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To cite this article: Ji Shu Zhou, Rui Wen Li, Ya Qiu Lin, Jian Gang Wang, Yang Li, Yuan Hao Yang, Enric Gisbert, Hai Bo Yu & Hong Ji (2018) LCFA Uptake and FAT/CD36: molecular cloning, tissue expression and mRNA expression responses to dietary oil sources in grass carp (*Ctenopharyngodon idellus*), *Journal of Applied Animal Research*, 46:1, 572-582, DOI: [10.1080/09712119.2017.1361828](https://doi.org/10.1080/09712119.2017.1361828)

To link to this article: <https://doi.org/10.1080/09712119.2017.1361828>



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Published online: 10 Aug 2017.



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## LCFA Uptake and FAT/CD36: molecular cloning, tissue expression and mRNA expression responses to dietary oil sources in grass carp (*Ctenopharyngodon idellus*)\*

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### ABSTRACT

The fatty acid translocase (FAT/CD36) plays an important role in trans-membrane uptake of long-chain fatty acids (LCFA) and has been identified and described in many organisms, while little is known about this gene in freshwater fish, and especially in the grass carp (*Ctenopharyngodon idellus*). In this study, the uptake of LCFA in hepatocytes of *C. idellus* was firstly determined and then *FAT/CD36* gene was cloned; then the gene was sequenced and mRNA levels from different tissues and the effect of dietary oils on its expression were determined. Present data showed that trans-membrane absorption of LCFA was related to the type and concentration of fatty acids (FAs) and it could be inhibited by specific membrane uptake inhibitors. The *FAT/CD36* isolated from grass carp contained 1422 bp and the open reading frame was predicted to have 1403 bp (GenBank access number: KU361231.1). *FAT/CD36* was highly expressed in the brain and its lowest expression was in the gills. *FAT/CD36* expression in white muscle was up-regulated when fish were fed with groundnut oil, whereas it was down-regulated in fish fed fish and linseed oils, respectively. These results indicated n-3 PUFA might inhibit the *FAT/CD36* mRNA expression and therefore the oxidation of FAs.

**Abbreviations:** Docosahexaenoic acid: DHA; Fatty acid: FA; Fatty acid translocase: FAT/CD36; Fish oil: FO; eicosapentaenoic acid: EPA; Groundnut oil: GO; Linseed oil: LO; Peroxisome proliferator-activated nuclear receptor: PPARs; Polyunsaturated fatty acid: PUFA; Unsaturated long-chain fatty acids: LCFA; Olive oil: OO

### ARTICLE HISTORY

Received 15 February 2017  
Accepted 26 July 2017

### KEYWORDS



*FAT/CD36*; cloning; tissue expression; dietary oil sources; grass carp (*C. idellus*)

## 1. Introduction

Long-chain fatty acids (LCFAs) are nutrients associated with many important functions of the organism, as they (i) are a main fuel for cellular energy production and a reserve energy source, (ii) are part of phospholipids constituting biological membranes, (iii) may covalently be attached to specific proteins to regulate their function, and (iv) act in selected signal transduction events, (v) and (vi) act to modify gene expression. (Jump 2004; reviewed by Glatz and Luiken 2017). Initially, it was considered that LCFAs were taken up by cells through passive diffusion mechanisms (Kamp et al. 2003; Pownall and Hamilton 2003; Hamilton and Brunaldi 2007), while evidence has shown that LCFA uptake is also facilitated by a protein-mediated mechanism (Abumrad et al. 1999; Bonen et al. 2007; Guo et al. 2013). In this sense, the intracellular transport of free FAs in mammals is facilitated by specific low-molecular-weight and highly conserved cytoplasmic proteins that bind both long-chain FAs and other hydrophobic ligands. These

fatty acid binding proteins (FABPs) are tissue specific and those from the liver, intestine, adipose tissue, brain and heart have been characterized extensively in mammals (Glatz and Luiken 2017). Similar studies have been reported in different fish species (Tocher 2003).

The fatty acid translocase (FAT/CD36), now officially being designated as the scavenger receptor B2 (SR-B2) (Prabhudas et al. 2014), is one of the most important FA transporters associated with LCFA trans-membrane uptake as it has been reported in humans (Luiken et al. 2016) and different teleost fish species (Torstensen et al. 2009; Zhou et al. 2010; Fink et al. 2015). The molecular and functional characterization of this protein has been described in zebra fish (*Danio rerio*), common carp (*Cyprinus carpio*) (Fink et al. 2015) and grass carp (*Ctenopharyngodon idellus*) (Tian et al. 2017). *FAT/CD36*-mediated FA transport into the skeletal muscle was reported to be critically coupled with muscle fuel selection and FA oxidation (McFarlan et al. 2012; Kim and Lim 2016), probably indicating that an increase in

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*FAT/CD36* stimulated fat lipolysis and oxidation (Zhou et al. 2012). In mammals, *FAT/CD36* (*SR-B2*) has been described to be implicated in lipid and FA disorders such as in the aetiology of obesity-induced or high fat diet-induced ectopic lipid accumulation and insulin resistance (Glatz et al. 2010; Liu et al. 2016; Glatz and Luiken 2017).

The grass carp is a herbivorous fish species and one of the most important freshwater carps cultured in China (Guo et al. 2015), as well as in other countries like Bangladesh, the Islamic Republic of Iran, the Lao People's Democratic Republic, Myanmar and the Russian Federation among others (FAO 2017). However, one of the main problems related with the culture of this species is the accumulation of excessive fat intra-peritoneally and in the hepatopancreas tissue, which affects the health and quality of fish fed diets with high lipid levels (Guo et al. 2015). Regardless of the fact that the dietary lipid levels can be easily managed by aquafeed manufacturers, how LCFAs are taken up by the hepatocyte and whether the excessive fat accumulation may be correlated with an excessive uptake process or excessive amount of *FAT/CD36* are not yet completely clear. In this regard, grass carp are generally fed with many types of dietary oil sources, depending on the cost of the feed, without any detrimental effect on growth performance (Cao et al. 1996). The inclusion of highly unsaturated fatty acids (HUFAs), including arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inclusion, was reported to suppress lipid accumulation in this species (Tian et al. 2014; Lei et al. 2016); thus, authors aimed to test whether the excessive fat accumulation normally found in grass carp could be suppressed by dietary oils through the regulation of *FAT/CD36* expression.

The molecular dynamics of LCFAs' uptake and of LCFAs on FA absorption and their impact on lipid accumulation was studied in *C. idellus*. For this purpose, the uptake of LCFAs in the hepatic tissue through the activity of *FAT/CD36*, as well as the cloning, specific-tissue expression and mRNA expression of *FAT/CD36* in response to different dietary oil sources were analysed in this study.

