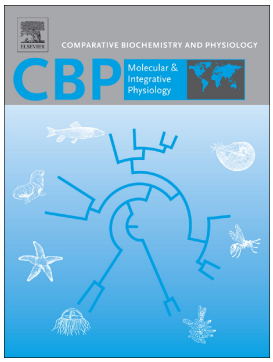


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# Forskolin reduces fat accumulation in Nile tilapia (*Oreochromis niloticus*) through stimulating lipolysis and beta-oxidation

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**ABSTRACT**

High fat diets are commonly used in aquaculture to reduce feed cost in Nile tilapia, but impair its lipid homeostasis. This study evaluated the role of forskolin on reducing fat accumulation in Nile tilapia (*Oreochromis niloticus*) by using in vitro and in vivo experiments. The use of 50 μM forskolin in vitro increased free fatty acid and glycerol release, but decreased triglyceride in adipocytes and hepatocytes. The adipose triglyceride lipase (ATGL), protein kinase cAMP-dependent type I regulatory subunit alpha (PKAR I) and other genes related to β-oxidation (peroxisome proliferator activated receptor alpha, PPARα and carnitine O-palmitoyltransferase 1, CPT1) were significantly up-regulated. After feeding a high-fat diet for six weeks, *O. niloticus* were fed with 0 (control), 0.5 and 1.5 mg/kg forskolin for two weeks to determine whether forskolin could reduce fat accumulation in vivo. Fish fed the two levels of forskolin decreased significantly the hepatosomatic and mesenteric fat indices. The total lipid in the whole fish and liver together with the serum glycerol content were lower in fish fed on forskolin than in the control. The fish fed on forskolin diets exhibited smaller areas of lipid droplets in adipose and liver tissues. Lipolysis related genes (ATGL, hormone-sensitive lipase, HSL; monoacylglycerol lipase, MGL; and protein kinase cAMP-activated catalytic subunit, PKAC) and β-oxidation genes (PPARα; fatty acid binding protein 1, FABP1; and CPT1) in the adipose were up-regulated. Similarly, in the liver lipolysis genes such as ATGL and PKAR I and β-oxidation genes (PPARα, FABP1, CPT1 and acyl-CoA oxidase, ACO) showed an increasing trend with the increase of forskolin doses. This study indicates that forskolin can reduce fat accumulation in the adipose and liver by stimulating lipolysis and β-oxidation in *O. niloticus*.

*Keywords: Oreochromis niloticus*, forskolin, lipolysis, β-oxidation

## 1. Introduction

Dietary fats play an important role in fish nutrition by providing essential fatty acid and energy. The lipid-rich diets are used widely because of their protein-sparing effects (Beamish and Medland, 1986; Watanabe, 1982). However, this practice increases fat deposition in cultured fish, compromises fish health, alters metabolic patterns (Lauriano et al., 2016; Rueda-Jasso et al., 2004) and lowers growth and feed efficiency (Du et al., 2006; Nath et al., 2018). Therefore, it is crucial to develop effective feeding strategies to reduce fat accumulation in cultured fish.

Different approaches including pharmacologic therapy, surgery and dietary supplements have been used as treatments to reduce overweight and obesity in humans (Colker et al., 1999; Van and F., 2008; Yamoneka et al., 2015). Dietary supplements offer several advantages over traditional therapy such as low toxic profile, easy accessibility to the general population and wide availability because they are sold over-the-counters (Ríos-Hoyo and Gutiérrez-Salmeán, 2016). Previous studies have investigated the effects of some dietary supplements that are derived from plants on reducing obesity by using in vitro and in vivo methods in various species (Hu et al., 2012; Oben et al., 2008; Park, 2015). African wild mango (*Irvingia gabonensis*) inhibited adipogenesis in adipocytes by down-regulating the adipogenic transcription factor (peroxisome proliferator-activated receptor gamma (PPAR-γ) (Oben et al., 2008). Raspberry ketone inhibited adipogenesis, adipocyte differentiation and increased transcriptional activities of genes involved in lipolysis and oxidation (Park, 2015). Fucoxanthin, a carotenoid present in edible brown seaweeds, exerted anti-obesity effects by regulating mRNA expression of enzymes related to lipid metabolism in white adipose tissue in diet-induced obesity rats (Hu et al., 2012). A few dietary supplements are also used in aquaculture to modulate lipid metabolism in fish. For example, silibinin from *Silybum marianum* inhibited lipid accumulation in zebrafish (Suh et al., 2015) and grass carp (Xiao et al., 2017) by reducing adipogenic factors and triglyceride levels. Although these ingredients have effects within preclinical or small-scaled clinical trials, the majority of these trials do not show health-related evidences, food-drug interactions and dose-related body fat reductions (Ríos-Hoyo and Gutiérrez-Salmeán, 2016).

