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Effect of Dietary Olaquindox on the Growth of Large Yellow Croaker (*Pseudosciaena crocea* R.) and the Distribution of Its Residues in Fish Tissues

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Abstract Olaquindox (OLA), one of quinoxaline-N, N-dioxides, has been put under ban. However it was used as a medicinal feed additive early; it promotes the growth of livestock and prevents them from dysentery and bacterial enteritis. In this study, we evaluated the effect of dietary OLA on the growth of large yellow croaker (*Pseudosciaena crocea* R.) and the histological distribution of OLA and its metabolite 3-methyl-quinoxaline-2-carboxylic acid (MQCA) in fish tissues. Four diets containing 0 (control), 42.5, 89.5 and 277.2 mg kg^{-1} OLA, respectively, were formulated and tested, 3 cages (1.0 m × 1.0 m × 1.5 m) each diet and 100 juveniles (9.75 ± 0.35 g) each cage. The fish were fed to satiation twice a day at 05:00 am and 17:00 pm for 8 weeks. The survival rate of fish fed the diet containing 42.5 and 89.5 mg kg^{-1} OLA was significantly higher than that of fish fed the diet containing 0 and 277.2 mg kg^{-1} OLA ($P<0.05$), while the weight gain rate of fish fed the diet containing 42.5 and 89.5 mg kg^{-1} OLA was significantly higher than that of fish fed the diet without OLA (control) ($P<0.05$), but similar to that of fish fed the diet with 277.2 mg kg^{-1} OLA. Fish fed the diet with 277.2 mg kg^{-1} OLA had the highest content of OLA and MQCA in liver (3.44 and 0.39 mg kg^{-1}, respectively), skin (0.46 and 0.09 mg kg^{-1}, respectively) and muscle (0.24 and 0.06 mg kg^{-1}, respectively). In average, fish fed the diet containing OLA had the highest content of OLA and MQCA in liver which was followed by skin and muscle ($P<0.05$), whereas OLA and MQCA were not detectable in control. Our findings demonstrated that OLA and MQCA accumulated in large yellow croaker when it was fed with the diet containing OLA, thus imposing a potential safety risk to human health.

Key words large yellow croaker; *Pseudosciaena crocea*; olaquindox; 3-methyl-quinoxaline-2-carboxylic acid

1 Introduction

Olaquindox (OLA), N-(2-hydroxyethyl)-3-methyl-2-quinoxalincarboxamide-1,4-dioxide, one of quinoxaline-N, N-dioxides, has been put under ban. However it was used as a medicinal feed additive early; it promotes the growth of livestock and prevents them from dysentery and bacterial enteritis (Baars *et al*., 1988; Anon, 1989). In 1980s, OLA was used in fish and shrimp farming (Xu *et al*., 1988; Ye *et al*., 1992). Unfortunately, it was cognized gradually that OLA is toxic to fish (Cao *et al*., 2001; Wang *et al*., 2004; Yang *et al*., 2005), thus been put under ban by many countries including China. However, its illegal use has never completely disappeared worldwide.

As was documented by the joint FAO/WHO Expert Committee on Food Additives, OLA is almost completely absorbable in the gastrointestinal tract of rat, dog and pig, and histological wide in distribution. The most of OLA is eliminated through urine and less through feces and by expiration. In urine, the original OLA accounts for 70%, while others include approximately 16 metabolites, of them 6 major metabolites have been fully characterized. MQCA is the main persistent residue of OLA in the edible tissues, thus is considered to be a marker of OLA in animals (FAO/WHO, 1995; MOA, 2002). To date, no study has been carried out on the histological distribution of OLA residue and its metabolites in fish. OLA may induce genotoxicity as was found in human hepatoma G2 (HepG2) cells (Zou *et al*., 2011) and cause phototoxicity to human (WHO, 1991; Emmert *et al*., 2007). We believe that OLA residue and its metabolites in fish tissues may affect human health.

Large yellow croaker (*Pseudosciaena crocea*) is one of the commercially important marine fish species in China, which has been widely cultured in recent years. Early studies in this fish species focused mainly on its nutrient

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requirement and feed modification (Duan *et al*., 2001; Mai *et al*., 2006; Ai *et al*., 2007; Li *et al*., 2007). In this study, the effect of dietary OLA on the growth of large yellow croaker and its histological residue were determined with an emphasis on OLA and its metabolite MQCA.

