Gossypol reduced the intestinal amino acid absorption capacity of young grass carp (Ctenopharyngodon idella)

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**ABSTRACT**

This study was conducted to evaluate the effects of gossypol on the growth, intestinal histology, intestinal amino acid (AA) absorption capacity and the safe upper limit of gossypol in the diets for young grass carp (Ctenopharyngodon idella) growth. The fish were fed six diets containing different levels of free gossypol (0, 125 (121.38), 250 (243.94), 375 (363.93), 775 (759.93) and 1175 (1162.06) mg kg\textsuperscript{-1} diet) from gossypol-acetic acid for 60 days. Results indicated that gossypol could reduce the growth performance and damage the intestines of young grass carp. Interestingly, gossypol modulated the absorptive abilities of various AAs, and mechanisms are not unified in fish. Firstly, gossypol decreased the contents of tryptophan (Trp), threonine (Thr), valine (Val), phenylalanine (Phe), glycine (Gly), serine (Ser) and tyrosine (Tyr) in the three intestinal segments of fish associated with down-regulating the mRNA levels of solute carrier (SLC) 6A19b, SLC7A5, SLC7A8, SLC1A5, SLC38A2 and peptide transporter 1 (PepT1) in the intestine. Secondly, gossypol reduced the contents of methionine (Met), isoleucine (Ile), proline (Pro), cysteine (Cys) and arginine (Arg) in the proximal intestine (PI) and mid intestine (MI) of fish by down-regulating the mRNA levels of SLC7A7, SLC7A6, SLC7A1, SLC6A19b, SLC7A5, SLC7A8, SLC1A5, SLC38A2 and PepT1 in the PI and MI. Thirdly, gossypol inhibited the aspartic acid (Asp) and taurine (Tau) contents in the PI and MI of fish by down-regulating the mRNA levels of SLC1A2a, SLC1A3, PepT1 and SLC6A6 in the PI and MI. Fourthly, gossypol suppressed the lysine (Lys) and histidine (His) contents in the MI and distal intestine (DI) of fish due to down-regulating the mRNA levels of SLC7A7, SLC7A5 and SLC38A2 in the MI, and SLC7A6, SLC7A1, SLC7A8 and PepT1 in the MI and DI. Gossypol down-regulated the above AA and peptide transporters in the intestine of fish partly related to suppressing the target of rapamycin (TOR) signalling pathway. Based on percent weight gain (PWG) and feed efficiency (FE), the safe upper limit of gossypol in the diets for young grass carp growth were estimated to be 182 and 179 mg kg\textsuperscript{-1} diet, respectively. In summary, gossypol reduced the intestinal AA absorption capacity of young grass carp partly relate to damaging the intestinal structure, down-regulating the mRNA levels of AA and peptide transporters, and inhibiting the TOR signalling pathway.

1. Introduction

Gossypol is a primary anti-nutritional factor present in the cotton-seed meal (Deng et al., 2014a). A study demonstrated that high levels of dietary gossypol decreased the growth performance and damaged the liver and spleen in rainbow trout (Salmo gairdneri) (Herman, 1970). The intestine is the main site for gossypol absorption in fish (Meric et al., 2011). It is well known that fish intestine with high levels of polyunsaturated fatty acids (PUFAs) are sensitive to oxidative damage (Jiang et al., 2013). However, studies have revealed that in phagocytic cells of channel catfish (Ictalurus punctatus), gossypol could stimulate the generation of reactive oxygen species (ROS) (Yildirimaksy et al., 2004), which could induce intestinal oxidative damage in fish (Wang et al., 2016a). These investigations indicated that gossypol might cause the intestinal damage in fish. However, Evans et al. (2009) reported that gossypol did not impact the intestinal histology of channel catfish.
The grass carp is one of the most important aquaculture species in the world (Wang et al., 2015). The intestinal PUFA content in grass carp (57.1% of total fatty acids) has been reported to be higher than that in channel catfish (32.1% of total fatty acids) (Houpe et al., 1997; Liu, 1991). Thus, gossypol might impact on the intestinal structure of grass carp. However, no studies have reported the effects of gossypol on the intestinal histology of grass carp.

