Full length article

Deoxynivalenol decreased the growth performance and impaired intestinal physical barrier in juvenile grass carp (Ctenopharyngodon idella)

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ARTICLE INFO

Keywords:
Deoxynivalenol
Grass carp (Ctenopharyngodon idella)
Intestine
Oxidative damage
Apoptosis
Tight junctions

ABSTRACT

Deoxynivalenol (DON) is one of the most common mycotoxin contaminants of animal feed worldwide and brings significant threats to the animal production. However, studies concerning the effect of DON on fish intestine are scarce. This study explored the effects of DON on intestinal physical barrier in juvenile grass carp (Ctenopharyngodon idella). A total of 1440 juvenile grass carp (12.17 ± 0.01 g) were fed six diets containing graded levels of DON (27, 318, 636, 922, 1245 and 1515 μg/kg diet) for 60 days. This study for the first time documented that DON caused body malformation in fish, and histopathological lesions, oxidative damage, declining antioxidant capacity, cell apoptosis and destruction of tight junctions in the intestine of fish. The results indicated that compared with control group (27 μg/kg diet), DON: (1) increased the reactive oxygen species (ROS), malondialdehyde (MDA) and protein carbonyl (PC) content, and up-regulated the mRNA levels of Kelch-like-ECH-associated protein 1 (Keap1: Keap1a but not Keap1b), whereas decreased glutathione (GSH) content and antioxidant enzymes activities, and down-regulated the mRNA levels of antioxidant enzymes (except GSTR in MI) and NF-E2-related factor 2 (Nrf2), as well as the protein levels of Nrf2 in fish intestine. (2) up-regulated cysteinyl aspartic acid-protease (caspase) -3, -7, -8, -9, apoptotic protease activating factor-1 (Apaf-1), Bcl2-associated X protein (Bax), Fas ligand (Fasl) and c-Jun N-terminal protein kinase (JNK) mRNA levels, whereas down-regulated B-cell lymphoma-2 (bcl-2) and myeloid cell leukemia-1 (Mcl-1) mRNA levels in fish intestine. (3) down-regulated the mRNA levels of ZO-1, ZO-2b, occludin, claudin-c, -4, -7a, -7b, -11 (except claudin-b and claudin-3c), whereas up-regulated the mRNA levels of claudin-12, -15a (not -15b) and myosin light chain kinase (MLCK) in fish intestine. All above data indicated that DON caused the oxidative damage, apoptosis and the destruction of tight junctions via Nrf2, JNK and MLCK signaling in the intestine of fish, respectively. Finally, based on PWG, FE, PC and MDA, the safe dose of DON for grass carp were all estimated to be 318 μg/kg diet.

1. Introduction

Intestinal health is crucial for the healthy growth of fish, which is related to intestinal physical barrier and immune function [1]. Intestinal physical barrier represents the first line of defense against pathogenic agents from the external environment of gut lumen in animal [2]. However, the intestinal physical barrier of fish is very easy to be damaged [3]. Over recent years, due to the fast growing of aquaculture worldwide, increasing use of cereals for replacement of fishmeal as a source of protein in commercial aquaculture feeds has led to an increase in the risk of mycotoxin contamination [4]. The intestinal tract is the first physiological barrier against feed-borne contaminants, as well as the first target for those contaminants in animal [5]. Previous studies had showed that mycotoxins (such as aflatoxin B1 and T-2 toxin) disrupted intestinal structure of fish [6,7]. Deoxynivalenol (DON) is a Fusarium mycotoxin, and occurs widely in animal feed ingredients and feed products [8], especially in cereals, such as maize, wheat, barley, oat, soybean and so on [9]. It was reported that DON could be detected in more than 80% of commercial fish feed, with the maximum concentration of it up to 825 μg/kg [10]. However, until now, there is only

https://doi.org/10.1016/j.fsi.2018.06.013
Received 24 March 2018; Received in revised form 1 June 2018; Accepted 7 June 2018
Available online 12 June 2018
1050-4648/ © 2018 Published by Elsevier Ltd.
one study referring to the effect of DON on the intestine of fish. Ma-
tejova et al. [11], reported that DON caused gastrointestinal hemor-
rhage in rainbow trout. Therefore, it is imperative to conduct sys-
tematic and in-depth study about the effect of DON on intestinal physical barrier and its potential mechanisms in fish.

It has been shown that the impairment of fish physical barrier is partly related to oxidative damage [12]. Oxidative damage usually correlates with the disruption of Nrf2 signaling pathway, which results in the declining antioxidant enzyme activity, such as glutathione per-
oxidase (GPx) and catalase (CAT) [13,14]. Up to now, there was no study regarding the effects of DON on oxidative status, as well as the possible signaling pathways in the intestine of fish. A study observed that in the RAW 264.7 macrophage cell line, DON enhanced the produc-
tion of H2O2 [15] which could induce oxidative damage in rat erythrocyes [16]. In the rat, DON increased blood glucose content [17], and glucose could decrease activities of GPx and CAT in cultured mouse podocytes [18]. Besides, in mouse, DON could reduce the plasma in-
duction of H2O2 which could induce oxidative damage in rat erythrocyes [16]. In the rat, DON increased blood glucose content [17], and glucose could decrease activities of GPx and CAT in cultured mouse podocytes [18]. Besides, in mouse, DON could reduce the plasma in-
sulin-like growth factor 1 (IGF-1) levels [19], and lack of IGF-1 was reported to exacerbate Nrf2 dysfunction in liver [20]. Thus, we hy-
pothesized that DON might cause fish intestinal oxidative damage and decrease antioxidant enzyme activities through the inhibition of Nrf2 signaling, which is worthy of investigation.