2 Materials and Methods

2.1 Diet Preparation

Four diets were formulated to contain 0 (control, Diet 1), 42.5 (Diet 2), 89.5 (Diet 3) and 277.2 (Diet 4) mg kg^{-1} OLA with 1% OLA premix (Animal Disease Control Center, Qingdao, China) (Table 1). The protein and lipid content of these diets was about 44% and 11%, respectively, which are considered sufficient to support optimal growth of large yellow croaker (Duan *et al*., 2001).

Table 1 Formulation of experimental diets (dry weight basis)

Ingredient (g kg^{-1})	Diet no. (OLA: mg kg^{-1})			
	Diet 1 (0)	Diet 2 (42.5)	Diet 3 (89.5)	Diet 4 (277.2)
Fish meal[b]	500	500	500	500
Soybean meal[c]	90	90	90	90
Beer yeast meal	30	30	30	30
Wheat meal	255.5	250.5	245.5	225.5
Fish oil	30	30	30	30
Soybean oil	25	25	25	25
Attractant[d]	3	3	3	3
Mould inhibitors[e]	1	1	1	1
Ethoxyquin	0.5	0.5	0.5	0.5
Lecithin	25	25	25	25
Vitamin premix[h]	14.5	14.5	14.5	14.5
Mineral premix[i]	20	20	20	20
Ascorbic acid[f]	3	3	3	3
Choline[g]	2.5	2.5	2.5	2.5
Olaquindox (1% premix)[a]	0	5	10	30
Analyzed chemical Composition (% dry weight basis)				
Crude protein	44.4	43.6	43.3	44.4
Crude lipid	11.5	11.8	11.6	11.8
Olaquindox (mg kg^{-1})	0	42.5	89.5	277.2

Notes: [a], Olaquindox: 1% premix with 10 g olaquindox kg^{-1} (Qingdao Animal Disease Control Center, Shandong, China); [b], fish meal: 68.9% crude protein (dry matter) and 10.1% crude lipid (dry matter) (Cishan Fisheries, Shandong, China); [c], soybean meal: 46.4% crude protein (dry matter) and 1.9% crude lipid (dry matter) (Liulu Oli Lit., Heilongjiang, China); [d], attractant: dimethyl-β-propiothetin; [e], mould inhibitors: 50% calcium propionic acid and 50% fumaric acid; [f], ascorbic acid: 35% L-ascorbate-2-monophosphate obtained from Beijing Enhalor International Tech Co., Ltd. (Beijing, China); [g], Choline (60%): obtained from Zouping Jujia Choline Industrial Co., Ltd. (Shandong, China); [h] and [i], vitamin premix and mineral premix: according to Li *et al*. (2009).

To prepare the diets, the solid ingredients were grounded into fine powder, sieved through a 320-μm mesh and mixed thoroughly with menhaden fish oil. The mixture was made into stiff dough by adding water and then pelleted with an experimental feed mill (F-26 II, South China University of Technology, China). The pellets were dried in a ventilated oven at 60℃ for about 12 h, broken up and sieved. The prepared diets were 1.5 mm× 2.0 mm in size, which were sealed in plastic bags and stored at −15℃.

2.2 Feeding Trial

Large yellow croaker juveniles were obtained from a commercial hatchery and stocked in a floating sea cage (3.0 m×3.0 m×3.0 m) in Xihu Bay, Ningbo, China, for 2 weeks. The fish were fed with Diet 1 during the acclimation.

The fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10000; Shanghai Reagent Corp, China). Fish in similar size (9.75 g ± 0.35 g) were divided into 12 sea cages (1.0 m×1.0 m×1.5 m), 3 cages each diet and 100 individuals each cage, and fed to satiation twice a day at 05:00 am and 17:00 pm, respectively, for 8 weeks. During feeding trial, water temperature and salinity were maintained at 26.5–32.5℃ and 25.0–28.0 g L^{-1}, respectively, while dissolved oxygen was kept above 7 mg L^{-1}. The natural light rhythm was followed throughout the trial.