## 2. Materials and methods

### 2.1. Trans-membrane uptake of LCFA in hepatocyte of grass carp

#### 2.1.1. Preparation of hepatocytes

Healthy *C. idellus* (body weight, BW = 460 ± 100 g) were purchased from Kang Le fish market (Yangling, Shanxi Province, China) and transported to the laboratory. Once there, fish were sedated with 0.1 g L<sup>-1</sup> metacain (MS222, Sigma-Aldrich), and the hepatopancreas (2–3 g) removed. The protocol described by Yu et al. (2003) was followed for obtaining primary cell cultures. In particular, small pieces of liver were washed in a PBS solution three times, then the tissues were digested with 20 mL PBS buffer (8 g NaCl L<sup>-1</sup>, 0.2 g KCl L<sup>-1</sup>, 1.44 g Na<sub>2</sub>HPO<sub>4</sub> L<sup>-1</sup>, 0.24 g KH<sub>2</sub>PO<sub>4</sub> L<sup>-1</sup>) containing collagenase (0.1 mg mL<sup>-1</sup>). Then, the digested hepatopancreatic tissue was gently filtered through a 100 µm nylon gauze twice; cells were then collected by centrifugation (50 g for 5 min at 4°C, three times) and washed after each centrifugation. Finally, cells were re-suspended in 10 mL complete dulbecco's modified

eagle medium (DMEM) medium. The cell viability was routinely determined by the trypan blue excluded method and cells with viability values >85% were considered for further use. Cells were diluted into a density of 5–8 × 10<sup>5</sup> cells mL<sup>-1</sup> and 100 µL cell solution were put into each well of a 96-well plate (BD Falcon 96-well Flat-bottom ELISA PLATES from BD Biosciences-Discov-ery Labware) and cultured at 20°C for 8 h before *in vitro* assays.

#### 2.1.2. LCFAs' uptake-time course study by QBT kit

Firstly, working solutions of the QBT kit (QBT Fatty Acid uptake Assay Kit, Molecular Devices, Inc.) were prepared and then 100 µL of the working solution was added into each well containing hepatocytes and incubated for 5, 10, 15, 25, 35, 45 min, and the relative fluorescence units (RFUs; λ = 515 nm) were read by micro-plate reader (TECAN M200pro, Switzerland) with 515 nm exciting light. Figure 1 shows two images of hepatocytes' primary cell cultures incubating with and without QBT.

#### 2.1.3. LCFA uptake depending on oleic acid (OA) incubating time and concentration

The aim of the present experiment was to measure the LCFA uptake depending on OA incubating time and concentration in order to find the proper time and OA concentration to show specificity of the LCFA uptake process in *C. idellus*. A volume of 75 µM OA, dissolved into 0.05% dimethyl sulfoxide (DMSO)-complete DMEM medium, was incubated for 0, 0.5, 1, 2 and 4 h as described by Liao et al. (2005); then, 0.1 mL QBT working solution was added into each well for 30 s and the RFUs were read by micro-plate reader, as previously indicated. Each condition (incubation time) had six methodological replicates.

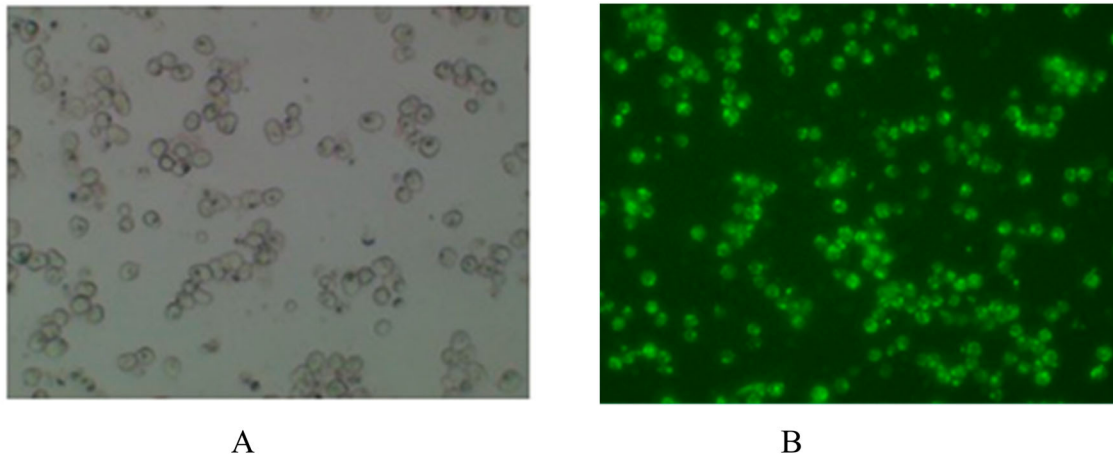
In order to evaluate the optimal OA concentration, 0, 37.5, 75, 150, 300 and 600 µM OA, previously dissolved into 0.05% DMSO-complete DMEM medium, respectively, were incubated with cells for 1 h (six replicates per OA concentration). Then, 0.1 mL QBT working solution was added into each well and the RFUs determined after 30 s.

#### 2.1.4. LCFA uptake

Five different LCFAs (OA, LA (18:2n-6), ALA (18:3n-3), EPA and DHA) were used for testing their uptake in primary hepatocytes' cell culture conditions. For this purpose, 75 µM of OA, LA, ALA, EPA and DHA (Fluka Chemicals, Sigma, USA) were initially dissolved into 0.05% DMSO-complete DMEM medium and incubated with cells for 1 h (six replications per LCFA). Then, 0.1 mL QBT working solution was added into each well and fluorescence read after 30 s as previously described. Cells without LCFA incubation were used as control groups.

#### 2.1.5. Inhibition of FA uptake

Hepatocytes were pre-incubated with specific FA uptake inhibitors according to Zhou et al. (2010): 250 µM phloretin, 250 µM 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid, disodium salt (DIDS), 200 µM Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SSM) and 10 mM cyclodextrin (Fluka Chemicals) were dissolved in 0.05% DMSO-complete DMEM medium for 0.5 h. Then, cells were incubated with QBT working solution kit and the RFUs read as previously described. Cells without the specific inhibitors were used as control groups. Each treatment had six methodological replicates.



**Figure 1.** The hepatocytes incubating with (A) and without (B) QBT, observed in the microscope ( $\times 10$ ).

## 2.2. Cloning and sequence analysis of FAT/CD36 gene in *C. idellus*

Six *C. idellus* (BW =  $500 \pm 16$  g) were collected from the local fish supermarket of Cheng Du (Si Chuan Province, China) and were gently anesthetized with metacain ( $0.1 \text{ g L}^{-1}$ ). The hepatopancreas was removed and total RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was quantified using a Gene-Quant spectrophotometer (Amersham Biosciences) and purity established by the absorbance ratio 260/280 nm. The integrity of the RNA was examined by gel electrophoresis. The cDNA was synthesized by Revert Aid First Strand cDNA Synthesis Kit (Fermentas Life Science, Hanover, MD, USA). The 25  $\mu\text{L}$  reaction volume was composed of  $2 \times$  Long Taq PCR Master Mix 12.5  $\mu\text{L}$ , forward primer ( $10 \mu\text{mol L}^{-1}$ ) 1.0  $\mu\text{L}$ , reverse primer ( $10 \mu\text{mol L}^{-1}$ ) 1.0  $\mu\text{L}$ , total RNA 1.0  $\mu\text{L}$  and 9.5  $\mu\text{L}$  ddH<sub>2</sub>O. Real-time qPCR was performed using a fluorescence temperature cycler (Bio-Rad, Hercules, CA, USA) and the qPCR programme started at 95°C for 4 min to pre-denature the cDNA, then it was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 1 min with a final extension at 72°C for 10 min. A final dissociation reaction (melting curve) was performed (95°C for 15 s, 60°C for 20 s, 95°C for 15 s and 60°C for 15 s) to evaluate the specificity of the amplicon. The PCR fragments were separated by 1% agarose gel electrophoresis, and then purified for cloning and subsequent sequencing. The sequences of FAT/CD36 gene primers were referred to that of common carp (*Cyprinus carpio*), whose GenBank accession number was KM 030422 (Table 1, Fink et al. 2015).