Reactive oxygen species-triggered oxidative damage could promote cell apoptosis in aquatic organism [21]. As we all know, caspase-3 is a typical hallmark of cell apoptosis, which could be regulated by JNK signaling pathway in human DU145 prostate cancer cells [22]. Up to now, there was no study concerning the effects of DON on cell apop-
tosis, as well as the possible signaling pathways in fish. A study ob-
erved that in the murine macrophage RAW 264.7 cell lines, DON up-
regulated the mRNA expression of INF-γ [23], and INF-γ increased caspase-3 protein expression in rabbit placentas [24]. Besides, a study in pig showed that DON ingestion decreased liver selenium concentration [25], and low levels of selenium could aggravate cell apoptosis through activation of JNK signaling pathways in HT 1080 cells [26]. Based on these data, we hypothesized that DON might aggravate fish intestinal cell apoptosis by activating JNK signaling, which needs to be in-
vestigated.

Cell apoptosis could lead to the disruption of tight junctions (TJs) with fragmentation of occludin, ZO-1 and ZO-2 proteins in human breast epithelial cell lines H184A1 [27]. In Atlantic salmon (Samo-
salar), the tight junctional complexes (such as claudins and ZOas) were regulated by myosin light chain kinase (MLCK) [28]. Up to present, there was no research referring to the effects of DON on tight junctions, as well as the possible signaling pathways in fish. Previous study has shown that in macrophage RAW 264.7 cells, DON enhanced the secre-
tion of TNF-α protein [29], and TNF-α down-regulated the protein content of ZO-1 and claudin-5 in bovine retinal endothelial cells [30]. It was reported that DON up-regulated mRNA expression of mouse liver IL-1β [31] which caused a progressive increase in MLCK protein ex-
pression in Caco-2 cells [32]. According to these studies, we put for-
ward the assumption that DON might result in the destruction of fish intestinal TJs through activating MLCK signaling, which is worthy of investigation.

In this study, we first investigated the effect of DON on oxidative status, cell apoptosis and tight junctions as well as related signaling pathway (Nrf2, JNK and MLCK signaling) in the intestine of grass carp, which could partially elucidate the effect of DON intestinal physical barrier and its potential mechanism in fish. Grass carp is the biggest contributor to the world’s aquaculture production (FAO, 2015) [33]. Thus, we determined the safe dose of DON for grass carp, which might provide a partial basis for the feed formulating of grass carp.

### Table 1

<p>| Composition and nutrients content of basal diet. |
|-------------------------------|----------------|</p>
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Nutrients content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>41.00 g/kg</td>
</tr>
<tr>
<td>Casein</td>
<td>270.00 g/kg</td>
</tr>
<tr>
<td>Gelatin</td>
<td>75.00 g/kg</td>
</tr>
<tr>
<td>DL-Met (99%)</td>
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</tr>
<tr>
<td>L-Try (98%)</td>
<td>0.30 g/kg</td>
</tr>
<tr>
<td>Th (98.5%)</td>
<td>1.40 g/kg</td>
</tr>
<tr>
<td>Fish oil</td>
<td>26.20 g/kg</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>18.10 g/kg</td>
</tr>
<tr>
<td>α-starch</td>
<td>230.00 g/kg</td>
</tr>
<tr>
<td>Corn starch</td>
<td>202.80 g/kg</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.00 g/kg</td>
</tr>
<tr>
<td>Ca(H2PO4)2</td>
<td>33.10 g/kg</td>
</tr>
<tr>
<td>Vitamin premix&lt;sup&gt;x&lt;/sup&gt;</td>
<td>10.00 g/kg</td>
</tr>
<tr>
<td>Mineral premix&lt;sup&gt;x&lt;/sup&gt;</td>
<td>20.00 g/kg</td>
</tr>
<tr>
<td>Ethoxyquin (30%)</td>
<td>0.50 g/kg</td>
</tr>
<tr>
<td>Choline chloride (50%)</td>
<td>10.00 g/kg</td>
</tr>
<tr>
<td>DON premix&lt;sup&gt;x&lt;/sup&gt;</td>
<td>10.00 g/kg</td>
</tr>
</tbody>
</table>

<sup>a</sup> Per kilogram of vitamin premix (g/kg): retinyl acetate (500,000 IU/g), 0.39; cholecalciferol (500,000 IU/g), 0.40; D, α-tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.09; ascorbyl acetate (95%), 9.77; niacin (99%), 4.04; meso-inositol (98%), 19.39; calcium-o-pan-
tothenate (98%), 3.85; riboflavin (80%), 0.73; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with corn starch to 1 kg.

<sup>b</sup> Per kilogram of mineral premix (g/kg): MnSO4·H2O (31.8% Mn), 2.6590; MgSO4·H2O (15.0% Mg), 200.0000; FeSO4·H2O (30.0% Fe), 12.2500; ZnSO4·H2O (34.5% Zn), 8.2460; CuSO4·5H2O (25.0% Cu), 0.9560; KI (76.9% I), 0.0650 g; Na2SeO3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1 kg.

<sup>c</sup> Per kilogram of DON premix (g/kg): the DON was diluted with corn starch to 1 kg premix was added to obtain graded levels of DON.

<sup>d</sup> Crude protein and crude lipid content were measured value.

<sup>e</sup> n-3 and n-6 content were referenced to Zeng et al. [106], and calculated according to NRC (2011).

<sup>f</sup> Available phosphorus content was referenced to Liang et al. [107], and calculated according to NRC (2011).

### 2. Materials and methods

#### 2.1. Experimental diet preparation

The formulation and proximate composition analysis of the diets are presented in Table 1. Fish meal, casein and gelatin were used as main dietary protein sources. Fish oil and soybean oil were used as main dietary lipid sources. DON (purchased from Pribolab Pte, Ltd. Singapore; purity > 98%) was added to the other ingredients at six differ-
cent concentrations (0 μg/kg, 1200 μg/kg, 3000 μg/kg, 6000 μg/kg, 900 μg/kg, 1200 μg/kg, 1500 μg/kg final feed, respectively) by the method of Pietsch et al. [34]. The approximate compositions of the diets were analyzed according to the standard methods of AOAC [35]. In brief, crude protein was determined by Kjeldahl nitrogen determina-
method, and crude lipid was analyzed by the Soxhlet extraction method. The dietary actual DON concentrations of the six diets were determined to be 27 (un-supplemented), 318, 636, 922, 1243 and 1515 μg/kg diet, by the method of high performance liquid chromatography (HPLC) according to Pietsch et al. [34]. The diets were prepared and stored at -20°C until feeding, which was similar to Sanden et al. [36].