As we know, intestinal structural damage could reduce the intestinal absorption and transport capacity for nutrients in animals (Ghishan and Kiela, 2014). Amino acids (AAs) and peptides are important nutrients for absorption in the intestines of fish (Counsel, 2011). In the intestines of animals, the absorption and transport of AAs and small peptides depends on the AA and peptide transporters, respectively (Bröer; Liu et al., 2012b). AA transporters (AATs) are mainly classified into the neutral AAT (e.g., solute carrier (SLC)38A2), the neutral and cationic AAT (e.g., SLC6A14), the cationic AAT (e.g. SLC7A1) and the anionic AAT (e.g., SLC1A2/-3), while the peptide transporter 1 (Pept1) is the major small peptide transporter presence in the intestines of animals (Lu and Klaassen, 2006; Xiao et al., 2015). However, there have been no reports concerning the effect of gossypol on the intestinal AAs and small peptide absorption ability in fish. Free-gossypol has been reported to bind with the lysine in feed, which in turn leads to the lysine deficiency in channel catfish (Robinson et al., 1995). In gilthead see bream muscle cells, lysine deficiency decreased the insulin-like growth factor (IGF)-1 mRNA level (Azizi et al., 2016). Studies have reported that IGF-1 inhibition could reduce the activity of SLC38A2 in human placenta (Jansson et al., 2006), down-regulate the SLC7A1 mRNA level in the placentas of Romney ewes (Walt et al., 2012) and decrease the activity of SLC6A14 activity in HEK 293 cells (Samul et al., 2012). Gossypol could also inhibit cyclic adenosine monophosphate (cAMP) formation in bovine granulosa cells (Lin et al., 1994), while the inhibition of cAMP decreased the activity of PepT1 in COS-7 cells (Galdrat et al., 2005). The above data indicates that gossypol might impact the AAs and small peptide absorption capacity in the intestines of fish via regulating the specific AA and peptide transporters, which warrants further exploration.

The AA and peptide transporter activities were found to be related to their gene expression in the intestines of animals (Zhang et al., 2013c, 2014). Studies have reported that AA and peptide transporters were regulated by the target of rapamycin (TOR) pathway in the plasma membrane of humans (Rosario et al., 2016) and in the intestine of C. elegans (Benner et al., 2011), respectively. Inhibition of the mTOR phosphorylation at Ser 2448 has been reported to inhibit the activity of p70 ribosomal protein S6 kinase 1 (S6K1) and activate eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Inoki et al., 2002). However, no study has addressed the relationship between gossypol and the TOR signalling pathway in the intestine of animals. In broiler chickens, gossypol binds iron in the bloodstream, causing iron deficiency (Watkins et al., 1993). Our previous study observed that iron deficiency down-regulated the mRNA levels of TOR and S6K1 and up-regulated 4E-BP1 in the head kidney and spleen of young grass carp (Guo et al., 2017). A study has demonstrated that gossypol may down-regulate the gene expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) in human breast adipocytes (Zhong et al., 2013), while the inhibition of PPAR-γ suppressed the activation of mTOR signalling in the adipose tissue of rats (Blanchard et al., 2012). These data implied that gossypol might regulate the AA and peptide transporters in the intestines of fish through the TOR signalling pathway, and the possibility warrants investigation.

The present study was performed to investigate the effect of gossypol on the growth performance and the safe upper limit of gossypol in the diets of young grass carp, which might provide a reference for cottonseed meal utilization in commercial fish feed. Based on the
2.2. Feeding trial and sampling

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. After obtaining from Tong Wei hatchery (Sichuan, China), grass carp were acclimated to experimental conditions for 4 weeks according to Ji et al. (2011). Then, 540 fish (mean weight 230.93 ± 0.50 g) were randomly assigned to 18 experimental groups (1.4 L × 1.4 W × 1.4 H m), resulting in 30 fish per cage as described in our laboratory study (Feng et al., 2016). Each cage was equipped with a disc of 100 cm diameter in the bottom to collect the uneaten feed, according to our laboratory study (Ni et al., 2016). For the feeding trial, fish were fed with their respective diets to apparent satiation 4 times daily according to Tu et al. (2015) for 60 days. Thirty minutes after feeding, uneaten feed was collected, dried and weighed to calculate the feed intake according to our laboratory study (Xu et al., 2016b). During the experiment, water temperature was averaged at 27 ± 2 °C, and pH value was maintained at 7.0 ± 0.4. The dissolved oxygen was not < 6.0 mg L⁻¹ according to our laboratory study (Xu et al., 2016a). The experimental units were under natural light and dark cycle.