Fragments of interest were cloned into pMD19-T vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* DH5a. For each fragment, five clones were selected and sequenced by Qing Ke Zhi Xin Biotechnology Co. Ltd. (Cheng Du, Si Chuan Province, China). This sequence was used to predict the amino acid sequence, isoelectric point and molecular weight of FAT/CD36 by ExpAsy-Tools (<http://www.expasy.org/tools>). Putative protein domains were characterized by Interproscan (<http://www.ebi.ac.uk/interpro/>). Signal peptides were predicted by SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al. 2011). The amino acid sequence homology was identified by BioEdit software version 5.0.6 (Hall 2001).

Phylogenetic analysis of FAT/CD36 among *C. idellus*, common carp (accession number: KM030422), zebra fish (NM\_001002363), *Homo sapiens* (KR710356) and *Macaca mulatta* (AY600441) has been used to study the evolutionary relationship by amino acid sequences extensively.

## 2.3. Analysis of FAT/CD36 mRNA levels in different tissues of *C. idellus* by qPCR

Different tissues, including the heart, liver, red and white muscle, intraperitoneal fat tissue, hepatopancreas, spleen, kidney, intestine and brain were sampled from six fish (BW =  $500 \pm 16$  g); then total RNA of these tissues were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with Rnase-free DNase in order to remove genomic DNA. RNA integrity and quality were determined by means of agarose gel electrophoresis and spectrophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, USA); then RNA (1  $\mu\text{g}$ ) was reverse-transcribed into cDNA by PrimeScript<sup>®</sup> RT reagent kit (TaKaRa, Dalian, China), respectively. The volume of amplification mixture for Real-Time PCR contained total RNA 1  $\mu\text{L}$ , SYBR<sup>®</sup> Premix Ex Taq TM ( $2 \times$ ) (TaKaRa, Dalian, China) 10  $\mu\text{L}$ , primers ( $10 \mu\text{mol/L}$ ) 0.5  $\mu\text{L}$  and ddH<sub>2</sub>O 8.5  $\mu\text{L}$ . The threshold cycle (CT) was analysed using the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen 2001). Real-time analysis was performed by a fluorescence temperature cycler (Bio-Rad, Hercules, CA, USA) according to the following parameters: pre-denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 54–63.5°C for 30 s and then extension at 72°C for 30 s. Primers for Real-Time PCR are given in Table 1.

## 2.4. Effect of different dietary oil sources on the relative expression of FAT/CD36

### 2.4.1. Preparation of experimental diets

Four different dietary oil sources, linseed oil (LO), groundnut oil (GO), olive oil (OO) and fish oil (FO), were added into a basal diet for *C. idellus*. Ingredient list and proximate composition of experimental diets are shown in Table 2. Feed ingredients were purchased from Huaqin Husbandry and Technology Co., Ltd. (Yangling, Shaanxi, China). Diets were prepared by mixing

**Table 1.** Primer sequence for molecular cloning and quantitative real-time PCR of *FAT/CD36* in grass carp (*C. idellus*). All sequences are presented as 5'-3'.

Names		Primer sequences (5'-3')	Annealing temperature (°C)	Use
FAT/CD36	F	ATGACCTGCTGTGATCTGAAAT	60	For cloning of cDNA
	R	CAAGCATCCCTCCCTTTATT		
FAT/CD36	F	GTGTTCAAGGGCTTCAATGA	60	For qPCR
	R	GTGGTAAAGGGGTTAAACAGTCATA		
$\beta$ -Actin	F	ATCCTCCGTCTGGACTTGG	55	Endogenous control
	R	TCCGTCAGGCAGCTCATAG		

all dry ingredients for 30 min; then four oils and sufficient distilled water were added respectively to form soft mixtures that were mechanically extruded to obtain pellets of ca. 2 mm in size. Pellets were air-dried at 25°C and stored in re-sealable plastic bags at -20°C until use.

Lipids from these 4 diets were extracted by homogenization using the chloroform:methanol (2:1 v/v) method as previously described (Folch et al. 1957). FAs were saponified and methylated using 12% boron trifluoride (BF<sub>3</sub>) in methanol and analysed by gas chromatography using the methods described previously by Lie and Lambertsen (1991). The gas chromatograph (HP model 6890) was equipped with a carbowax capillary column and a flame ionization detector, with a 50-m CP-sil 88 (Chromopack) fused silica capillary column (i.d., 0.32 mm). Injection and detection temperatures were 250°C, nitrogen was the carrier gas and the column temperature was programmed to rise from 100°C to 220°C at a rate of 25°C/min. The FAs were identified by retention time using standard mixtures of methyl esters (Nu-Chek-Prep) and quantified using Totalchrom software (version 6.2, Perkin Elmer). The FA composition of the diets is shown in Table 3.

**Table 2.** Composition and nutrient levels of the experimental diets (air-dry basis, %) for grass carp (*C. idellus*) used in the *in vivo* study.

Raw material	Fish oil (FO)	Olive oil (OO)	Groundnut oil (GO)	Linseed oil (LO)
<i>Ingredients</i>				
Fish meal	3	3	3	3
Soybean Meal	33.92	33.92	33.92	33.92
Rapeseed meal	19	19	19	19
Cottonseed meal	12	12	12	12
Wheat middling and red dog	15.96	15.96	15.96	15.96
Wheat meal	10	10	10	10
Linseed oil				3
Groundnut oil			3	
Olive oil		3		
Fish oil	3			
NaCl	0.3	0.3	0.3	0.3
Calcium dihydrogen phosphate	1.5	1.5	1.5	1.5
Mineral <sup>a</sup> and vitamin <sup>b</sup> mix	1.3	1.3	1.3	1.3
Envelope VC	0.02	0.02	0.02	0.02
Total	100	100	100	100
<i>Proximate composition<sup>c</sup></i>				
Crude protein	34.54	35.06	35.35	34.93
Crude fat	6.00	6.00	6.08	6.09
Moisture	8.11	7.91	7.94	8.50
Ash	6.34	6.49	6.60	6.36

<sup>a</sup>Minerals: Fe (iron sulphate) 140 mg/kg; Cu (copper sulphate) 2.5 mg/kg; Zn (zinc oxide) 65 mg/kg; Mn (manganese oxide) 19 mg/kg; Mg (magnesium sulphate) 230 mg/kg; Co (cobalt sulphate) 0.1 mg/kg; I (potassium iodine) 0.25 mg/kg; Se (sodium selenite) 0.2 mg/kg.

<sup>b</sup>Vitamins: vitamin A 4000 IU/kg, vitamin D<sub>3</sub> 800 IU/kg, vitamin E 50 IU/kg, vitamin B<sub>1</sub> 2.5 mg/kg, vitamin B<sub>2</sub> 9 mg/kg, vitamin B<sub>6</sub> 10 mg/kg, vitamin C 250 mg/kg, nicotinic acid 40 mg/kg, pantothenic acid 30 mg/kg, biotin 100 µg/kg, choline 1000 mg/kg.

<sup>c</sup>Results are means ± SD (n = 2).