Forskolin is a labdane diterpene produced from the root of *Coleus forskohlii*

(Bhat et al., 1977). Its extract has been recorded in Ayurveda medicine since ancient times and its medicinal value has been studied since early-1980s (Bristow et al., 1984; Kavitha et al., 2010). Early studies have suggested that forskolin is a potential agent for management and treatment of obesity. Forskolin promoted lipolysis by regulating the production of cyclic adenosine monophosphate levels in membranes, cells, or tissues (Insel and Ostrom, 2003) through activation of cAMP-dependent protein kinase (PKA) and hormone-sensitive lipase (HSL) (Belfrage et al., 1982). Previous studies have also shown that, forskolin regulates lipolysis by stimulating perilipin A in mice adipocytes (Miyoshi et al., 2006) and adipose triglyceride lipase (ATGL) in human multipotent adipose-derived stem (hMADS) cells (Bezaire et al., 2009). In small-scaled clinical trials, the in vivo use of forskolin supplements for 12 weeks twice a day decreased significantly the body fat in obese men (Godard et al., 2005). Its supplement has also been shown to increase significantly the high-density lipoprotein-cholesterol together with reduction of waist and hip circumference in humans (Loftus et al., 2015). However, compared to the knowledge on the use of forskolin for regulation of lipolysis in mammals, information on its effects in fish is currently unknown. Considering the potential effect of forskolin on reducing fat accumulation, there is a need for further exploration on its physiological role in fat reduction and the underlying mechanisms in cultured fish.

Nile tilapia (*Oreochromis niloticus*) is an important species farmed worldwide (Doan et al., 2018). Lipid-rich diets are commonly used in Nile tilapia aquaculture to reduce feed cost, but impair lipid homeostasis (Huang et al., 2016). Therefore, it is important to determine whether forskolin can stimulate lipolysis and reduce fat accumulation in cultured fish where high lipid deposition is undesired. Currently, no research has been conducted to investigate the potential role of forskolin on lipolysis in cultured fish species such as Nile tilapia. The primary objectives of this study were to determine whether 1) forskolin in vitro stimulates lipolysis on Nile tilapia adipocytes (APCs) and hepatocytes (HPCs) and its associated mechanisms; and 2) forskolin (0.5 mg/kg BW and 1.5 mg/kg BW) in vivo has a positive effect on the reduction of fat accumulation in Nile tilapia fed on high fat diet.

## 2. Materials and methods

### *2.1. Ethical statement*

This research was approved by the Animal Ethics Committee of East China Normal University and all experiments were conducted according to the protocols and procedures of the Laboratory Animal Management Ordinance of China.

*2.2. In vitro experimental designs*

*2.2.1. Fish source, isolation of adipocytes and hepatocytes*

Four Nile tilapia (above 500 g) were purchased from Yueqiang Company (Guangzhou, China). Animals were anesthetized with MS-222 (25 mg/L) and then killed by a lethal blow on the head, followed by immersion in 75% ethanol for 30 s. The APCs and HPCs were isolated as described previously by Albalat et al. (2005) with some modifications. Individual fat or liver tissues from the four fish were pooled and cut into thin pieces. Tissues were incubated in a shaking water bath at 28 °C for 60 min with the Dulbecco's Modified Eagle's medium (DMEM, Thermo Fisher, China) buffer containing collagenase type II and IV for the fat and liver tissue, respectively. The cell suspension was filtered through a nylon strainer and then washed three times by using the Krebs–Hepes buffer containing 1% bovine serum albumin (BSA). Cells were centrifuged at 1000 rpm for 10 min and resuspended with the DMEM buffer containing 10% fetal bovine serum (FBS). Cells were incubated in a petri dish at 28 °C with 5% $CO_2$ overnight and then washed three times by using phosphate-buffered saline (PBS). Finally, cells were carefully resuspended at approximately $10^6$ cells / mL with FBS free DMEM and incubated for 1 h in a 12-cell dish at 28 °C containing 0 (control), 50, 100 μM forskolin (APExBIO, USA) in four replicates in order to establish the effective dose. Both forskolin doses stimulated HPCs lipolysis and increased free fatty acids (FFAs) release while only 50 μM was effective in APCs (Fig. 1S). Based on this preliminary experiment, 50 μM forskolin was chosen as the dose for further experiments, thereafter cells were incubated at 0 (control) and 50 μM forskolin.