The fish from each cage were weighed and counted at the initiation and termination of the feeding trial. Twelve fish were randomly selected from each treatment, anesthetized in a benzocaine bath as described by Deng et al. (2014b) then sacrificed. The intestines of fish were quickly removed, weight, frozen in liquid nitrogen and stored at −80 °C according to Betancor et al. (2017). Parts of intestinal samples from six fish were used for detection of the gossypol and FAA contents. Additionally, at the end of the growth trial, the three intestinal segments (proximal intestine (PI), middle intestine (MI) and distal intestine (DI)) of three fish from each cage were sampled to examine the intestinal histology according to Wu et al. (2016).

2.3. Analysis of gossypol content in tissues

The gossypol contents in the three intestinal segments were determined according to Bian et al. (2016). Briefly, tissue samples were homogenised with extraction reagent (2% 2-amino-1-propanol and 10% glacial acetic acid in N, N dimethylformamide) for 1 min. The homogenate was heated at 95 °C for 30 min, cooled on ice and then centrifuged at 1500 × g for 10 min, 1 ml of supernatant was filtered through a 0.45 μm filters before HPLC analysis. A Waters Alliance 2695 Separations Module (Waters, Milford, USA) with a Zorbax Eclipse XDB-C18 column (4.6 × 250-mm, 5 μm) was used. The mixture was chromatographed on a Waters Altek 486 UV-Vis detector. The samples were detected at 254 nm, with a flow rate of 1.0 ml min⁻¹; (+) and (−) gossypol-acetic acid (Sigma: G4382) was used as a standard.

2.4. Histological analysis

For histological evaluation, fish were dissected to remove the intestine tissues and fixed with 4% paraformaldehyde (Morán et al., 2013). Tissues were dehydrated in a graded ethanol series, equilibrated in xylene and embedded in paraffin according to standard histological techniques (Zhang et al., 2018). The tissues were further dissected into 5-μm sections, mounted on slides, and stained with haematoxylin and eosin (H&E) (Betancor et al., 2013). The morphological structures of these tissues were examined by a Nikon TS100 light microscope (Nikon corporation, Tokyo, Japan).

2.5. FAA analysis

The FAA contents in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of fish were analysed by an automated AA analyser (L-8900, Hitachi) with a lithium high-performance column (Mente et al., 2003). Briefly, intestine samples (400 mg) were homogenised in 1.2 ml of 10% sulfosalicylic acid solution. After centrifugation at 12, 000 rpm for 10 min, 1 ml of supernatant was filtered through 0.22-μm filters for FAA measurement.

2.6. Quantitative real-time PCR (qRT-PCR)

In this study, the quantitative real-time PCR was similar to the previously described in another study conducted in our laboratory (Jiang et al., 2015). Briefly, the total RNAs were extracted from the PI, MI and DI tissues using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer’s instructions followed by DNase I treatment. The RNA quantity and quality were assessed by agarose gel (1%) electrophoresis and spectrophotometric analysis (A260:280 nm ratio). Subsequently, cDNA was synthesized using a PrimeScript™ RT reagent kit (TaKaRa) according to the manufacturer’s protocols. The quantitative real-time PCR (qPCR) reactions were performed in triplicate on a GFX96TM Real Time PCR Detection System (Bio-Rad, Hercules, CA) using a SYBR® Green I Supermix (TaKaRa) based on the manufacturer’s protocol. For qPCR, 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 used as a reference gene to normalize cDNA.

### Table 2: Real-time PCR primer sequences.

<table>
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<th>Protein name</th>
<th>Gene name</th>
<th>Primer sequence forward (5′→3′)</th>
<th>Primer sequence reverse (5′→3′)</th>
<th>Accession number</th>
<th>Temperature (°C)</th>
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loading. The target and housekeeping gene amplification efficiency were calculated according to the specific gene standard curves generated from 10-fold serial dilutions. The 2−ΔΔCT method was used to calculate the expression results after verifying that the primers amplified with an efficiency of approximately 100% as described by Wang et al. (2016b).

2.7. Western blotting

The processes for intestines protein extract preparation, antibodies and western blotting are the same as those described in our previous studies (Hu et al., 2015). Briefly, after extraction the PI, MI and DI protein concentrations were determined, using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (40 μg protein per lane) were separated by SDS-PAGE and transferred to 0.45 μm PVDF membrane for western analysis. The membrane was blocked for 1 h at room temperature (RT) and then incubated with primary antibody overnight at 4 °C. We used the same anti-total TOR, p-TOR Ser2448, and β-actin antibodies as those in our previous studies (Dong et al., 2017). The western bands were quantified using NIH Image 1.63 software.