2.3 Sampling and Content Assaying

2.3.1 Sampling

The fish were fasted for 24 h ahead of harvest. Total number of fish in each cage was counted and their body weights were measured. Twenty fish per cage were randomly collected and stored frozen at −20℃ to determine whole-body proximate composition and the content of OLA and its metabolite. The skin, liver and muscle were sampled under caliginous light carefully and freeze-dried in ALPHA 1-2, a freeze dryer (Martin Christ, Germany) for 24 h, then immediately stored frozen at −20℃.

2.3.2 Analysis of ingredients, diets and fish body composition

Analyses of ingredients, diets and fish body composition were made following the usual procedures (AOAC, 1995): samples were dried to a constant weight at 105℃ to determine the dry matter content; crude protein was determined by measuring nitrogen (N×6.25) using the Kjeldahl method; crude lipid was measured by ether extraction using Soxhlet method; ash by combustion at 550℃ for 16 h.

The content of OLA and MQCA was determined with a liquid chromatography (LC) equipped with ultraviolet (UV) detector set at 260 nm and 320 nm, respectively.

2.3.3 Content assaying

For content determination, the skin, liver and muscle were homogenized with 5 mL saturated ammonium sulfate, and then 10 mL mixed solution of acetonitrile-ethyl acetate (3:2) was added to homogenize for 1–2 min following with a 10-min centrifugation at 4200 r min^{-1}. The supernatant were evaporated to dryness under a stream of nitrogen

(Organomation Associates, Berlin, MA, USA) at 50℃ water-bath. The residue were dissolved with 2 mL acetonitrile and degreased with 3 mL n-hexane for twice and centrifuged. The stock solution were evaporated to dryness under a stream of nitrogen at a water bath of 50℃ and 0.5 mL methanol was added following a 1–2 min shaking mixture and centrifugation, of which 10 μL supernatant was injected into the liquid chromatography (LC) equipped with ultraviolet (UV) detector set at 260 nm to determine the concentration of OLA. The skin, liver and muscle were hydrolyzed by 5 mL 3 mol L^{-1} NaOH at a 95−100℃ water bath for 40–45 min. The alkaline hydrolysate was acidified to pH≤1 with 3 mL HCl and extracted with 15 mL ethyl acetate following with a 10-min centrifugation at 4200 r min^{-1}. The supernatant was washed with 5 mL distilled water by shaking to clarify and rejecting the lower water phase twice. Then 5 mL 0.5 mol L^{-1} citric acid buffer (pH 6.0) was added into the extraction and mixed to get the lower buffer phase. Then, the aqueous extraction was acidified with 3 mL HCl. The acidified aqueous extraction was transferred into a cation exchange column (AG MP-50 resin) for ion-exclusion chromatography. The column was washed with 50 mL 1 mol L^{-1} HCl and 70 mL mixed solution of methanol with water (50 + 50) to collect the eluate, respectively. The eluate was extracted with 25 mL $CHCl_3$ triplicate after adding 2 mL HCl and rotary evaporated to dryness at 45–50℃. The residue were dissolved with 2 mL methanol twice and evaporated to dryness under a stream of nitrogen at a water bath of 50℃, then 0.5 mL methanol was added following a 1–2 min shaking mixture and centrifugation, of which 10 μL supernatant was injected into the liquid chromatography (LC) equipped with ultraviolet (UV) detector set at 320 nm to determine the concentration of MQCA.

2.4 Data Processing

The weight gain rate (WGR) of fish was determined with the equation

$$WGR\,(\%) = \frac{Wt - Wo}{Wo} \times 100\,,$$

where *Wt* and *Wo* are final and initial mean body weight of fish, respectively. Data corresponding to each diet were subjected to one-way ANOVA with SPSS 10.0 for windows. When overall difference was significant ($P<0.05$), Tukey's test was used to compare the means between diets (Zar, 1984).

3 Results

3.1 Survival and Growth

Fish fed the diet with 277.2 mg kg^{-1} OLA were slow in feed intaking and nervous to any stimulation (*e.g.*, mad jumping). Occasionally, hyperemia and hemorrhage were observed at the base of fins and the corner of the mouth and in abdomen. The survival rate ranged from 86.0% to 92.0% was significantly influenced by dietary treatment (Table 2). Fish fed diet with 42.5 and 89.5 mg kg^{-1} OLA survived significantly higher than those fed with control diet and 277.2 mg kg^{-1} OLA diet ($P<0.05$); while fish fed diet with 277.2 mg kg^{-1} OLA had a significantly lower survival rate than the control) ($P<0.05$). The WGR of fish was significantly influenced by the dietary OLA. Fish fed diets with 42.5 and 89.5 mg kg^{-1} OLA had signify-cantly higher WGR (173.0% and 184.0%, respectively) than control (133.3%) ($P<0.05$), and the fish fed with control diet and 277.2 mg kg^{-1} OLA diet had similar WGR (Table 2).