## 2.4.2. Feeding and management of fish

A total of 80 juvenile grass carps (BW = 56.9 ± 4.2 g) were divided into 4 groups, 20 fish per group and were reared in 4 indoor tanks (112 cm × 34 cm × 54 cm) and fed with the above-mentioned experimental diets (feeding rate: 3%). Fish were fed by hand 3 times per day (8:30, 12:30 and 16:30). Water in each tank was aerated 24 h per day with airstones and 2/3 of the water in the tank was exchanged with fresh water every morning. The water temperature and dissolved oxygen were 22–25°C and 5–8 mg L<sup>-1</sup>, respectively. The nutritional trial lasted for 60 days. Fish BW was measured every 20 days in order to adjust feeding rates with grass carp somatic growth.

## 2.4.3. Sampling procedures and *FAT/CD36* expression in white muscle by qPCR

At the end of the feeding trial, fish were gently anesthetized with metacain (0.1 g L<sup>-1</sup>) and their final body weight (BWF) measured to the nearest 0.1 g. Then, eight fish per tank were slaughtered and their white muscles were sampled. Total RNA was extracted from white muscle and the expression of *FAT/CD36* in this tissues was determined by qPCR analysis as previously described.

## 2.5. Statistical analysis

Data are shown as mean ± standard deviation (SD). Statistical differences were analysed by one way ANOVA, followed by a

**Table 3.** FA composition of diets (% of total FA) used in the *in vivo* study with grass carp (*C. idellus*).

	Fish oil (FO)	Olive oil (OO)	Groundnut oil (GO)	Linseed oil (LO)
14:0	5.06	0.40	0.46	0.44
16:0	18.78	12.22	13.27	9.54
18:0	3.13	3.23	3.59	3.40
Sum sat <sup>a</sup>	26.96	15.85	17.32	13.37
16:1 n-7	5.52	1.09	0.75	0.71
18:1 n-9	18.14	55.62	33.27	23.84
20:1 n-9	4.18	0.94	1.29	1.07
MUFA <sup>b</sup>	27.85	57.65	35.31	25.62
18:2 n-6	19.09	19.87	38.53	34.36
20:4 n-6	nd <sup>c</sup>	nd	nd	nd
20:3 n-6	0.57	nd	nd	nd
Total n-6 HUFA	19.66	19.87	38.53	34.36
18:3 n-3	4.27	3.06	2.58	22.75
20:5 n-3	6.87	0.79	2.31	0.94
22:6 n-3	8.48	0.48	0.55	0.46
Total n-3 HUFA	19.62	4.33	5.44	24.15
n-3/n-6	1.00	0.22	0.14	0.70

<sup>a</sup>SFA, saturated fatty acids.

<sup>b</sup>MUFA, monounsaturated fatty acids.

<sup>c</sup>nd: not detected.

Tukey's *post-hoc* test when statistically significant differences were found between experimental groups. All data were checked for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test) prior to the ANOVA test. All statistical analyses were performed using SPSS13.0 for Windows Software (SPSS, Chicago, IL, USA). Results were considered significant at  $P < .05$ .

### 3. Results

#### 3.1. Trans-membrane uptake of LCFA in grass carp hepatocytes

##### 3.1.1. LCFA uptake-time course study

LCFAs' uptake-time course study showed that the QBT was progressively taken up by hepatocytes between 5 and 45 min, whereas it gradually reached a steady state (uptake saturation) after 20 min. The relationship between LCFA uptake and time was linear and represented by the following curve:  $y = 1724.2 \ln(x) + 16533$  ( $R^2 = 0.9065$ ), where  $x$  corresponds to the incubation and  $y$  the RFUs; (Figure 2). As the uptake of QBT increased very fast, the RFU determination using the QBT could be done within 5–15 min of incubation time.

##### 3.1.2. Establishing optimal LCFA uptake depending on experimental conditions

Uptake of OA by hepatocytes progressively increased with increasing OA concentration as it is shown in Figure 3 ( $P < .05$ ). Considering these results and the FA concentration in fish blood the concentration of 75  $\mu\text{M}$  was chosen for the following experiments. The OA uptake kinetics in hepatocytes is shown in Figure 4. Results indicated that LCFA uptake with incubating hepatocytes with 75  $\mu\text{M}$  OA was maximum during the first hour, whereas it decreased later on at 2 and 4 h post-incubation ( $P < .05$ ). Thus, incubation time was set up at 1 hour for further *in vitro* trials. LCFA uptake varied depending on the FA considered. The highest absorption rates (RFUs) were observed when hepatocytes were incubated with 18:3n-3 and 8:2n-6, followed by 18:1n-9 and 20:5n-3, whereas the lowest uptake was observed when hepatocytes were incubated with 22:6n-3 (Figure 5;  $P < .05$ ).

##### 3.1.3. Modulation of LCFAs' uptake by specific inhibitors

To clarify whether LCFAs' uptake into hepatocytes of grass carp was mediated by membrane proteins, the effects of several

membrane protein inhibitors (phloretin, DIDS, SSM and cyclodextrin) on FA uptake was studied (Figure 6). The exposure of hepatocytes to SSM and phloretin resulted in the highest inhibition of LCFAs' uptake ( $14.0 \pm 10.3\%$  and  $10.19 \pm 6.4\%$ , respectively) compared to the control group ( $P < .05$ ). DIDS showed lower inhibition levels ( $3.8 \pm 2.5\%$ ), whereas cyclodextrin showed no inhibition on LCFAs' uptake, since values of RFUs were similar in comparison to the control group ( $P > .05$ ).

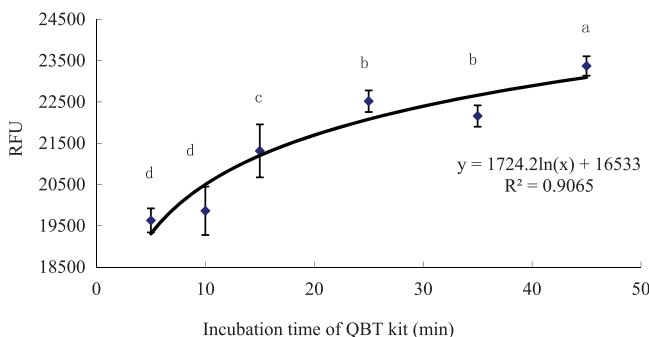
#### 3.2. Cloning and characterization of FAT/CD36 gene in *C. idellus*

The FAT/CD36 cDNA of *C. idellus*, being cloned and sequenced, was found to be composed of 1422 nucleotides with an open reading frame (ORF) of 1403 nucleotides. This ORF was postulated to encode a protein with 468 amino acids and a molecular weight of 52.47 kDa. This gene sequence was submitted to GenBank and the accession number was KU361231.1.

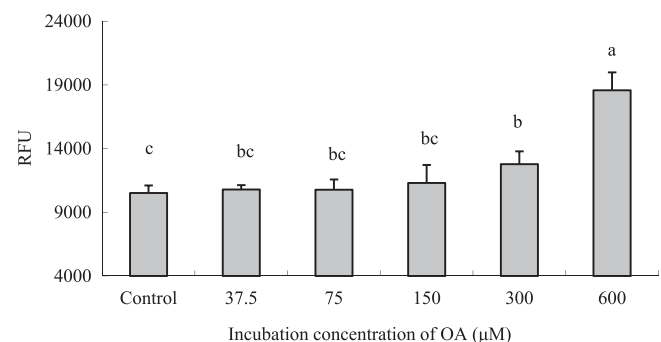
The multiple sequence alignments showed that the amino acid sequence of FAT/CD36 in *C. idellus* had a very high identity with that of other cyprinid species like *Cyprinus carpio* and *Danio rerio*, with similarity values of 82% and 76% respectively (Figure 7). By further analysis of the post-translational modifications, it was postulated that there were 6 serine phosphorylation sites, 7 threonine phosphorylation sites, 3 tyrosine phosphorylation sites, 2 oxygen-glycosylation sites and 7 nitrogen-glycosylation sites along the gene sequence (Figure 8).