#### 2.2. Feeding trial and sample collection

All protocols were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. The grass carp obtained from fishers (Sichuan, China) was acclimated to the experimental
environment for 4 weeks as described by Zhang et al. [37]. Then, 1440 fish (mean weight 12.17 ± 0.03 g) were randomly assigned to 18 experimental cages (1.4 length × 1.4 width × 1.4 height in meters), resulting in 80 juvenile grass carp per cage. A disc of 100 cm diameter was equipped in the bottom of each cage to collect the uneaten feed, according to Tang et al. [38]. In the feeding trial, fish were fed with their respective diets to apparent satiation, four times per day for 60 days. After feeding 30 min, uneaten feed was collected, dried and weighted to calculate the feed intake (FI) as described by Tian et al. [39]. During the experimental period, dissolved oxygen was above 6.0 mg/L. The water temperature was at 28.5 ± 2.0 °C, pH value was at 7.5 ± 0.3. The feeding trial were conducted under natural light and dark cycle, which was similar to Chen et al. [40].

At the termination of feeding trial, fish from each cage was weighted and counted. Forty-five fish were randomly selected from each group, anaesthetized in a benzocaine bath as described by Geraylou et al. [41]. After sacrifice, the intestines of fish were quickly separated, measured and weighted for calculating the index of intestine, then segmented proximal intestine (PI), mid intestine (MI) as well as distal intestine (DI), frozen in liquid nitrogen and stored at -80 °C for later analysis as described by Xu et al. [42]. Parts of the PI, MI and DI were drenched with physiological saline and preserved in 10% neutral buffered formalin for histological examination according to Wu et al. [43].

2.3. Histological examination

Preserved intestinal tissue were embedded in paraaffin wax after dehydration and clear. Tissue was sectioned to 4 μm. Sections were stained using standard haematoxylin and eosin (H & E) and examined by a Nikon TS100 light microscope according to Kokou et al. [44].

2.4. Sample preparation and biochemical analysis

Intestinal samples were homogenized in 10 vol (w/v) of ice-cold physiological saline and centrifuged at 6000 g for 20 min at 4 °C as described by Luo et al. [45], then the supernatants were used for biochemical analysis. The reactive oxygen species (ROS) production was assayed according to Soraya et al. [46]. The content of malondialdehyde (MDA) was determined according to Yu et al. [47]. Total antioxidant capacity (T-AOC) was measured by the method of Zhou et al. [48]. The activities of superoxide dismutase (SOD) was measured according to the method of Dawood et al. [49]. The content of glutathione (GSH), glutathione reductase (GR) as well as glutathione peroxidase (GPx) were measured according to Deng et al. [50]. The catalase (CAT) activity was analyzed as described by Zhou et al. [51]. The content of protein carbonyl (PC), and the activities of anti-superoxide anion (ASA), anti-hydroxyl radical (AHR) and glutathione-S-transferase (GST) were measured as described by Jiang et al. [52]. The content of DON in the three intestinal segments were analyzed by ELISA (ELISA kit purchased from JiangSu SUWEI Microbiology Research Co., Ltd; china) according to Pestka et al. [53]. The analyzed DON concentrations are exhibited in Table 3.

2.5. Analysis of DNA fragmentation

The fragment DNA of the intestine tissue was isolated by the method of Li et al. [3]. DNA fragmentation was determined by electrophoresis for 1.5 h at 40 V using 2% agarose gel. The gel was evaluated and photographed using a Gene Genius Bio-Imaging system (Syngene, Frederick, MD, USA).

2.6. Real-time PCR analysis

The RNA extraction and real-time quantitative PCR [performed on the CFX96TM Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.)] were similar to Silveyra et al. [54]. The total RNA was extracted from PI, MI and DI using RNAiS Plus kit (TaKaRa, Dalian, Liaoning, China). RNA quality and quantity were assessed by using agarose gel (1%) electrophoresis and spectrophotometric (A260:280 nm ratio) analysis, respectively. Then, the cDNA was synthesized with reverse transcription of RNA by using the PrimeScript™ RT reagent Kit (TaKaRa) according to the manufacturer’ instructions. For quantitative real-time PCR, specific primers were designed according to the sequences cloned in our laboratory and the published sequences of grass carp (Table 2). According to the results of our preliminary experiment concerning the evaluation of internal control genes (data not shown), β-actin was elected as a reference gene to normalize cDNA loading. The target and housekeeping gene amplification efficiency was calculated according to the specific gene standard curves generated from 10-fold serial dilutions. The \(2^{-\Delta\DeltaCT}\) method was used to calculate the expression results after verifying that the primers were amplified with an efficiency of approximately 100% as described by Livak and Schmittgen [55].

2.7. Western blot analysis

Protein homogenates preparation from intestines, antibodies and western blotting were processed according to Jiang et al. [56]. Protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (40 μg per lane) were separated by SDS-PAGE and transferred to a PVDF membrane for western analysis. The membrane was blocked for 1 h at room temperature, and then incubated with primary antibody overnight at 4 °C. We used the same anti-total Nrf2, Lamin B1 and β-actin antibodies as those in our previous laboratory studies [57]. Lamin B1 and β-actin were used as reference proteins for nuclear Nrf2 and cytosolic Nrf2, respectively. After being washed, the PVDF membrane was incubated for 2 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST. The immune complexes were visualized using ECL reagents (Beyotime Biotechnology Inc., China). The western bands were quantified using NIH Image 1.63 software. Different groups were expressed relative to the level of control group. This experiment was repeated at least three times, and similar results were obtained each time.