Table 2 Survival and growth performance of large yellow croaker

Diet no. (mg kg^{-1} OLA)	Survival rate (%)	Final body weight (g)	Weight gain rate (%)
Diet 1 (0)	89.3 ± 0.7^{b}	22.8 ± 0.7^{b}	133.3 ± 6.6^{b}
Diet 2 (42.5)	92.0 ± 0.6^{a}	26.6 ± 1.0^{a}	173.0 ± 9.8^{a}
Diet 3 (89.5)	92.0 ± 0.6^{a}	27.7 ± 0.5^{a}	184.0 ± 5.2^{a}
Diet 4 (277.2)	86.0 ± 0.6^{c}	24.9 ± 0.9^{ab}	155.6 ± 9.3^{ab}

Notes: The values are means ± SD (n=3). The initial mean body weight of fish was 9.75 g ± 0.35 g. Means in the same column with different superscripts are significantly different (Tukey's test, $P<0.05$).

3.2 Body Composition

The dietary OLA did not significantly influence the composition of fish body. Water (69.2%–70.3%), lipid (8.1%–8.7%) and ash (4.1%–4.3%) content was within a normal range (Table 3). Although body protein content (18.4%) was higher in fish fed diet with 89.5 mg kg^{-1} OLA than that of control (16.5%) ($P<0.05$), but such a difference was not significant among other dietary OLA treatments ($P>0.05$) (Table 2).

Table 3 Effect of dietary olaquindox on the body composition (% wet weight) of large yellow croaker

Diet no. (mg kg^{-1} OLA)	Moisture	Crude protein	Crude lipid	Ash
Diet 1 (0)	70.3 ± 0.9	16.5 ± 0.5^{b}	8.6 ± 0.3	4.1 ± 0.2
Diet 2 (42.5)	70.3 ± 0.6	17.5 ± 0.4^{ab}	8.4 ± 0.2	4.1 ± 0.1
Diet 3 (89.5)	69.2 ± 0.7	18.4 ± 0.4^{a}	8.1 ± 0.0	4.3 ± 0.1
Diet 4 (277.2)	70.2 ± 0.2	17.1 ± 0.2^{ab}	8.7 ± 0.2	4.2 ± 0.1

Notes: The values are means ± SD (n=3). Means in the same column with different superscripts are significantly different (Tukey's test, $P<0.05$).

3.3 Histological Distribution OLA and MQCA

OLA and MQCA were not detectable in the fish tissues of control group; however, OLA residue and its metabolite MQCA significantly increased in skin, liver and muscle in a dose-dependent pattern ($P<0.05$). Fish fed diet with 277.2 mg kg^{-1} OLA had significantly higher OLA and MQCA in their liver (3.44 and 0.39 mg kg^{-1}, respectively), skin (0.46 and 0.09 mg kg^{-1}, respectively) and muscle (0.24 and 0.06 mg kg^{-1}, respectively) than those fed other diets. In average, fish fed diet with 42.5, 89.5 and 277.2 mg kg^{-1} OLA had the highest content of OLA and MQCA in their liver followed by skin and muscle (Table 4).

Table 4 Content of OLA and MQCA in different tissues of large yellow croaker

Diet no. (mg kg^{-1} OLA)	OLA content (mg kg^{-1})			MQCA content (mg kg^{-1})		
	Skin	Liver	Muscle	Skin	Liver	Muscle
Diet 1 (0)	ND	ND	ND	ND	ND	ND
Diet 2 (42.5)	0.15 ± 0.01^{c}	1.43 ± 0.09^{c}	0.05 ± 0.00^{c}	0.03 ± 0.00^{c}	0.15 ± 0.01^{c}	0.01 ± 0.00^{c}
Diet 3 (89.5)	0.32 ± 0.01^{b}	2.73 ± 0.11^{b}	0.11 ± 0.01^{b}	0.07 ± 0.00^{b}	0.30 ± 0.01^{b}	0.03 ± 0.00^{b}
Diet 4 (277.2)	0.46 ± 0.04^{a}	3.44 ± 0.01^{a}	0.24 ± 0.02^{a}	0.09 ± 0.00^{a}	0.39 ± 0.00^{a}	0.06 ± 0.00^{a}

Notes: ND, below detectable limits. The values are means ± SD (n=3). Means in the same column with different superscripts are significantly different (Tukey's test, $P<0.05$).