*2.2.2. Free fatty acids (FFAs) and glycerol analysis*

At the end of incubation period with forskolin, cells were centrifuged at 1000 rpm for 10 min at 4 °C. Cell-free aliquots of the media were taken to measure the concentrations of FFAs and glycerol as an index of lipolysis using commercial kits (Jiancheng Biotech CO., China). The remaining medium was removed and 1-mL

Trizol reagent or 1-mL chloroform/methanol (1:1, v/v) was added for transcriptomic analysis by using quantitative PCR (RT-PCR) and measurement of TG concentration in cells by thin-layer chromatography (TLC).

2.2.3. *Lipolysis and β-oxidation transcriptomic analysis*

The total RNA was extracted to measure the expression of ATGL, HSL, PKA, peroxisome proliferator-activated receptor alfa (PPARα), fatty acid-binding protein 1 (FABP1) and carnitine palmitoyltransferase 1 (CPT1) genes. Total RNA extraction was carried out using Trizol reagent (RN0101, Aidlab, China). The RNA quantity and quality were checked on a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, USA). Total RNA (1000 ng) was reversely transcribed using a PrimeScript$^{TM}$ RT Master mix (RR047A, Takra, Japan) at 42 °C for 2 min to remove genomic DNA and at 37 °C for 15 min and 85 °C for 5 sec to complete reverse transcription. The cDNA was unified according to the measured concentration before quantitative PCR (RT-PCR). The RT-PCR amplification (in duplicate) was performed using 2 × ultra SYBR mixture (CW0957, Kangwei, China). The elongation factor 1-alpha (EF1α) was used as the internal reference gene.

The PCR samples were prepared in a final volume of 20 μL consisting of 10 μL of 2 × ultra SYBR mixture, 0.5 μL of 10 mM forward and reverse primers, 2 μL of cDNA template and 7 μL of $H_2O$ with following cycling conditions: initial denaturation at 95 °C for 30 sec, followed by 40 cycles of 94 °C for 15 sec and 72 °C conducted in the CFX96 Real-time PCR system (Bio-rad, Richmond, CA). The cycle time (Carmen and Víctor) values of different treatments were compared to their corresponding internal control and then converted to fold change values by comparing the control group (without forskolin). All the primers for the amplification of genes used in this study are listed in Table 1.

*2.2.4. Triglyceride content analysis*

The TG content was quantified as described previously by Blank et al. (1964) and Skipski et al. (1964) with some modifications. Briefly, the total lipid obtained in section 2.2.2. above were separated by TLC using heptane/ether/acetic acid (55:45:1 by volume). Plates were heated in an oven for 20 min at 60 °C to remove the adsorbents. Different doses of TG standards 2, 4 and 6 μg (ZZStandard, China) were

applied carefully to the plates with 5 μL standardized micropipettes. The plates were developed in the iodine chambers and analyzed for TG content by using thin-layer chromatogram scanner (KH-3000, kEZHE, Shanghai). Peak area of TG standard was calculated from a standard curve. The TG content was quantified by using a standard curve.

*2.3. In vivo experimental designs*

*2.3.1. Experimental fish and feeding management procedures*

About 350 Nile tilapia were purchased from Yueqiang Company (Guangzhou, China). They were acclimated in one (500 L) tank for 10 days. Fish were supplied with compressed air via air-stones from air pumps at a 10 h/14 h light/dark cycle and water quality parameters were maintained at optimal levels for Nile tilapia growth and survival. During this period, the fish were hand-fed using a commercial diet (Chengdu, China) containing > 33% protein and > 5% lipid. After 10 days of acclimation 35 fish (weight 4.41 ± 0.32 g) were randomly distributed into nine 300 L tanks. To determine whether forskolin had a positive effect on reducing fat accumulation on Nile tilapia, fish were first fed with a high fat diet (Table 2, total lipid 15.31%) to accumulate body fat for six weeks. During the first six weeks, all fish were fed thrice daily (at 0800, 1200 and 1700 h) at a feeding rate of 5% body weight using the high fat diet. Two hours after each feeding occasion, the uneaten diets were removed by siphon, dried and weighed for determination of feed conversion ratio (FCR).

