contents in liver tissue of fish fed MeHg containing diets. Syversen and Parvinder (2012) reported that MeHg mainly accumulated in the brain as MeHg-GSH complex and rest of the part found in liver and kidney. It has been proposed that MeHg binds to the blood and tissue protein SH-groups to lessen the SH-groups of cysteine and glutathione (GSH) (Syversen and Parvinder, 2012). Moreover, Moniruzzaman et al. (2017) reported the higher level of MeHg accumulation in all the tissue levels compared to $HgCl_2$ of the present study. Moreover, the experiment showed higher mortality compared with the present experiment which attributed to the more toxic effect of MeHg than did $HgCl_2$. The results of the present study also suggest the low bioaccumulation of dietary inorganic Hg in fish may be due to low digestion, absorption or high excretion of dietary Hg (Wang et al., 2013). In light of the results of the present study, we assume that Hg content in liver, kidney and muscle tissues of fish might be reduced by vitamin E due to its scavenging ability on reactive oxygen species (ROS) that were produced by tissue level Hg intoxication. The possible route of $HgCl_2$ elimination could be through urine and feces (Manahan, 2003). However, the mechanism of toxicity of Hg is still unveiled (Manahan, 2003), where most of them are speculative (Lihm et al., 2013; Wang et al., 2015).

Mercury compounds commonly generate oxidative stress in tissues with the generation of ROS such as H_2O_2, O^{2-} and OH^-, and electrophilic free-radical metabolites (NRC, 2011). In general, ROS are reported to damage the PUFAs of the membrane phospholipids of the cells causing impairments of cellular functions (Halliwell, 1999) by damaging cellular biomolecules. In the present study, Hg did not show any significant degradation of omega-3 fatty acids in the corresponding diets possibly because of supplementation of dietary inorganic Hg. Moreover, the whole body proximate composition of fish also was not affected by dietary Hg inclusions might be because of its inorganic nature, which agrees with the results found by Lee et al. (2016). In this experiment, whole body lipid as well as fatty acid contents of fish were increased may be due to the fat-soluble nature of vitamin E (NRC, 2011).

5. Conclusions

In conclusion, the results of the present study suggest that dietary supplementation of vitamin E in terms of TA (100 or 200 mg/kg diet) can enhance the growth performance and effectively reduce the accumulation and toxicity of $HgCl_2$ in muscle, liver and kidney tissues of juvenile olive flounder. Our findings also indicate that dietary vitamin E is more effective against inorganic Hg than it is against organic Hg (Moniruzzaman et al., 2017) in terms of toxicity and bioaccumulation of Hg in different tissues of olive flounder. However, further research can be conducted on the metabolism of the vitamin E and Hg to understand the mechanism of their actions in the tissue levels.

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