4 Discussion

OLA is a synthetic antimicrobial chemical, which can promote the growth of animals and prevent them from the infection of pathogens. In this study, we found that the survival rate of large yellow croaker was significantly influenced by dietary OLA, indicating that OLA played a role as an antimicrobial agent. However, such a scenario was not appeared when dietary OLA increased to 277.2 mg kg^{-1}, of which the survival rate was lower than that of control. A similar trend was also reported early in grass carp (Ye *et al*. 1992). We believed that the toxicity of OLA (Cao *et al*., 2001; Wang *et al*., 2004; Yang *et al*., 2005), especially when its content is high, caused such a pattern of the influence of OLA on the survival rate of fish.

It is believed that OLA can inhibit the growth of harmful bacteria and influence the metabolism of body nutrients, thus promoting the synthesis of nutrients, especially protein, of animal (Ye *et al*., 1992; Shen *et al*., 1995). In this study, the content of protein of the fish fed diet with 42.5 mg kg^{-1} OLA was significantly higher than that of control, which may be explained by these early understandings. Nevertheless, high content of OLA may disrupt such a scenario; the fish fed diet with 277.2 mg kg^{-1} OLA has lower content of protein than control.

The residue of OLA and its metabolites have been documented early (WHO, 1991; FAO, 1991). As reported early, 70% of OLA administrated orally may be eliminated through urine unchanged, and 16 metabolites of OLA have been detected in the urine, of which 6 major metabolites are fully characterized (WHO, 1991). Among the metabolites of OLA, MQCA was the last major remaining detectable metabolite in animal, has been designated as the marker substance (FAO/WHO, 1995; MOA, 2002). In this study, OLA and MQCA were high in content in the tissues of fish fed diet with 42.5, 89.5 and 277.2 mg kg^{-1} OLA but not in control, demonstrating that OLA and MQCA accumulated in the tissues of large yellow croaker. The content of OLA in diet significantly affected the histological concentration of its residue and its metabolite MQCA ($P<0.05$), and showed an obvious dose-dependent relationship with the residues in tissues. A similar finding was also reported in mirror carp (Ye *et al*., 2003) and pig (Zhang *et al*., 2011a). The distribution of OLA and its metabolite MQCA was histologically different. In average, the content of OLA and MQCA was the highest in liver which was followed by that in skin and muscle. This pattern was similar to the report early in carp (Wang *et al*., 2003; Ye *et al*., 2003; Li *et al*., 2012) and tilapia (Chen *et al*., 2011). The metabolism pathway of OLA in animals may explain such a pattern. First, the liver is the major metabolizing places for OLA (WHO, 1991), in which more OLA is transformed than in skin and muscle. Second, liver damage and metabolic disorder caused by OLA may slow the elimination of residues in liver (Zeng *et al*., 1995; Yang *et al*., 2005). These factors may explain why the content of residual OLA and its metabolite MQCA in liver is the highest.

The toxicity of OLA and their metabolites to fish have been documented intensively, which included cumulative toxicity (Wang *et al*., 2004; Yang *et al*., 2005), photosensitive toxicity (De Vries *et al*., 1990) and genotoxity (WHO, 1991; Cao *et al*., 2001; Zou *et al*., 2011; Zhang *et al*., 2011b; Zhao *et al*., 2013). Despite OLA has been put under ban in many countries, its illegal use in aquaculture has never vanished from sight all over the world. In this study, we found that OLA residue and its metabolite MQCA were detectable in diverse tissues of large yellow croaker when it was fed with the diet containing OLA. The OLA residue and its metabolites in farmed large yellow croaker is a potential risk to human health, thus we should keep watch on its use in order to ensure the safety of our food.

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