On the first day of the $7^{th}$ week, all fish (weight 11.57 ± 1.81 g) were redistributed randomly into three groups: control (0), medium forskolin (0.5 mg/kg) and high forskolin (1.5 mg/kg) each group containing 35 fish/tank in triplicate. The forskolin diet was prepared by dissolving forskolin in dimethyl sulphoxide (DMSO) (Table 2). The resulting forskolin solution was mixed with a given amount of wheat flour to make a wet dough. Wet dough was made by hand into small pellets of approximately 1.5 mm based on size of the fish. When fish ingested the forskolin particles at 1% body weight, the amount of forskolin consumed was equivalent to 0.5 or 1.5 mg/kg body weight (BW), depending on the amount of forskolin added to the dough pellets. Every morning, the fish were fed with a small wheat flour dough containing the control, medium and high forskolin diets at 1% body weight for 30

min before normal feeding with the high fat diet. Afterwards, the same amount of high fat diet was fed to the fish at 4% body weight. One hour later, the uneaten diets were removed by siphon, dried and weighed for determination of FCR. The formulations of the high fat diet and wheat flour-dough particle are listed in Table 2. The water quality in all experimental tanks were relatively similar to those used during acclimation.

*2.3.2. Growth performance and feed efficiency*

At the end of eight-week trial, all fish were fasted for 12 hours, collected by using a scoop net and their individual final weights measured for determination of weight gain and FCR using the following formulae:

Weight gain (WG, %) = [final weight – initial weight] × 100 / initial weight

Feed conversion ratio (FCR) = dry feed weight / wet weight gain.

*2.3.3. Serum biochemical analysis and hepatosomatic and mesenteric fat indices*

A sample of 20 fish from each treatment were anesthetized by using MS-222 (25 mg/L) as described above for blood sampling. The blood was drawn from the caudal vein using 2 mL syringes (Klmedical, China). Blood samples were immediately centrifuged at 1000 rpm for 10 min at 4 $^{\circ}$C, the serum was placed into polypropylene tubes. Serum TG and FFAs concentrations were determined by using specific commercial assay kits (Jiancheng Biotech CO., China).

The remaining fish after blood sampling were dissected to obtain muscle, liver and adipose tissues. Each liver or adipose tissue was weighed to determine the hepatosomatic index (HSI) and mesenteric fat index (MFI) by using the following formulae:

Hepatosomatic index (HSI, %) = wet liver weight × 100 / wet body weight;

Mesenteric fat index (MFI, %) = wet mesenteric fat weight × 100 / wet body weight.

*2.3.4. Proximate analysis of whole fish and muscle, liver and adipose tissues*

A sample of six fish were collected randomly from each treatment and stored at -20 $^{\circ}$C for whole body composition analysis. Whole body composition, muscle and liver tissues were analyzed according to the methods described by Bligh and Dyer

(1959) with some modifications. Each whole fish was dried at 70 °C to constant weight and then ground to powder. About 60 mg fish powder, muscle, liver or adipose tissues were homogenized in 5 mL chloroform/methanol (1:1, v/v) and stored at 4 °C for overnight after blending for 30 sec, followed by adding 1 mL 0.28 mM KCl and blending for 30 sec. After centrifugation at 1200 rpm for 10 min, subnatant was imbibed and filtered through filter paper into pre-weighed glass tubes by Pasteur pipettes. Total lipid was quantified by gravimetry using a vacuum drying oven (DZH-6050, Jinghong, Ltd, Shanghai, China). The total protein of whole fish body was determined by a semi-automatic Kjeldahl System (FOSS, Sweden) after acid digestion.