2.8. Statistical analysis

The data of initial body weight (IBW), final body weight (FBW) and feed intake (FI) were used to calculate the percent weight gain (PWG), feed efficiency (FE), intestinal somatic index (ISI) and intestinal length index (ILI) according to Li et al. (2015).

\[
PWG = 100 \times \left( \frac{FBW \ (g \ fish^{-1}) - IBW \ (g \ fish^{-1})}{IBW \ (g \ fish^{-1})} \right)
\]

\[
FE = 100 \times \left( \frac{FBW \ (g \ fish^{-1}) - IBW \ (g \ fish^{-1})}{FI \ (g \ fish^{-1})} \right)
\]

\[
ISI = 100 \times \left( \frac{1}{wet \ intestine \ weight \ (g)/wet \ body \ weight \ (g)} \right)
\]

\[
ILI = 100 \times \left( \frac{intestine \ length \ (cm)/total \ body \ length \ (cm)} \right)
\]

The general linear models (GLM) procedure of SAS software (SAS Institute Inc., 2006) was used to determine treatment effects, and considered significant when P < 0.05. Orthogonal polynomial contrasts were used to test linear and quadratic effects of gossypol. Abscissa is the logarithmic value of the level of variation (Log gossypol levels), and the ordinate is the statistical value of the relevant parameter, which were used for regression analysis according to Tomonaga (2008). The two-slope broken line model (Yang et al., 2002) was used to evaluate the safe upper limit of gossypol in the diets of young grass carp growth.

3. Results

3.1. Effects of gossypol on the growth performances of fish

The effect of dietary gossypol on the growth performances parameters of young grass carp are shown in Table 3. At the start of the experiment, there were no significant differences between the means and range of young grass carp initial body weights for the six diets (P > 0.05). Increasing the level of dietary gossypol resulted in a linear (P < 0.05) and quadratic decrease (P < 0.05) in FBW, PWG, FI and FE of fish. In addition, ISI and ILI were linearly decreased (P < 0.05) by increasing dietary gossypol concentration.

3.2. Dietary gossypol accumulations in the three intestinal segments of fish

The gossypol accumulation in the PI, MI, and DI of young grass carp is shown in Table 4. In the three intestinal segments of fish, gossypol content was not detected in the control group. The (−), (+) and total gossypol contents were linearly increased (P < 0.05) in the three intestinal segments by increasing dietary gossypol levels.

3.3. Histopathological examination

The histological results showed that gossypol induced the damage of the intestine in young grass carp. In the PI, the intestinal morphology of fish was found to be normal when dietary gossypol levels were lower than 243.94 mg kg−1 diet. However, the minor necrosis in the mucosal layer of fish was observed in the group fed with gossypol of 363.89 mg kg−1 diet (Fig. 1 PI DI). Moreover, the blood capillary hyperaemia, the nuclear migration and the goblet cell hyperplasia were found in the group fed with gossypol of 759.93 mg kg−1 diet (Fig. 1 PI E), and the enterocyte nuclear migration and necrosis in the mucosal layer were observed in the group fed with 1162.06 mg gossypol kg−1 diet (Fig. 1 MI C and E). The nuclear migration was observed in the MI of fish fed with 363.89, 759.93 and 11,642.06 mg gossypol kg−1 diet (Fig. 1 MI D, E and F). Moreover, the goblet cell hyperplasia in the intestinal folds and the necrosis in the mucosal layer were observed in the mid intestine of fish fed with 759.93 and 1162.06 mg gossypol kg−1 diet (Fig. 1 MI E and F), respectively. In the DI, the nuclear migration was observed in fish fed with 243.94, 363.89, 759.93 and 1162.06 mg gossypol kg−1 diet (Fig. 1 DI E), respectively.

3.4. Effects of different dietary levels of gossypol on the FAA profile of various intestinal segments of young grass carp