The secondary protein structure of FAT/CD36 suggested that this protein contains 139 amino acids (blue line domain), 109 amino acids (red line domain) and 220 amino acids (purple line domain), occupying 29.7%, 23.3% and 47.0% of total amino acids, respectively, which may be attributed to be  $\alpha$ -helices,  $\beta$ -brands and random coils, respectively (Figure 9). Two trans-membrane domains were postulated to be located in this protein without a messenger peptide structure (Figure 10).

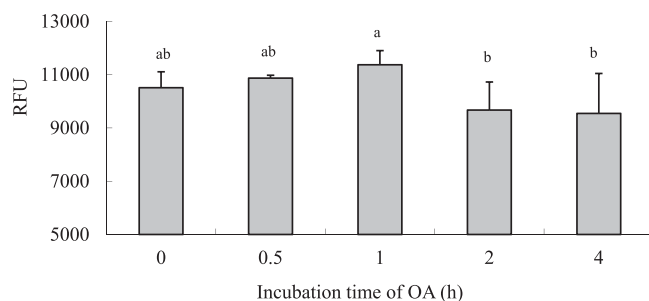
The phylogenetic analysis based on the protein sequences of FAT/CD36 showed that the genetic relationship between *C. idellus*, *C. carpio* and *D. rerio* was very similar (99% similarity; Figure 11). However, the genetic relationship between grass carp and higher vertebrates like *Homo sapiens* or *Macaca mulatta* was very different, where the homology of ITS was less than 87% comparing their ITS sequences of FAT/CD36 (Figure 12).



**Figure 2.** Time-course uptake of LCFAs in hepatocytes determined by means of the QBT kit from grass carp (*C. idellus*).



**Figure 3.** LCFA uptake depending on the OA concentration in hepatocytes from grass carp (*C. idellus*).



**Figure 4.** Effects of incubating time on LCFA uptake in hepatocytes from grass carp (*C. idellus*).

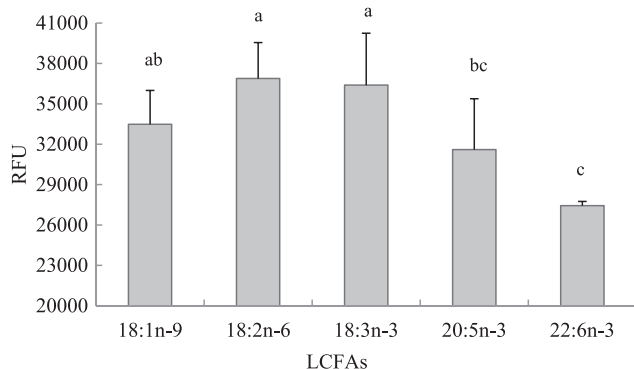
### 3.3. Gene expression patterns of FAT/CD36 in *C. idellus*

Among the 10 different tissues where *FAT/CD36* was expressed, the brain and hepatopancreas were the organs where this gene was mainly expressed, whereas the lowest expression values were found in the gills (Figure 13;  $P < .05$ ). Diets containing different types of oils statistically affected the expression of *FAT/CD36* in the white muscle of *C. idellus* (Figure 13;  $P < .05$ ). The highest mRNA levels of *FAT/CD36* in the white muscle were found in grass carp fed the diet containing GO, followed by those measured in fish fed the diet containing OO, whereas fish fed the LO and FO diets showed the lowest levels in *FAT/CD36* gene expression.

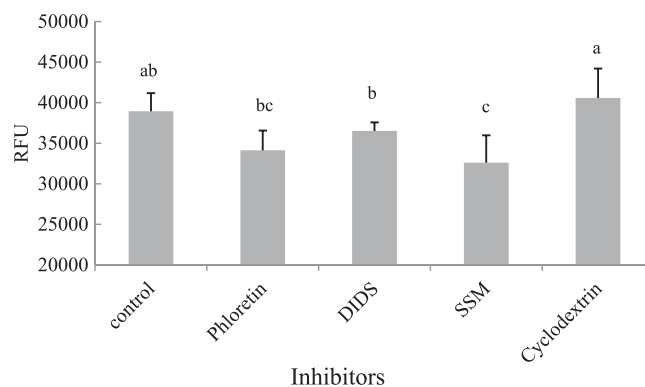
## 4. Discussion

### 4.1. LCFA trans-membrane uptake in the hepatocytes of *C. idellus*

Hepatocytes of grass carp are an established *in vitro* system that has been used in a range of lipid metabolic studies (Lu et al. 2012). The present study is the first one focused on trying to elucidate the mechanisms underlying the LCFAs uptake in *C. idellus* hepatocytes. Traditionally, the experimental approaches used for studying the FA uptake in cells and their metabolism were based on the use of a  $^{14}\text{C}$  labelled FA and then measuring the intracellular levels of radioactivity (Torstensen and Stubhaug 2004; Stubhaug et al. 2005; Vegusdal et al. 2005; Zhou et al. 2010). The quantification of FA uptake using fluorescence by means of the QBT kit, where



**Figure 5.** Effects of LCFA uptake depending on the type of FA in hepatocytes from grass carp (*C. idellus*).



**Figure 6.** Effects of non-specific inhibitors on LCFA uptake in hepatocytes from grass carp (*C. idellus*).

QBT is a kind of LCFA being fluorescently labelled, is a quite recent and environmental-friendly procedure and a safer protocol from the point of view of the user (Liao et al. 2005). In the present study, QBT was efficiently taken up by grass carp hepatocytes of grass carp, being highest during the first 45 min post LCFA incubation, whereas this uptake occurred through a saturable transport mechanism (Sorrentino et al. 1989), which was in line with the results reported in other Teleost species like in rainbow trout (Richards et al. 2003), Atlantic salmon (Zhou et al. 2010) as well as in different tissues from higher vertebrates [i.e. skeletal muscle (Turcotte et al. 1991), mammalian adipocytes (Abumrad et al. 1981; Schwieterman et al. 1988), cardiac myocytes (Sorrentino et al. 1988) and hepatocytes (Stremmel and Berk 1986)]. Under current experimental conditions, LCFA uptake was stimulated in hepatocytes of *C. idellus* when incubated 18:2n-6 and 18:3n-3, while it was suppressed in 22:6n-3. These results differed with those already reported in Atlantic salmon (Stubhaug et al. 2005; Zhou et al. 2010), where 20:5n-3 and 22:6n-3 were predominantly taken up by hepatocytes in comparison to 18-C FAs. Such differences between both studies may be attributed to the different essential FA requirements between both studies. The grass carp is a warm water fish species, whereas the main essential FAs for this species are 18:2n-6 and 18:3n-3 (Takeuchi et al. 1991) while the Atlantic salmon is mainly a marine fish species with important requirements in n-3 HUFA, especially EPA and DHA (NRC 1993). In general terms, the membrane proteins, FABPm, FAT/CD36 and fatty acid transport protein (FATP), are responsible for the cellular uptake of LCFAs (Frohnert and Bernlohr 2000) and phloretin was reported to be a potent non-specific inhibitor of membrane transport proteins (Lefevre 1975; Andersen et al. 1978). This meant that the buffer containing this inhibitor would suppress the efflux or influx of FAs across the cell membrane by blocking the activity of these proteins (Abumrad et al. 1981). In this study, LCFAs' uptake was differently inhibited by the five inhibitors tested, although any of the tested inhibitors resulted in a complete inhibition of LCFA uptake across the hepatocytes' membrane. In particular, hepatocytes exposed to phloretin showed  $10.1 \pm 6.4\%$  inhibition compared with the control, whereas DIDS and SSM inhibited LCFA transport by  $3.8 \pm 2.5\%$  and  $13.9 \pm 10.3\%$ , respectively. These values were lower than those reported