2.8. Calculations and statistical analysis

Data on initial body weight (IBW), final body weight (FBW) and feed intake (FI) was used to calculate percentage of weight gain (PWG), specific growth rate (SGR) and feed efficiency (FE), as described by Tang et al. [38]. The length and weight of intestine were used to calculate the intestinal length index (ILI) and intestinal somatic index (ISI) respectively according to Jiang et al. [58].

\[
\text{PWG} (%) = 100 \times \frac{\text{FBW (g/fish)} - \text{IBW (g/fish)}}{\text{IBW (g/fish)}}
\]

\[
\text{SGR} (%) = \frac{\ln(\text{mean final weight}) - \ln(\text{mean initial weight})}{\text{days}}
\]

\[
\text{FE} (%) = \frac{\text{FBW (g/fish)} - \text{IBW (g/fish)}}{\text{FI (g/fish)}}
\]

\[
\text{ILI} (%) = \frac{100 \times \text{intestine length (cm)}}{\text{total body length (cm)}}
\]

\[
\text{ISI} (%) = \frac{100 \times \text{wet intestine weight (g)}}{\text{wet body weight (g)}}
\]

The results were shown as the mean ± standard deviation (SD). All data above was subjected to a one-way analysis of variance (ANOVA) followed by the Duncan’s multiple-range test to evaluate significant differences among treatments at \( P < 0.05 \) with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Based on PWG, SGR, PC and MDA, the safe dose of DON for grass carp were determined by according to the method of Patterson et al. [59].

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The effects of DON on the growth parameters of juvenile grass carp are shown in Table 3. The IBW was not significantly different among the groups. The growth performance (FBW, PWG, SGR, FI, and FE) and intestinal growth parameters (IL, II, IW, and ISI) of juvenile grass carp were decreased as increasing dietary DON levels. Compared to control group, the FBW, PWG, SGR, FI, and FE of II, IW, and ISI were significantly lower with increasing dietary DON levels up to 636 μg/kg and 1515 μg/kg. In comparison with control group, the intestine of juvenile grass carp from 636 μg/kg diet group showed obvious hyperemia phenomenon (Fig. 2A). After further histopathological observation (Fig. 2B), we found that DON caused the edema in the lamina propria in the 1515 μg/kg diet group. In the MI of juvenile grass carp, the edema in the lamina propria were both observed in the 636 μg/kg, 1243 μg/kg, and 1515 μg/kg diet groups. In the DI of juvenile grass carp, the necrosis of the epithelial cell were both observed in the 636 μg/kg, 1243 μg/kg, and 1515 μg/kg diet groups. In the MI and DI of juvenile grass carp, the necrosis of the epithelial cell were both observed in the 636 μg/kg and 1515 μg/kg diet groups. In the DI of juvenile grass carp, the edema in the lamina propria were both observed in the 636 μg/kg and 1515 μg/kg diet groups, and the necrosis of epithelial cell was only observed in the 1515 μg/kg diet group.

3.3. DON increased ROS production, caused oxidative damage and decreased antioxidant ability-related parameters, and related signaling molecules mRNA levels in the PI, MI and DI of juvenile grass carp

In comparison with control group, the intestine of juvenile grass carp from 636 μg/kg and 1515 μg/kg diet groups showed obvious hyperemia phenomenon (Fig. 2A). After further histopathological observation (Fig. 2B), we found that DON caused the edema in the lamina propria and the necrosis of epithelial cell in the three intestinal segments of juvenile grass carp. In the PI and MI of juvenile grass carp, the necrosis of the epithelial cell were both observed in the 636 μg/kg and 1515 μg/kg diet groups. In the DI of juvenile grass carp, we also found the edema in the lamina propria in the 1515 μg/kg diet group. In the MI of juvenile grass carp, the edema in the lamina propria were both observed in the 636 μg/kg and 1515 μg/kg diet groups. In the DI of juvenile grass carp, the edema in the lamina propria were both observed in the 636 μg/kg and 1515 μg/kg diet groups, and the necrosis of epithelial cell was only observed in the 636 μg/kg diet group.

3.4. DON increased ROS production, caused oxidative damage and decreased antioxidant ability-related parameters, and related signaling molecules mRNA levels in the PI, MI and DI of juvenile grass carp

As is shown in Table 4, the ROS, PC as well as MDA content of three intestinal segments of juvenile grass carp were increased by DON in a
dose-dependent manner. Compared with control group, the content of ROS, PC and MDA in three intestinal segments were all significantly higher with increasing dietary DON levels up to 636 μg/kg diet (P < 0.05). The activities of ASA, AHR, T-AOC, CuZnSOD, MnSOD, CAT, GPx, GST as well as GR, and GSH content in the PI of juvenile grass carp were significantly lower with increasing dietary DON levels up to 636, 922, 636, 318, 922, 636, 636, 922 and 636 μg/kg diet (P < 0.05), respectively. Similarly, as the increasing dietary DON levels increased to 318, 922, 636, 318, 318, 1243, 318, 922, 636 and 636 μg/kg diet, the activities of ASA, AHR, T-AOC, CuZnSOD, MnSOD, CAT, GPx, GST as well as GR, and GSH content in the MI of juvenile grass carp were significantly decreased (P < 0.05), respectively. Besides, the activities of ASA, AHR, T-AOC, CuZnSOD,
MnSOD, CAT, GPx, GST as well as GR, and GSH content in the DI of juvenile grass carp were significantly lower, when the dietary DON levels increased up to 636, 922, 318, 636, 636, 636 and 318 μg/kg diet (P < 0.05), respectively.