The effects of dietary gossypol on the intestinal FAA parameters of young grass carp are shown in Table 5. In the PI, the contents of methionine (Met), tryptophan (Trp), valine (Val), phenylalanine (Phe), aspartic acid (Asp), glycine (Gly), cysteine (Cys), threonine (Thr), isoleucine (Ile), arginine (Arg), proline (Pro), serine (Ser), tyrosine (Tyr) and tryptophan (Trp) in the mid intestine of young grass carp showed no significant differences (P > 0.05) among different dietary gossypol levels. In the MI, the contents of Lys, Thr, Ile, Val, His, Phe, Asp, Pro, Gly, Pro, Ser, Tau, Met, Arg, Tyr and Cys were linearly and quadratically (except Thr, Ile, Arg, Pro, Ser, Tyr and Tau) decreased (P < 0.05) by increasing dietary gossypol concentrations. However, the contents of tyrosine (Tyr), histidine (His), leucine (Leu), alanine (Ala) and glutamic acid (Glu) in the PI of young grass carp showed no significant differences (P > 0.05) among different dietary gossypol levels. In the MI, the contents of Lys, Trp, Thr, Ile, Val, His, Phe, Asp, Pro, Gly, Pro, Ser, Tau, Met, Arg, Ser, Tyr and Cys were linearly and quadratically (except Met, Arg, Ser, Tyr and Cys) decreased (P < 0.05) by increasing dietary gossypol levels. No differences occurred in the contents of Leu, Ala and Glu (P > 0.05) occurred among different dietary gossypol levels. In the DI, the contents of Trp, His and Tyr were linearly and quadratically decreased (P < 0.05) with increasing the dietary gossypol levels. Increasing dietary levels of gossypol linearly reduced (P < 0.05) the contents of Lys, Thr, Val, Phe, Gly and Ser. However, different dietary levels of gossypol did not affect (P > 0.05) the contents of Met, Ile, Leu, Arg, Ala, Asp, Glu, Pro, Cys and Tau in the DI of fish.

3.5. Relative mRNA levels of AA and peptide transporters, and related signalling molecules in the intestines of fish

The effect of dietary gossypol on AA and peptide transporters, and related signalling molecule gene expression in the PI, MI and DI of young grass carp are presented in Fig. 2. In the PI and MI, the mRNA levels of LC7A7, SLC7A6, SLC7A1, SLC1A5, SLC6A19b, SLC7A5, SLC7A8, SLC38A2, SLC1A2a, SLC1A3, SLC6A6 and PepT1 were linearly down-regulated (P < 0.05) with increasing dietary gossypol levels. In the DI, the mRNA levels of SLC7A6, SLC7A1, SLC1A5, SLC6A19b, SLC7A8 and PepT1 were linearly down-regulated (P < 0.05) by increasing the dietary gossypol levels. However, the dietary gossypol had no effect (P > 0.05) on the mRNA levels of SLC614 and SLC7A9 in the three intestinal segments, and SLC7A7, SLC7A5, SLC38A2, SLC1A2a, SLC1A3 and SLC6A6 in the DI of young grass carp. Compared with the
Regression analysis of growth performance indicator responded to dietary gossypol. 

control group, increasing levels of dietary gossypol linearly down-regulated (P < 0.05) the TOR and S6 K1 (rather than DI) mRNA levels in the three intestinal segments of fish. In addition, the mRNA levels of 4E-BP1 were linearly up-regulated (P < 0.05) in the three intestinal segments by increasing the dietary gossypol levels.

3.6. Effects of gossypol on the protein levels of TOR in the intestine of fish

As shown in Fig. 3, although similar quantities of proteins were loaded onto the gels, the p-TOR Ser2448 protein levels were lower in the three intestinal segments of fish in a dose-dependent manner. Compared with the control group, increasing levels of dietary gossypol linearly reduced (P < 0.05) the phosphorylation levels of TOR protein in the PI, MI and DI of fish.

4. Discussion

Cottonseed meal (CM) is an important plant protein source used in aquaculture (Zheng et al., 2012). However, the CM contains gossypol, an anti-nutritional factor, which could be toxic to aquatic animals (Robinson, 2006). Therefore, our study used gossypol-acetic acid to provide the anti-nutritional factor, which could be toxic to aquatic animals.

In this study, increasing the dietary gossypol levels by increasing the dietary gossypol increased the phosphorylation levels of TOR protein in the PI, MI and DI of fish.

Table 3
Growth performance and intestinal growth of young grass carp (Ctenopharyngodon idella) fed diets containing different levels of gossypol (mg kg⁻¹ diet) for 60 days. Regression analysis of growth performance indicator responded to dietary gossypol.