Ctenopharyngodon_idellus	MTCCDLKCALITGAVLGALIAVLGGILIPVGNMFIENTVHKETVLES	60
Danio_rerio	MTCCDQRCALITGAVLGALIALLLGGILIPVGDMLIKNTVHKETVLENGTLAFDWTWTSVDI	60
Cyprinus_carpio	MTCCDLKCGLITGTVLGALIALLLGVILIPVGNMFIENTVHTETVLENGTLAFDWTWTSVDI	60
Consensus	mtccd c litg vlgalia lg ilipvg m i ntvh etvle gtlafdtwtsvd	
Ctenopharyngodon_idellus	AIYRQFWLFDVQNPDEIVLSOGAKPVLVQKGPYTYRTRFIPKTNITFNDNFTVSVFLPAGA	120
Danio_rerio	AMYRQFWIFNVENPDKVLSEGSKPVLVQKGPYTYRVRVYIPKTNITFNDNFTVSVFLPAGA	120
Cyprinus_carpio	PMYRQFWLFDVQNPDEIVLSOGAKPVLVQKGPYTYRTRFIPKTNITFNDNFTVSVFLPAGA	120
Consensus	yrfqw f v npd s g kplvlvqkgytyr r ipktnitfndn t sfvlp ga	
Ctenopharyngodon_idellus	IFEPGMSVGTEDIFTSLNLAVAGTYGLLDHKLANLIFQRYDATLFOQNRVTKELLWGYRD	180
Danio_rerio	TFEFSMSVGSSEDEVFTSLNLAVAGVYRLIGPKLADWLIRSSGSSLFQNRVTKELLWGYKD	180
Cyprinus_carpio	IFEFSMSVGTEDIFTSLNLAVAGVYRLDHRILANLLIQRSNSTLFOQNRVTKELLWGYKD	180
Consensus	fep msvg eed ftslnlavag y l la lfq rvtkellwgy d	
Ctenopharyngodon_idellus	PMLNSMVGVFYFPYNNITDGPYTVFTGKDDIKKVAIIERWQGETSLSYWNDFYCNKINGTD	240
Danio_rerio	PMLNSLVCAFYPYNGITVDGPYTVFTGKDDINKVAIIERWQGETSVNYWNDSYCDKINGSD	240
Cyprinus_carpio	PMLGSTLGVFYPYNDITDGPYTVFTGKDDINKVAIIERWAGEPSSLSYWNDFYCNKINGTD	240
Consensus	pml s g fypyn t dgpvtvftgkddi kva ierw ge s ywnd yc king d	
Ctenopharyngodon_idellus	GSSFHPFLDKKKEPLYFFSSDICRSISAKYEYRTVNLKGDVYRYMLFAEALASFAVNPNDQ	300
Danio_rerio	GSSFHPFLDKKKEPLYFFSEFDICRSISAEYEATVNLKGDVYRYLLPVDALASFVSNPNDM	300
Cyprinus_carpio	GSSEHPFLNKKETLFFSSDICRSISAEYEATVNLKGDVYRYMLPEALASEVENPNDQ	300
Consensus	gssfhpfl kk l ffs dicrsisa ye tv lkgidvryry lp alasp npdn	
Ctenopharyngodon_idellus	CYCTNTVITKNCTMAGLLDMTPCRGTPVFTSLPHFLYGTSDLVQAMTGLNPNFDEHSIFV	360
Danio_rerio	CYCTDHEITRNCTLAGLLDITSCRGTPVFTSLPHFLYASIELQGGVGMNPNLDEHSIFL	360
Cyprinus_carpio	CYCTDPVITRNCTTAGLLDLTACRGTPVFTSLPHFLYGSNDLHQGVIGLNPNDDEHSIFL	360
Consensus	cyct it nct aglld t c gtpvf slphfly l q g npn dehsif	
Ctenopharyngodon_idellus	DVEPITGFTLRFQAKRLQVNMMLHGPSADIVLNLKIKNYTTFPILWVNETAVLDDETAADIFK	420
Danio_rerio	DVEPITGFTLRFQSKRLQVNMMLYGPSDDIALNLKIKEHTIIPILWVNETAVLDDETAADIFK	420
Cyprinus_carpio	DVEPITGFTLRFQAKRLQVNMMLYGPSDSIVLNLKIKDYTFPILWVNETAVLDDETAADIFK	420
Consensus	dvepitgftlrf krlq nm gps i lnkik t pilw netavlddeta fk	
Ctenopharyngodon_idellus	KELISRMDLLEGGFQIGLLTVGLIIFVSCITGLLLVCRKQEKSKHSFI	467
Danio_rerio	NELISRMDLLEGLQIGLLVTVGSAIFLGCMLGLIVVCSKPSKTNLS..	465
Cyprinus_carpio	KELIARMDLLEGIQIGLIVGGLVLFASCLIGLIVVCTKRSNNKKNLH	467
Consensus	eli rmd leg qigl g f c igl vc k	

**Figure 7.** Comparison of FAT/CD36 amino acids' homology in grass carp (*C. idellus*), common carp (*C. carpio*) and zebrafish (*D. rerio*). The line of consensus shows the conserved residues of FAT/CD36 among these fish and these identical amino acid residues are shown in white words with black background. The trans-membrane domain of FAT/CD36 is boxed. The alignment was generated using vector DNAMAN software.