*2.3.5. Histology analysis*

The liver and adipose tissues from three fish in each group were cut into 2-mm pieces and stored in paraformaldehyde. After dehydrated, the samples were embedded in paraffin and sliced into 4 μm-thick sections. Each section was stained with hematoxylin and eosin, examined microscopically and photographed on a Nikon Microscope ECLIPSE N*i* (Nikon Corporation, Tokyo, Japan). The lipid droplet area was measured and calculated by Image J (LOCI, University of Wisconsin, U.S.A).

*2.3.6. Lipolysis and β-oxidation mRNA expression in liver and adipose tissues*

The liver and adipose tissue samples from 12 fish per treatment were used for molecular analyses as described previously in section 2.2.2 above. Before analysis, samples were collected and stored at -80 °C until the time for analyses. All the primers for the amplification of genes used in this study are listed in Table 1.

*2.4. Statistical analyses*

All results are presented as mean ± S.E.M. Data were tested for normality and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively. The in vitro results were analyzed by independent-samples *t*-test to evaluate the significant difference ($P < .05$) and extremely difference ($P < .01$) for measured variables between the control and 50 μM forskolin cells. One – way analysis of variance (ANOVA) was used to detect the possible differences in measured

parameters among control and the different forskolin concentrations for the in vivo experiments. When differences were detected, Tukey's post hoc test was performed to determine specific differences. All analyses were conducted using the SPSS Statistics version 23 software (IBM, USA).

## 3. Results

### *3.1. In vitro results*

#### *3.1.1 Lipolysis in adipocytes and hepatocytes*

We determined the ability of forskolin to stimulate lipolysis by using APCs and HPCs (Fig. 1). The 50 µM forskolin stimulated lipolysis both in APCs and HPCs by increasing FFAs (Fig. 1A) and glycerol release (Fig. 1B; $P < .05$). However, the 50 µM forskolin decreased TG content in the APCs cells but not in HPCs (Fig. 1C).

#### *3.1.2. Lipolysis and β-oxidation genes expression*

The mRNA expressions showed a similar tendency between APCs and HPCs (Fig. 2). The forskolin treatment up-regulated ATGL and PKAR I mRNA genes expression ($P < .05$; Fig. 2). However, forskolin doses did not affect the mRNA expression of HSL gene ($P > .05$). The forskolin treatment elevated the genes related to β-oxidation such as PPARα and CPT1 ($P < .05$) except FABP1 ($P > .05$; Fig. 2). These results indicate that, in the in vitro experiments, forskolin stimulated lipolysis in both APCs and HPCs by regulating lipases and β-oxidation genes.

### *3.2. In vivo results*

#### *3.2.1. Growth performance, feed efficiency and body indices*

The forskolin doses did not affect the WG of Nile tilapia compared to the control ($P < .05$). However, fish fed on 0.5 mg/kg forskolin had significantly lower FCR (by nearly 40%) compared to those fed on the control diet ($P < .05$; Fig. 3). When fish were fed with 1.5 mg/kg forskolin, the FCR was statistically comparable to the control and 0.5 mg/kg forskolin ($P > .05$). The HSI and MFI in fish fed the different forskolin doses were reduced significantly in comparison to the control ($P < .05$), but did not differ between the two doses ($P > .05$; Fig. 3).

#### *3.2.2. Body proximate composition, liver and muscle and serum biochemical indices*

The forskolin diets decreased significantly lipid in the whole fish and the liver compared to control (Fig. 4A and 4B; $P < .05$), but not in the muscle (Fig. 4C; $P > .05$). The fish fed on the forskolin doses decreased significantly the glycerol content in the serum compared to the control ($P < .05$; Fig. 4D). The FFAs content increased by two folds in the 1.5 mg/kg forskolin dose compared to the 0.5 mg/kg forskolin and the control ($P < .05$; Fig. 4E). The forskolin doses did not affect protein content in the whole fish and the liver among the different treatments ($P > .05$; Fig. 4F and 4G).

*3.2.3. Histology*

To determine whether forskolin could reduce fat accumulation in Nile tilapia, the liver and adipose tissues were sectioned, stained and photographed. The liver tissue sections for the fish fed on forskolin doses exhibited vacuolar degeneration and an increase in hepatic parenchyma (Fig. 5E and 5F). The fish fed on the 0.5 mg/kg forskolin treatment decreased the lipid droplet area in adipose compared to control ($P < .05$; Fig. 5G). Similarly, the fish fed on forskolin doses decreased the lipid droplet area in the liver in a dose-dependent manner compared to control ($P < .05$; Fig. 5H).