Table 4
Total and (+ and −) isomers of gossypol accumulation in the PI, MI and DI of young grass carp fed diets containing various levels of gossypol for 60 days. P-values indicate a significant linear or quadratic dose response relationship (P < 0.05).
Fig. 1. The histology of the PI, MI and DI (HE ×100) in young grass carp fed diets containing different levels of gossypol (mg kg⁻¹ diet). (A) Control (0). (B) 121.38 mg kg⁻¹ diet. (C) 243.94 mg kg⁻¹ diet. (D) 363.89 mg kg⁻¹ diet. (E) 759.93 mg kg⁻¹ diet. (F) 1162.06 mg kg⁻¹ diet. In each panel, N: necrosis; NM: nuclear migration; GH: goblet cell hyperplasia; B: blood capillary hyperemia.
higher gossypol caused blood capillary hyperaemia, goblet cell hyperplasia and displacement of enterocyte nuclei in the three intestinal segments, and the highest level of gossypol caused mucosal layer necrosis in the PI and MI of young grass carp (Fig. 1), suggesting that gossypol could damage the fish intestine. These negative influences were partly due to the accumulation of gossypol in the PI, MI and DI of fish, especially for the (−) gossypol. Gossypol is a mixture of two enantiomers, (+) and (−) gossypol, while the (−) gossypol enantiomer is the most biologically active form (Gadela et al., 2014). Current results displayed that the contents of gossypol (+) and (−) were significantly increased in the three intestinal segments of young grass carp (Table 4). In turbot, gossypol could accumulate in the liver and cause fibrosis (Bian et al., 2016). Liu et al. (2016) reported that, diets containing high levels of cottonseed meal could damage the mucosal folds of the intestine and reduce growth.
of the intestines of pre-adult grass carp. Thus, these results suggest that gossypol impaired the intestinal histological structure, which might be partially related to the (−) gossypol accumulation in the intestinal tissues of young grass carp.

All the data above indicated that gossypol decreased the growth and damaged the intestine of young grass carp. Meanwhile, these results support our speculation that gossypol might impact the intestinal structure of grass carp. Additionally, the growth performance of fish is closely related to the intestinal FAA absorption capacity (Dabrowski et al., 2010). Thus, we next investigated the effect of dietary gossypol on the intestinal FAA absorption capacity of fish.

Fig. 2. Relative expression of AATs and signalling molecules in the PI(A), MI(B) and DI(C) of young grass carp (Ctenopharyngodon idella) fed diets containing different levels of gossypol for 60 days. Values are presented as the mean ± S.E., n = 6. *P-values underlined with a solid line indicate a significant linear dose response relationship (P < 0.05).
4.2. Gossypol reduced the intestinal FAA absorption capacity referring to the AA and peptide transporters in fish

AAs are efficiently absorbed by epithelial cells along the digestive tract in animals (Webb, 1990). A study reported that the intestinal AA absorption capacity could be reflected by the intestinal FAA contents in Atlantic salmon (Berge et al., 2004). The present study observed that increasing the level of dietary gossypol could decrease the FAA contents (except Lys and His in the PI, and Met, Ile, Arg, Asp, Pro, Cys and Tau in the DI) in the three intestinal segments of fish, suggesting that gossypol suppressed the FAA absorptive capacity in the intestines of young grass carp.

The fact that dietary gossypol reduced the FAA contents in the intestine of young grass carp may be partly explained in two ways. First, dietary gossypol decreased the FAA contents in the three intestinal segments of fish, which might be related to the mRNA levels of AATs. As we know, intestinal AAs are mainly transported by their corresponding AATs in animals (shown in supplementary Table S1) (Bröer, 2008; Hyde et al., 2003; Zhang et al., 2013a). The current results displayed that increasing dietary levels of gossypol could down-regulate the FAA contents (except SLC7A7, SLC7A5, SLC38A2, SLC1A2a, SLC1A3 and SLC6A6 in the DI) in the three intestinal segments of fish, suggesting that gossypol suppressed the FAA absorptive capacity in the intestines of young grass carp.

Second, the dietary gossypol decreased the FAA contents in the intestines of young grass carp, which might be partially related to the PepT1 mRNA levels. It is well known that the intestinal PepT1 mediates electrogenic proton-coupled influx of dietary di- and tripeptides into epithelial cells (Daniel and Stelzl, 2016). Some of these small peptides could be further hydrolysed by peptidases at the surface of the epithelial cells to produce FAAs (Adibi, 2003; Shimizu, 2004). In this study, compared with the control group, the dietary gossypol down-regulated the mRNA levels of PepT1 in fish intestines. Further correlation analysis indicated that the FAA contents (except Leu, Ala and Glu) were positively correlated with the PepT1 mRNA levels in the intestines of young grass carp (supplementary Table S2). All the data above imply that gossypol decreased the intestinal FAA contents, which might be partially related to down-regulating the mRNA levels of AATs and PepT1.