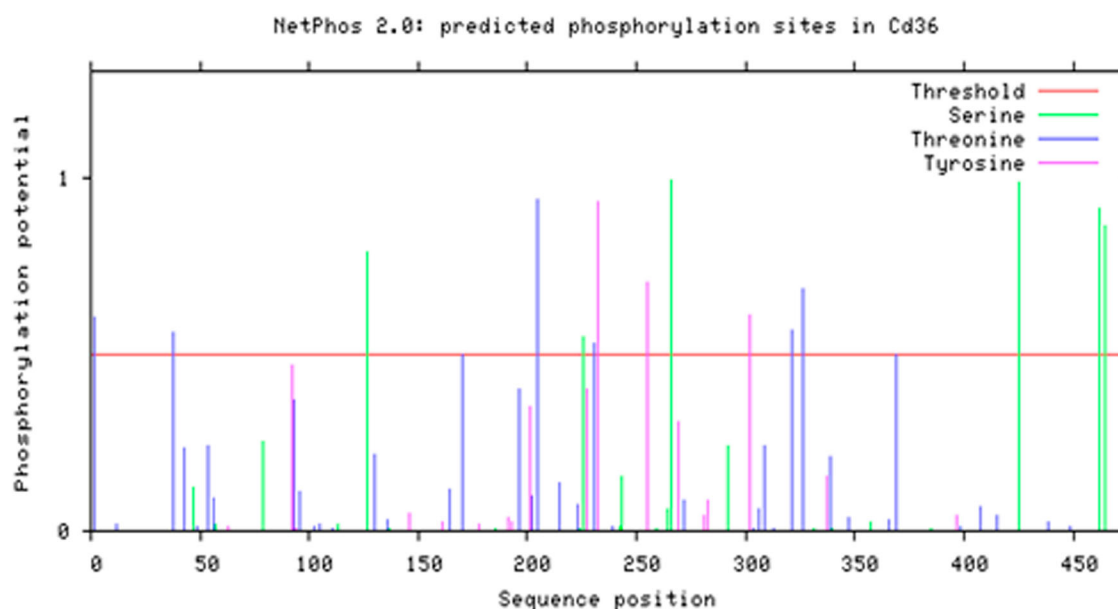
earlier in Atlantic salmon and rainbow trout, where LCFA uptake was inhibited by 28% in hepatocytes (Zhou et al. 2010) and up to 40% in muscle sarcolemma (Richards et al. 2003), respectively. In contrast, inhibition values were higher in higher vertebrates (rodents) and ranged from 60% to 9% in cardiac myocytes (DeGrella and Light 1980; Stremmel 1988; Luiken et al. 1997) and adipocytes (Abumrad et al. 1984; Harmon et al. 1991). These discrepancies between different studies may suggest that the mechanisms of LCFAs' uptake in the hepatocytes of *C. idellus* grass might be not so reliable on a protein-mediated transport compared to other fish and mammalian cell lines. In addition, the inhibitor of cyclodextrin was previously reported to disturb the lipid raft by lowering the cholesterol levels in the lipid raft (Ikonen 2001), and no QBT uptake inhibition by cyclodextrin was found in the present study, being in line with previous results in hepatocytes of Atlantic salmon (Zhou et al. 2010), probably indicating that lipid raft was not included in FA uptake in hepatocytes of grass carp or the contribution of lipid raft to FA uptake was little. More research is required

to explore the mechanisms of LCFAs' uptake by lipid raft in grass carp.

#### 4.2. Molecular cloning of FAT/CD36 in *C. idellus*

Characterization of FAT/CD36 in the hepatopancreas of grass carp showed that FAT/CD36 is an integral membrane glycoprotein with two trans-membrane domains, a large extracellular loop and two short intracellular tails. This topology is similar to the mammalian CD36 (Febbraio et al. 2001) as well as the CD36 described in common carp and zebrafish (Fink et al. 2015). Although the grass carp FAT/CD36 sequence seems relatively similar to that of mammals, common carp and zebrafish CD36, there were a number of intriguing differences among them. Contrary to the findings in the human, common carp and zebrafish CD36 proteins that they did not have O-glycosylation sites (Febbraio et al. 2001; Fink et al. 2015), there were two predicted O-glycosylation sites in FAT/CD36 of grass carp. However, the significance of these potential O-glycosylation sites in FAT/CD36 in fish is not yet known (Fink et al. 2015).





**Figure 8.** Phosphorylation sites predicted in FAT/CD36 protein.

Grass carp FAT/CD36 was predicted to be a 52.47 kDa protein, which was similar to the molecular weight of this protein described in pigeons (52.7 kDa; Xie et al. 2012), while it had a lower molecular weight than in rats (88 kDa; Abumrad et al. 1993; Baillie et al. 1996). These differences may be attributed to species-specific differences rather than to their functional properties.

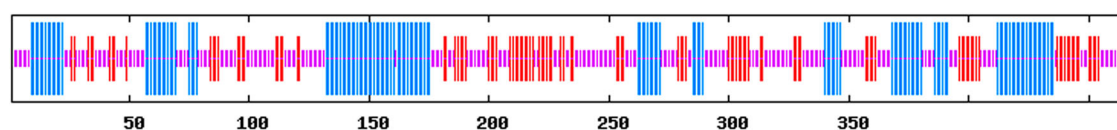
#### 4.3. Tissue distribution of FAT/CD36 measured by qPCR in *C. idellus*

The amount of FAT/CD36 mRNA in different tissues may be attributed to its role of facilitating FA transport in tissues with a high capacity for FA metabolism, which may fluctuate depending on developmental, hormonal and environmental conditions (Silverstein and Febbraio 2009). In grass carp, FAT/CD36 was expressed in all analysed tissues, which was in accordance with previous results in mammals where FAT/CD36 was expressed in the skeletal and smooth muscle, the intestinal epithelium, adipocytes, endothelial cells, platelets, dendritic cells, and monocytes and macrophages (Febbraio et al. 2001). Results from the qPCR analyses indicated that the expression of this gene in the brain and the hepatopancreas was the highest in comparison to the other studied tissues; results that were in accordance with previous results in common carp and zebrafish (Fink et al. 2015) by their higher requirements of FAs due to specific-tissular metabolism. The lowest FAT/CD36 gene expression was found in the spleen and gill,

regardless of their importance as tissues involved in the immune defence of the organism (Han et al. 2010). Similar results were recently reported by Tian et al. (2017), who showed that levels *ciCD36* mRNA were absent in the kidney of grass carp. In mammals, it has been reported that FAT/CD36 (*SR-B2*) expression in the liver was absent or very low (Febbraio et al. 1999), while in the present study FAT/CD36 expression was relatively high in the hepatopancreas of grass carp, probably indicating an important role of this protein in the cellular FA uptake in the hepatocytes of grass carp, which may be different from mammals.

#### 4.4. FAT/CD36 mRNA expression responses to different dietary oil sources in *C. idellus*

The effect of different dietary oil sources and/or FAs on their cellular uptake and accumulation has received a lot of attention during the last decades. In the hepatopancreas of Atlantic salmon, the expression of FAT/CD36 was not affected by vegetable or FOs (Zhou et al. 2014), while in mammals, unsaturated LCFAs significantly increased FAT/CD36 mRNA levels in macrophages and ventricular myocytes (van der Lee et al. 2000; Vallvé et al. 2002), the small intestine, preadipocytes and neonatal cardiomyocytes (Chen et al. 2001), mature adipose cells (Nisoli et al. 2000) and abdominal fat of hypertensive rats (Aguilera et al. 2006). Furthermore, Yang et al. (2007) showed that FAs with a chain length longer than eight carbons could effectively inhibit FAT/CD36 expression in adipocytes, but unsaturated



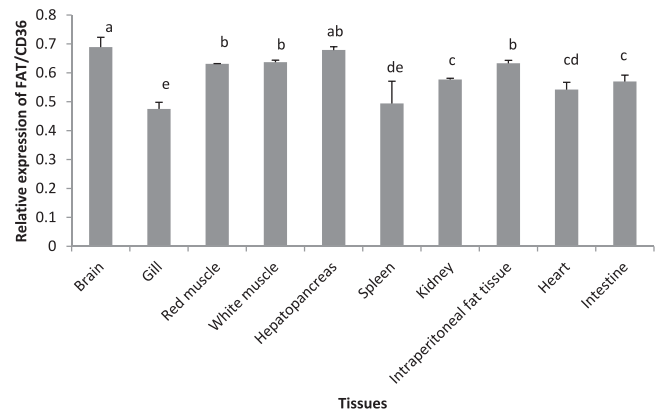
**Figure 9.** Secondary structure of FAT/CD36 protein is predicted. The domain of amino acids in blue line are  $\alpha$ -helices, the domain of amino acids in red line are  $\beta$ -brands and the amino acids left in purple line are random coils.