The mRNA levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR, GSTP1, GSTP2, GSTO1, GSTO2, GR as well as Nrf2 in the PI of juvenile grass carp, were significantly down-regulated with the increasing dietary DON levels up to 636, 1243, 1515, 636, 636, 636, 636, 922, 1243, 636, 922, 922 and 636 μg/kg diet (P < 0.05), respectively. The mRNA levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTP1, GSTP2, GSTO1, GSTO2, GR as well as Nrf2 in the MI of juvenile grass carp, were significantly down-regulated when the dietary DON levels were reached 318, 636, 922, 318, 318, 318, 318, 318, 318, 318, 318, 318, 1243, 636 and 318 μg/kg diet (P < 0.05), respectively. Similarly, as dietary DON levels increased to 636, 1243, 318, 318, 636, 318, 636, 318, 636, 636, 922, 636 and 318 μg/kg diet, the mRNA levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR, GSTP1, GSTP2, GSTO1, GSTO2, GR as well as Nrf2 in the DI of juvenile grass carp, were significantly down-regulated (P < 0.05), respectively. Besides, the mRNA levels of Keap1a in the PI, MI as well as DI of juvenile grass carp were significantly up-regulated with increasing dietary DON levels up to 1243, 1243 and 1515 μg/kg diet (P < 0.05), respectively. Specifically, DON had no effect on the mRNA expression of Keap1b in the PI, MI and DI, and GSTR in the MI of juvenile grass carp (P > 0.05) (Fig. 3A).

3.4. DON decreased the protein levels of nuclear Nrf2 and cytosolic Nrf2 in the PI, MI and DI of juvenile grass carp

The nuclear Nrf2 and cytosolic Nrf2 protein levels in the PI, MI and DI of juvenile grass carp were presented in Fig. 3B. The protein levels of nuclear Nrf2 and cytosolic Nrf2 in the three intestinal segments of juvenile grass carp were decreased as increasing dietary DON levels. In comparison of control group, the nuclear Nrf2 and cytosolic Nrf2 protein levels of three intestinal segments of juvenile grass carp were all significantly lower with increasing dietary DON levels up to 636 μg/kg diet (P < 0.05).

3.5. DON resulted in DNA fragmentation and decreased the mRNA expression of cell apoptosis-related genes and signaling molecules in the PI, MI and DI of juvenile grass carp

The effects of DON on DNA fragmentation in three intestinal segments of juvenile grass carp are presented in Fig. 4A. This study confirmed that the ladder-like pattern of DNA fragments was visibly observed in the PI, MI and DI of grass carp as the dietary DON levels reached up to 922, 922 and 1243 μg/kg, respectively. The effects of DON on cell apoptosis-related genes and signaling molecules mRNA expression were presented in Fig. 4B. The results showed that the mRNA expression of caspase-3, caspase-8, caspase-9, Bax, Bcl-2, Bid, Fas, FasL, and p53 were significantly down-regulated with increasing dietary DON levels (P < 0.05).
levels in the intestine of juvenile grass carp are presented in Fig. 4B. The mRNA levels of caspase-3, caspase-7, caspase-8, caspase-9, Apaf-1, Bax, Fasl, and JNK in the PI of juvenile grass carp, were significantly up-regulated with the increasing dietary DON levels up to 1515, 922, 922, 1515, 922, 1515 and 922 μg/kg diet (P < 0.05), respectively. Similarly, the mRNA levels of caspase-3, caspase-7, caspase-8, caspase-9, Apaf-1, Bax, Fasl, and JNK in the MI of juvenile grass carp, were significantly up-regulated when the dietary DON levels were up to 1243. 922, 922, 1243, 922, 922, 1243 and 922 μg/kg diet (P < 0.05), respectively. Besides, the mRNA levels of caspase-3, caspase-7, caspase-8, caspase-9, Apaf-1, Bax, Fasl, and JNK in the DI of juvenile grass carp, were significantly up-regulated as the increasing dietary DON levels reached up to 636, 922, 1243, 922, 636, 922, 1243 and 636 μg/kg diet (P < 0.05), respectively. For Bcl-2 and Mcl-1, the mRNA expression were significantly down-regulated with increasing dietary DON levels up to 636 and 318 μg/kg diet in the PI, 636 and 922 μg/kg diet in the MI, as well as 636 and 1515 μg/kg diet in the DI (P < 0.05), respectively.

### Table 4

<table>
<thead>
<tr>
<th>Dietary DON level (μg/kg diet)</th>
<th>27</th>
<th>318</th>
<th>636</th>
<th>922</th>
<th>1243</th>
<th>1515</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ROS</td>
<td>0.00 ± 8.92</td>
<td>104.77 ± 8.61</td>
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</tr>
<tr>
<td>MDA</td>
<td>13.80 ± 0.90</td>
<td>14.52 ± 0.97</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PC</td>
<td>2.00 ± 0.17</td>
<td>2.05 ± 0.15</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ASA</td>
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<td>40.92 ± 3.61</td>
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Values are means ± SD (n = 6), and superscripted different letters in the same row are significantly different (P < 0.05). ROS, reactive oxygen species (% DCF fluorescence); MDA, malondialdehyde (nmol/g tissue); PC, protein carbonyl (nmol/mg protein); ASA, anti-superoxide anion (U/g protein); AHR, anti-hydroxy radical (U/mg protein); T-AOC, total antioxidant capacity (U/mg protein); CuZnSOD, copper/zinc superoxide dismutase (U/mg protein); MnSOD, manganese superoxide dismutase (U/mg protein); CAT, catalase (U/mg protein); Gpx, glutathione peroxidase (U/mg protein); GST, glutathione-S-transferase (U/mg protein); GR, glutathione reductase (U/mg protein); GSH, glutathione (mg/g protein).

3.6. DON decreased the mRNA levels of tight junctions and related signaling molecules in the PI, MI and DI of juvenile grass carp