Interestingly, our data showed that dietary gossypol had no effect on the contents of Ala, Leu and Glu in the three intestinal segments, and Lys and His in the PI (rather than MI and DI), as well as Arg, Met, Ile, Asp, Pro, Cys and Tau in the DI (rather than in the PI and MI) of young grass carp. The possible reasons for these differences were analysed as follows. First, the dietary gossypol had no effect on the contents of Ala and Leu in the three intestinal segments, and Lys and His in the PI of fish, which might be related to the SLC6A14 and SLC7A9 mRNA levels. Study revealed that in the intestines of animals, the Ala, Leu, Lys and His could be transported by the SLC6A14 and SLC7A9 (Bröer, 2008). Current results showed that gossypol did not affect the mRNA levels of SLC6A14 and SLC7A9 mRNA levels in the intestines of young grass carp. However, the Lys and His contents in the PI are inconsistent with the MI and DI remains unclear, which...
requires further investigation. Second, the dietary gossypol did not influence the contents of Glu in the three intestinal segments of fish, which might be due to the contents of Ala in the intestine. The Ala and α-ketoglutarate could be catalysed by alanine aminotransferase to form Glu (Schousboe et al., 2003). In this study, dietary gossypol had no effect on the Ala content in the three intestinal segments of fish, which supports our hypothesis. Finally, the dietary gossypol had no effect on the contents of Met, Ile, Pro, Cys, Arg, Asp, Glu and Tau in the DI of fish, which might be related to the mRNA levels of SLC7A7, SLC7A5, SLC38A2, SLC1A2a, SLC1A3 and SLC6A6 in the DI. It has been reported that the SLC7A5 and SLC38A2 are responsible for the neutral AA (such as Met, Ile, Pro and Cys) transportation, the SLC7A7 for the cationic AA (Such as Arg), the SLC1A2 and SLC1A3 for the anionic AA (Such as Asp and Glu), and the SLC6A6 for the Tau in the intestine of animals (Bröer, 2008). In this study, the dietary gossypol did not influence the mRNA levels of SLC7A7, SLC7A5, SLC38A2, SLC1A2a, SLC1A3 and SLC6A6 in the DI (rather than PI and MI) of fish, which supports our hypothesis.

We also found that dietary gossypol had no effect on the mRNA levels of SLC6A14 and SLC7A9 in the PI, MI and DI of young grass carp, which might be related to iron and INF-γ mRNA levels. The gossypol could bind with the iron in the bloodstream of broiler chickens, causing iron deficiency (Watkins et al., 1993). Guo et al. (2017) reported that iron deficiency had no influence on the mRNA levels of IFN-γ in head kidney, spleen and skin of young grass carp. In human colonic epithelial cells, IFN-γ could up-regulate the SLC7A9 and SLC6A14 mRNA levels (Bhutta et al., 2015). Hence, we speculated that dietary gossypol had no influence on the SLC7A9 and SLC6A14 mRNA levels in the intestine of young grass carp, which might be partially associated with the unchanged mRNA levels of IFN-γ. However, this hypothesis requires further investigation. In addition, gossypol did not alter the mRNA levels of SLC7A7, SLC7A5, SLC38A2, SLC1A2a, SLC1A3 and SLC6A6 in the DI of young grass carp, which might be associated with the contents of (−) gossypol in the DI of fish. A study revealed that the (−) gossypol was more cytotoxic than the (+) isomer (Joseph et al., 1986). The current results showed that the (−) gossypol contents in the DI was lower than that in the PI and MI of young grass carp. Interestingly, there are large differences among the intestinal absorption capacity of various AAs inhibited by gossypol, as well as the related mechanisms. Firstly, gossypol decreased the contents of neutral AAs (Trp, Thr, Val, Phe, Gly, Ser and Tyr) in the three intestinal segments of fish partly related to down-regulating the mRNA levels of neutral AA transporters (SLC6A19b, SLC7A5, SLC7A8, SLC1A5 and SLC38A2) and PepT1 in the intestine of fish. Secondly,

4.3. Gossypol suppressed the mRNA levels of AA and peptide transporters might be partially related to TOR signalling in different intestinal segments of fish