**Figure 10.** Three-dimensional model of FAT/CD36 shown by cartoon and surface models. This protein included many  $\alpha$ -helices,  $\beta$ -strands and random coils. It was generated by PHYRE2 Server.

LCFAs had no effect. Dietary oil sources, such as lard oil, FO or sunflower-seed oil, differently influenced *FAT/CD36* expression in the liver and muscle of rats (Feillet-Coudray et al. 2013) and in the mammary secretory tissue and mental adipose tissue of goats (Toral et al. 2013), whereas *FAT/CD36* expression in the hepatopancreas was not affected by diets containing lard oil diet or FO in grass carp (Tian et al. 2015). These previous studies give scarcely identical information on different long-chain unsaturated FAs affecting *FAT/CD36* expression.

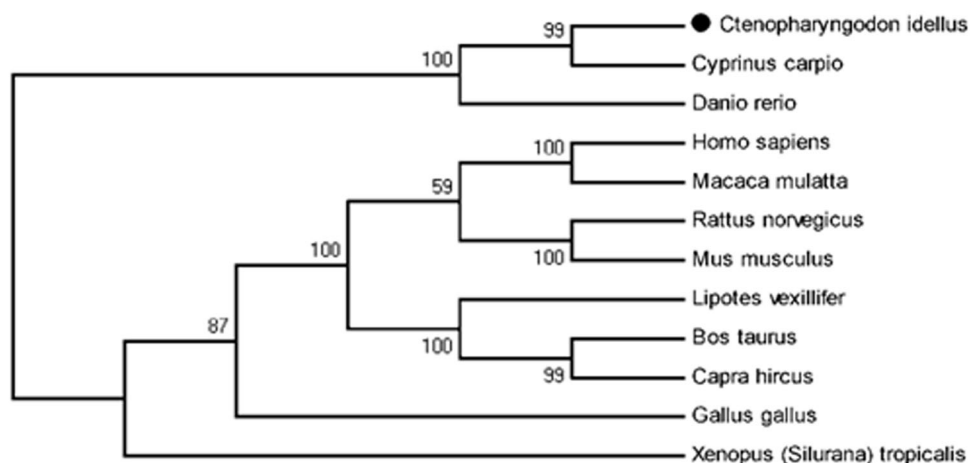
In the present study, the highest *FAT/CD36* expression in the white muscle was found in the GO group, a diet rich in 18:2n-6, whereas the lowest *FAT/CD36* expression levels were found in fish fed the LO and FO diets, which contained high contents



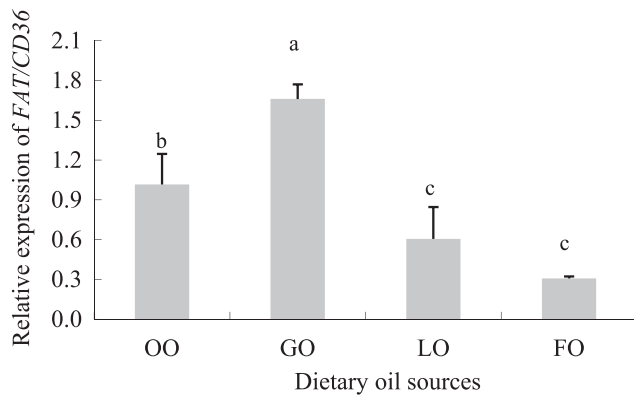
**Figure 12.** Expression of *FAT/CD36* in different tissues of grass carp (*C. idellus*) by real-time quantitative PCR analysis. Values are mean  $\pm$  SD ( $n = 3$ ). Different letters denote statistically significant differences among tissues ( $P < .05$ ).

of 18:3n-3, 20:5n-3 and 22:6n-3. These results indicated that n-3 PUFAs inhibited *FAT/CD36* expression in the white muscle, and therefore reduced or even suppressed LCFA uptake in this tissue, which was in agreement with the above-mentioned results from the *in vitro* study conducted in hepatocytes from grass carp. These findings are of special significance when formulating compound diets for grass carp, since selecting the proper type of oil source with a particular FA profile may be of interest for reducing the adiposity in this herbivorous species, once the nutritional requirements are met in terms of growth performance and health condition. Nevertheless, further research is required to determine the molecular mechanisms of LCFAs' uptake and how LCFAs regulate the activity of transport membrane proteins in grass carp.

In conclusion, results from the *in vitro* studies revealed that the uptake of FAs by grass carp hepatocytes was shown to be partly membrane protein associated, since LCFAs were found to be absorbed by cells regardless of the transport protein inhibitor tested. *FAT/CD36* gene in grass carp has high homology with other fish, especially cyprinid species, and it is primarily expressed in the brain and in the hepatopancreas,



**Figure 11.** Phylogenetic analysis of *FAT/CD36*. Phylogenetic tree based on protein sequences was constructed by the neighbour-joining method with Mega 5.0 software. The strength of branch relationships was assessed by bootstrap replication ( $N = 1000$  replicates). Grass carp *FAT/CD36* was indicated by '●'. Accession numbers of protein sequences for *FAT/CD36* are as follows: *Cyprinus carpio* (KM030422); *Danio rerio* (NM\_001002363); *Homo sapiens* (KR710356); *Macaca mulatta* (AY600441); *Rattus norvegicus* (NM\_031561); *Mus musculus* (NM\_001159558); *Lipotes vexillifer* (XM\_007467039); *Bos taurus* (NM\_001278621); *Capra hircus* (JF690773); *Gallus gallus* (NM\_001030731); *Xenopus (Silurana) tropicalis* (NM\_001113679).



**Figure 13.** Effect of dietary oil sources on *FAT/CD36* expression in grass carp (*C. idellus*). Values are expressed as means  $\pm$  SD (n = 8). Different letters denote statistically significant differences among dietary groups ( $P < .05$ ).

whereas its lower expression levels were found in the gill and spleen, differences that may be attributed to tissue-specific differences in FA requirements and metabolism. Dietary oil sources, containing different LCFAs, have a significant impact on *FAT/CD36* expression in the white muscle of grass carp, where mRNA levels were highest in fish fed the GO diet, being rich in 18:2n-6, and lowest in fish fed the LO and FO diets that were rich in 18:3n-3, 20:5n-3 and 22:6n-3, showing a regulatory role of FAs on *FATA/CD36* expression and FA absorption in cells.

## Acknowledgements

Thanks are due to Xiao Ling Chen and XiaoYan Chen for their help in sampling and preparing the cells.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by the National Natural Sciences Foundation of China under Grant [No. 31201990]; Applied Basic Research Program of Sichuan Province China under Grant [No. 2014JY0088], the Fundamental Research Funds for the Central Universities under Grant [Northwest A&F University, QN2011104] and the Effect of prickly ash seed oil on experimental lipid liver of grass carp and its mechanism [2017JM3026].

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