The effects of DON on mRNA levels of intestinal TJs and MLCK in the three intestinal segments of juvenile grass carp are shown in Fig. 5. The mRNA levels ofZO-1, ZO-2b, occludin, claudin-c, claudin-f, claudin-7a, claudin-7b and claudin-11 in the PI of juvenile grass carp, were significantly down-regulated with the increasing dietary DON levels up to 636, 636, 318, 922, 636, 1243, 636 and 922 μg/kg diet (P < 0.05), respectively. Similarly, the mRNA levels ofZO-1, ZO-2b, occludin, claudin-c, claudin-f, claudin-7a, claudin-7b and claudin-11 in the MI of juvenile grass carp, were significantly down-regulated with the increasing dietary DON levels up to 922, 1243, 922, and 922 μg/kg diet (P < 0.05), respectively. Besides, the mRNA levels ofZO-1, ZO-2b, occludin, claudin-c, claudin-f, claudin-7a, claudin-7b and claudin-11 in the MI of juvenile grass carp, were significantly down-regulated when the dietary DON levels were reached 318, 636, 318, 636, 636, 318 and 636 μg/kg diet, (P < 0.05), respectively. Besides, The mRNA levels ofZO-1, ZO-2b, occludin, claudin-c, claudin-f, claudin-7a, claudin-7b and claudin-11 in the DI of juvenile grass carp, were significantly down-regulated as the increasing dietary DON levels reached up to 636, 922, 922, 636, 922, 1243, 922, 636, 318, 922 and 318 μg/kg diet (P < 0.05), respectively.
However, DON had no impact on the mRNA expression of claudin-b, claudin-3c and claudin-15b in the PI, MI and DI of juvenile grass carp (P > 0.05). For claudin-12, claudin-15a and MLCK, the mRNA expression were significantly up-regulated with increasing dietary DON levels up to 1243, 922 and 1243 μg/kg diet in the PI, 1243, 1243 and 922 μg/kg diet in the MI as well as 1515, 636 and 922 μg/kg diet in the DI of juvenile grass carp, respectively.

Fig. 3. A. Relative levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTP1, GSTP2, GSTO1, GSTO2, GR, Nrf2, Keap1a and Keap1b in PI (A), MI (B) and DI (C) of juvenile grass carp fed diets containing graded levels of deoxynivalenol. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different (P < 0.05).

Fig. 3B. Western blot analysis of nuclear Nrf2 and cytosolic Nrf2 in the PI(A), MI(B), and DI(C) of juvenile grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of DON for 60 days.
4. Discussion

4.1. DON causes growth retardation and body malformation in fish

The present study showed that DON level up to 636 μg/kg diet resulted in poor growth performance and body malformation in grass carp. The malformation (mainly skeleton malformation) of DON-treated fish might be related to the inhibition of osteoclastic and osteoblastic activities by melatonin. As we all know, osteoblasts and osteoclasts play a pivotal role in the formation and architecture of skeleton in animal [60,61]. It was documented that in pineal cell of rat, DON increased the concentration of melatonin [62] which suppressed osteoclastic and osteoblastic activities in goldfish [63]. These results help to explain our findings.

4.2. DON caused the histopathological lesions in intestine of fish

In this study, we observed that DON caused the edema in the lamina propria and necrosis of epithelial cell in the three intestinal segments of grass carp. These two histopathological phenomena could be explained as follows. Firstly, the appearance of edema in lamina propria of intestine might be related to the inhibition of protein synthesis by DON. Study showed that DON caused the inhibition of protein synthesis in the plasma of pig [64]. A study reported that a lack of plasma protein resulted in the edema in kidney of human [65]. Secondly, the necrosis of intestinal epithelial cell might be related to the DNA damage caused by DON. Research reported that in hepatocyte of mice, DON induced DNA damage [66] which led to the necrotic cell death in human prostate epithelial cell [67]. Thus, these results help to support our assumptions.

4.3. DON increases ROS production, causes oxidative damage and decreases antioxidant capacity partly relating to the inhibition of Nrf2 signaling pathway in the intestine of fish

As we all know, excess ROS has been considered to be an important causative factor for oxidative damage (indicated by MDA and PC) in fish [68]. This study firstly found that DON levels up to 636 μg/kg diet significantly increased the content of ROS, MDA and PC in the intestine.
Fig. 4. A. Effects of different dietary DON levels on DNA fragmentation in PI, MI and DI of juvenile grass carp using agarose gel electrophoresis. Lane 1: control group. Lane 2 - Lane 6: levels of dietary DON were 318, 636, 922, 1243 and 1515 μg/kg, respectively. This experiment was repeated three times with similar results achieved.

Fig. 4B. Relative levels of caspase-3, caspase-7, caspase-8, caspase-9, Apaf-1, Bax, FasL, Bcl-2, Mcl-1 and JNK in the PI (A), MI (B) and DI (C) of juvenile grass carp fed diets containing graded levels of DON. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different (P < 0.05).
of grass carp, suggesting that DON caused lipid peroxidation and protein oxidative damage in the intestine of fish. Besides, studies observed that excessive ROS could decrease antioxidant capacity by decreasing antioxidant enzyme activities (such SOD and GPx) in the breast muscle of broiler [69], and depleting GSH in rat hepatoma H5-6 cells [70]. In this study, we observed that DON level up to 318 μg/kg diet decreased antioxidant enzyme activities, as well as GSH content in the intestine of grass carp, suggesting that DON could attenuate antioxidant capacity in the intestine of fish. A study in rat reported that antioxidant enzyme activities were closely related to their mRNA levels [71]. Our data firstly showed that DON level up to 318 μg/kg diet suppressed the antioxidant enzyme gene expression (except GSTR in MI) in the intestine of grass carp. Correlation analysis revealed that antioxidant enzyme activities were positively related to their corresponding mRNA levels in

![Bar chart](image)

**Fig. 5.** Relative levels of ZO-1, ZO-2b, occludin, claudin-b, claudin-c, claudin-f, claudin-3C, claudin-7a, claudin-7b, claudin-11, claudin-12, claudin-15a, claudin-15b and MLCK in PI (A), MI (B) and DI (C) of juvenile grass carp fed diets containing graded levels of DON. Data represent means of six fish in each group, error bars indicate S.D. Values having different letters are significantly different (*P* < 0.05).
the intestine of grass carp (Table 5), which confirmed that DON decreased antioxidant enzyme activities might partly through the down-regulation of their corresponding mRNA levels.

It was reported that Nrf2 is a key factor for the promotion of gene transcriptions of antioxidant enzymes which was suppressed by Keap1 in EPC cell [72]. The nuclear translocation of cytosolic Nrf2 (indicated transcriptions of antioxidant enzymes which was suppressed by Keap1 regulation of their corresponding mRNA levels.