AATs and PepT1 are regulated by mTOR (Benner et al., 2011; Rosario et al., 2016), which could phosphorylate S6 K1 and inhibit 4E-BP1 in humans (Hay and Sonenberg, 2004). The current results showed that, increasing the level of dietary gossypol could down-regulate the mRNA levels of TOR and S6 K1 (rather than DI), and decrease the TOR phosphorylation levels, as well as up-regulate the 4E-BP1 mRNA levels in the three intestinal segments of young grass carp. Correlation analysis (supplementary Table S3) indicated that the mRNA levels of PepT1 and AATs (except SLC7A7, SLC7A5, SLC38A2, SLC1A2a, SLC1A3 and SLC6A6 in the DI) were positively correlated with TOR and S6 K1, respectively, and were negatively related to 4E-BP1 in the three intestinal segments. All the data above suggest that gossypol down-regulated a majority of AATs mRNA levels, which may be related to [TOR/(4E-BP1, S6 K1)] signalling in the intestines of fish.

Interestingly, the dietary gossypol had no significant effect on the S6 K1 mRNA level in the DI of young grass carp. The possible reasons for the diversity might be partially associated with the (−) gossypol contents in the DI. In human lung cancer cells, gossypol could activate AMP-activated protein kinase (AMPK) (Youl Kim et al., 2015), which might inhibit the S6 K1 (Liu et al., 2012a). The current results showed that the (−) gossypol content in the DI was lower than that in the PI and MI of fish. Thus, we presume that dietary gossypol had no effect on the S6 K1 mRNA level in the DI might be partially related to the lower contents of (−) gossypol in the DI of young grass carp. However, the underlying mechanism by which dietary gossypol cannot alter the S6 K1 gene expression in the DI of fish requires further investigation.

4.4. The safe upper limit of dietary gossypol for young grass carp growth

The current results indicated that dietary gossypol could decrease the growth performance of young grass carp. Thus, it is necessary to evaluate the safe upper limit of gossypol in the diet for young grass carp growth. As shown in Fig. 4, based on the two-slope broken line model analysis for growth performance (PWG and FE), the safe upper limit of dietary gossypol for young grass carp growth was estimated to be 182 and 179 mg kg⁻¹ diet.

5. Conclusions

In summary (Fig. 5), we found for the first time that dietary gossypol suppressed the intestinal AA absorption capacity to inhibit the growth of young grass carp. Interestingly, there are large differences among the intestinal absorption capacity of various AAs inhibited by gossypol, as well as the related mechanisms. Firstly, gossypol decreased the contents of neutral AAs (Trp, Thr, Val, Phe, Gly, Ser and Tyr) in the three intestinal segments of fish partly related to down-regulating the mRNA levels of neutral AA transporters (SLC6A19b, SLC7A5, SLC7A8, SLC1A5 and SLC38A2) and PepT1 in the intestine of fish. Secondly,
gossypol reduced the contents of neutral AAs (Met, Ile, Pro and Cys) and cationic AA (Arg) in the PI and MI of fish partly related to down-regulating the mRNA levels of neutral and cationic AATs (SLC7A7 and SLC7A6), cationic AAT (SLC7A1), neutral AATs (SLC6A19b, SLC7A5, SLC7A8, SLC1A5 and SLC38A2) and PepT1 in the PI and MI. Thirdly, gossypol inhibited the anionic AA (Asp) and Tau contents in the PI and MI of fish partly related to down-regulating the mRNA levels of anionic AATs (SLC1A2a, SLC1A3), cationic AAs (SLC7A1), neutral AAs (SLC7A5, SLC7A8, SLC1A5 and SLC38A2) and PepT1 in the PI and MI. Fourthly, gossypol suppressed the cationic AA (Lys and His) contents in the MI and DI in fish partly related to down-regulating the mRNA levels of neutral and cationic AA (SLC7A7) and neutral AAs (SLC7A5 and SLC38A2) in the MI, and neutral and cationic AAs (SLC7A6), cationic AAs (SLC7A1), neutral AAs (SLC7A8) and PepT1 in the MI and DI. Meanwhile, gossypol down-regulated the above AA and peptide transporters in the intestine of fish partly related to suppressing the TOR signalling pathway. In addition, gossypol had no influence on the contents of Ala, Leu, and Glu in the three intestinal segments of fish partly associated with the unchanged SLC6A14 and SLC7A9 mRNA levels. Finally, based on PWG and FE, the safe upper limit of gossypol in the diets for young grass carp growth was estimated to be 182 and 179 mg kg⁻¹ diet, respectively.

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Appendix A. Supplementary data

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