In the intestine of grass carp (Table 5), which con

Table 5

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<th>Independent parameters</th>
<th>Dependent parameters</th>
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<th>DI</th>
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<tr>
<td>Claudin-15a mRNA level</td>
<td>+0.971</td>
<td>&lt; 0.01</td>
<td>+0.944</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Claudin-15b mRNA level</td>
<td>+0.152</td>
<td>= 0.773</td>
<td>+0.639</td>
<td>0.172</td>
</tr>
</tbody>
</table>

study showed that DON decreased Nrf2 mRNA levels, cytosolic and nuclear Nrf2 protein levels in the intestine of grass carp, which manifested that DON suppressed the transcription and translation of Nrf2. Secondly, the other reason might involve the up-regulation of the mRNA expression of Keap1 (a negative regulator of Nrf2) caused by DON. Our study showed that DON up-regulated Keap1 mRNA levels in the intestine of grass carp, which might result in the inhibition of nuclear translocation of Nrf2.

Luckily, we found two interesting phenomena in the oxidative damage and reduced antioxidant capacity caused by DON in fish intestine. First, we found that DON up-regulated Keap1 (but not Keap1b) in the intestine of grass carp, which might be related to prostaglandin. As we all know, the cystine residues Cys-288 and Cys-273 in Keap1 of mouse intestine was respectively corresponding to Keap1a and Keap1b in zebrafish, and either Cys-288 in Keap1a or Cys-273 in Keap1b is sufficient for Keap1 to repress Nrf2 [75]. Study in mouse reported that DON enhanced the production of prostaglandin [76], and prostaglandin could result in the mutation of Cys-273 site (but not Cys-288 site) [77].
Hence, we supposed that DON enhanced the production of prosta-
glandin, and prostaglandin might activate Keap1a (rather than
Keap1b) in the intestine of fish. Second, DON down-regulated the GSTR
mRNA levels in the PI and DI (except in MI) of grass carp, which might
be related to lipid. Study had shown that DON increased lipid uptake in
human intestinal epithelial cells [78], and elevated lipid down-regu-
lated GSTR mRNA expression in the PI and DI (except in MI) of young
grass carp [79]. Thus, these results help to support our assumptions.

Research had shown that oxidative damage could contribute to cell
apoptosis in grass carp kidney cells [80]. Hence, we next investigated
the effects of DON on cell apoptosis in the intestine of fish.

4.4. DON aggravates apoptosis partly relating to JNK signaling pathway in the intestine of fish

DNA fragmentation has been utilized as a characteristic feature of
apoptosis in the hepatopancreas of Eriocheir sinensis [81]. This study
demonstrated that DON resulted in DNA fragmentation in the PI, MI
and DI of grass carp, suggesting that DON caused cell apoptosis in
the intestine of fish. However, the underlying mechanism is unclear, which
might be partially involved in the decreased folic acid. Study showed
that DON could reduce the tissue accumulation of folic acid in the je-
junal segment of mice [82], and folic acid deficiency could promote
apoptosis in fetal mouse brain [83]. These results help to explain our
findings.

As we all know that mitochondrial apoptotic pathway ([Bcl-2, Mcl-1
and Bax)/Apaf/caspase-9] and death receptor pathway (Fasl/caspase-
8) are two main pathways to cause apoptosis in mammalians, and
caspases participate in the process of these two pathways [84]. The apoptosis
caspases are further categorized into the initiator caspase-8, -9 and the effector caspase-3, -7 in fish [85]. Studies had indicated that JNK
could induce Bax-dependent apoptosis in 293 T kidney cells [86], and FasL-related apoptosis in human leukemia HL-60 cells [87]. In this
study, we observed that DON level up to 318 μg/kg diet up-regulated
caspases, Apaf-1, Bax, Fasl and JNK, and down-regulated Bcl-2 and
Mcl-1 mRNA levels in intestine of grass carp. Correlation analysis in-
dicated that caspase-9 mRNA levels were positively related to Apaf-1
and Bax mRNA levels, but negatively correlated with Bcl-2 and Mcl-1
mRNA levels in the intestine of grass carp. Besides, the caspase-8 mRNA
level was positively related to FasL mRNA level, and pro-apoptotic
Apaf-1, Bax and Fasl were positively correlated with JNK mRNA levels,
while anti-apoptotic Bcl-2 and Mcl-1 were negatively related to JNK
mRNA levels in the intestine of grass carp (Table 5). These data sug-
ggested that DON could aggravate mitochondrial apoptotic
pathway and death receptor pathway partly relating to JNK signaling in
the intestine of fish. The underlying mechanisms of the up-regulation
JNK mRNA levels in the intestine of grass carp induced by DON are still
unknown, but it might be partially involved in the decreased GSTO
content. In HeLa cells, GSTO could inhibit protein expression of JNK
[88]. Our study demonstrated that DON down-regulated mRNA level of
GSTO1 and GSTO2 in the intestine of grass carp, which supported our
hypothesis.

Research revealed that cell apoptosis resulted in the disruption of
tight junctions in endothelial cell of pig brain [89]. Therefore, we next
investigated the effects of DON on tight junctions in the intestine of fish.

4.5. DON disrupts tight junctions partly relating to MLCK pathway in the intestine of fish

According to previous research in fish, down-regulation of barrier-
forming TJ proteinsZO-1, occludin and claudins mRNA levels could
reflect the disruption of tight junctions in the intestine [90]. Moreover,
the increased mRNA expression of pore-forming TJ proteins, such as
claudin-12 [91] and claudin-15 [92], are likely to improve intestinal
epithelial paracellular permeability in mice. This study firstly observed
that DON level up to 318 μg/kg diet down-regulated the mRNA levels of
barrier-forming TJ proteins (except claudin-b, -3c), and up-regulated
pore-forming TJ proteins (except claudin-15b) mRNA levels in the in-
estine of grass carp, suggesting that DON could disrupt the tight
junctions in the intestine of fish. In mouse, MLCK played an important
role in promoting TJ disruption of intestinal epithelium [93]. Present
study showed that DON up-regulated the mRNA levels of MLCK in the
intestine of grass carp. Correlation analysis suggested that the mRNA
levels of barrier-forming TJ proteins (except claudin-b, -3c) were ne-
gatively related to MLCK mRNA levels, and pore-forming TJ proteins
(except claudin-15b) mRNA levels were positively related to MLCK
mRNA levels in the intestine of grass carp (Table 5), which indicated that
DON disrupted tight junctions might be related to the activation of
MLCK signaling in the intestine of fish. The potential mechanisms of the
activation of MLCK signaling in the intestine of grass carp caused by
DON are still unclear, but it might be partially involved in the en-
doplasmic reticulum (ER) stress and intracellular Ca^{2+} levels. In human
T lymphocyte cell line, DON could provoke endoplasmic reticulum
stress, resulting in the elevation of intracellular Ca^{2+} levels [94]. Study
reported that increasing cytosolic Ca^{2+} concentration promoted MLCK
phosphorylation in bovine tracheal smooth muscle cells [95]. These
results help to support our assumption.

Interestingly, DON had no impact on the mRNA levels of claudin-b,
claudin-3c and claudin-15b (rather than claudin-15a) in the intestine of
grass carp. There are several reasons for these interesting phenomena as
follows. Firstly, DON had no effects on the mRNA levels of claudin-b in
the intestine of grass carp, which might be correlated with cortisol in-
fluenced by IL-6. It was reported that in macrophage RAW 264.7 cells,
DON could enhance IL-6 protein secretion [29], which could increase
plasma cortisol level in human [96]. It was reported that cortisol had no
effect on the mRNA levels of claudin-b in the goldfish (Carassius aur-
atus) [97]. Secondly, DON had no impact on the mRNA levels of
claudin-3c in the intestine of grass carp, which might be dependent on
the concentration of DON. Study showed that 10 μM DON (corre-
sponding to 3 mg/kg feed) had no impact on protein expression of
claudin-3, whereas 30 μM DON (corresponding to 10 mg/kg feed) sig-
ificantly decreased claudin-3 protein expression in IPEC-1 cell lines
[98]. In our study, the maximum concentration of DON was 1.5 mg/kg
feed, which was much lower than 3 mg/kg feed, supporting our hy-
pothesis. Thirdly, DON up-regulated the mRNA levels of claudin-15a
(rather than claudin-15b) in the intestine of grass carp, which might be
partly relevant to the feedback mechanism for increasing Na^{+} transport
capacity in the intestine of fish. It has been reported that DON inhibited
the Na^{+} transport in the intestinal mucosa of laying hens [99]. In
the intestine of Atlantic salmon (Salmo salar), claudin-15 could enhance
the Na^{+} movement by increasing paracellular permeability [100]. In Ja-
panese medaka (Oryzias latipes) intestine, claudin-15a expression
were much higher than claudin-15b [101]. Thus, DON up-regulated claudin-
15a (rather than claudin-15b) gene expression, which might be a
feedback mechanism for increasing Na^{+} transport capacity in the fish
intestine.

4.6. The safe dose of DON for grass carp

In our study, based on PWG, FE, PC and MDA, the safe dose of DON
for grass carp was all estimated to be 318 μg/kg diet. Meanwhile, our
results showed that DON residue was not detected in the control group
and 318 μg/kg diet group, which agree with this evaluation results.
However, the recommended maximum acceptable level of DON for
piglet is 900 μg/kg diet (2006/576/EC) [102], which is much higher
than our results in fish, suggesting that grass carp is more sensitive to
DON than piglet. It might be related to the content of intestinal poly-
unsaturated fatty acid, which is susceptible to oxidative damage [103].
Our study showed that DON increased MDA content in the intestine of
ground carp. The content of intestinal polyunsaturated fatty acid in grass
carp was approximately 57.1% [104], which was much higher than
34% in ileum of piglet [105]. These data indicated grass carp is more
susceptible to DON than piglet.

5. Conclusions

Taken all together (summarized in Fig. 6), this study was for the first time to investigate the effects of DON on the intestinal physical barrier in fish. Our data showed that the impairment of intestinal physical barrier of fish by DON might partly be involved in (1) increasing ROS production resulting in oxidative damage and decreasing antioxidant capacity through down-regulating the mRNA levels of CuZnSOD, MnSOD, CAT, GPx1a, GPx1b, GPx4a, GPx4b, GSTR (not in MI), GSTO1, GSTO2, GSTP1, GSTP2 and GR in the intestine of fish, which might be associated with the activation of Nrf2/Keap1 (Keap1 but not Keap1b) signaling pathway. (2) aggravating cell apoptosis through the mitochondrial apoptotic pathway (Bax, Bcl-2 and Mcl-1/Apaf-1/caspase-9) and death receptor pathway (FasL/caspase-8), which might partly be regulated by activating JNK signaling pathway. (3) impairing the tight junctions might be related with the decreased ZO-1, ZO-2b, occludin, claudin-c, claudin-f, claudin-7a, claudin-7b and claudin-11 (except claudin-b and claudin-3c) mRNA levels, and the increased claudin-12 and claudin-15a (not claudin-15b) mRNA levels, which might partly be related to the activation of MLCK signaling pathway. Based on PWG, FE, PC and MDA, the safe dose of DON for grass carp were all estimated to be 318 μg/kg diet.

Acknowledgements

This research was financially supported by the National Basic Research Program of China (973 Program) (2014CB138600), National Department Public Benefit Research Foundation (Agriculture) of China (201003020), Outstanding Talents and Innovative Team of Agricultural Scientific Research (Ministry of Agriculture), Science and Technology Support Program of Sichuan Province of China (2014NZ0003), Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2013NC0045), The Demonstration of Major Scientific and Technological Achievement Transformation Project of Sichuan Province of China (2015CC0011) and the modern agricultural industry technology system of Sichuan freshwater fish innovation team. Foundation of Sichuan Youth Science and Technology Innovation Research Team (2017TD0002), and supported by the Earmarked Fund for China Agriculture Research System (CARS-45). The authors would like to thank the personnel of these teams for their kind assistance.

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