*3.2.4. Lipolysis and β-oxidation mRNA expressions in liver and adipose tissues*

The forskolin treatment influenced significantly the expression of ATGL, HSL and monoacylglycerol lipase, MGL genes. In adipose, lipase genes were up-regulated by both forskolin doses ($P < .05$; Fig. 6A). However, in the liver tissue, the ATGL gene increased while the MGL gene was down-regulated ($P < .05$; Fig. 6B). The PKAR I and PKAR II genes showed different tendencies between the adipose and liver tissues. The PKAC gene was up-regulated in both tissues ($P < .05$). The expressions of β-oxidation related genes such as PPARα, FABP1, CPT1 and acyl-CoA oxidase (ACO) were increased by four-fold in the liver of fish fed on forskolin compared with the control ($P < .05$). In the adipose, the 0.5 mg/kg forskolin dose up-regulated FABP1 and CPT1 expressions ($P < .05$), while no difference in expression was found between the 1.5 mg forskolin /kg treatment and the control ($P > .05$; Fig. 6A). The results on in vivo experiments show that similar to the results in vitro, oral administration of forskolin decreased lipid content,

glycerol content and influenced the mRNA of genes related to lipolysis and β-oxidation.

## 4. Discussion

Lipolysis is an important biochemical process, which breaks down TG into FFAs, for utilization by different body cells (Young and Zechner, 2013; Zechner et al., 2012). In mammals, ATGL, HSL and MGL are mainly involved in the lipolysis process from TG to diacylglycerols (Seamon et al.) , DG to monoacylglycerols (MG), and MG to FFAs, respectively (Chanda et al., 2010; Jenkins et al., 2004; Vaughan et al., 1964; Villena et al., 2004; Zimmermann et al., 2004). Similar to mammals, the three lipases are regulated by the levels of lipids, FFAs, hormones and bioactive ingredients in fish such as grass crap (*Ctenopharynogodon idella*) (Ji et al., 2012), rainbow trout (*Oncorhynchus mykiss*) (Kittilson et al., 2011), Japanese flounder (*Paralichthys olivaceus*) (Khieokhajonkhet et al., 2014), large yellow croaker (*Larimichthys crocea*) (Wang et al., 2013) as well as Nile tilapia (Ning et al., 2016; Ning et al., 2017; Tian et al., 2013). Although forskolin plays a positive role in reducing fat accumulation in mammals through PKA-dependent activation of HSL and ATGL (Belfrage et al., 1982; Bezaire et al., 2009), little is known about the effects of forskolin on fat metabolism in fish both in vivo and in vitro. In the present study, both doses of 0.5 and 1.5 mg/kg forskolin reduced fat accumulation in vivo by decreasing lipid contents in the whole fish and the liver. Moreover, the HSI and MFI in the fish fed on forskolin were also lower than those in the control. Furthermore, the histological observations confirmed vacuolar degeneration, hyperplasia of hepatic parenchyma and reduction of lipid droplet size in the adipose tissue of the fish fed on forskolin doses. The present study demonstrates that the forskolin can help to decrease fat accumulation in Nile tilapia fed on high fat diet.

To the best of our knowledge, the present study was the first to show that forskolin can stimulate lipolysis in APCs and HPCs in fish. Although HSL gene expression was not increased significantly by forskolin in APCs and HPCs for the in vitro experiments, the dietary supplementation of forskolin up-regulated significantly HSL mRNA expression in the Nile tilapia adipose tissues in the in vivo experiments. Moreover, the adipose tissue seems to be more sensitive to forskolin than the liver tissue because ATGL, HSL and MGL mRNA expressions were all

increased in the adipose tissue but not in the liver tissue. This may be due to the fact that, the adipose rather than the liver is the main tissue for lipid deposition and lipolysis (Ahmadian et al., 2007). These results indicate that, similar to mammals, forskolin can stimulate lipolysis by up-regulating the mRNA expression of PKAR I, II and PKAC as well as the lipases genes such as ATGL, HSL and MGL.

Presently, there is no evidence which shows toxicity of forskolin in animal trials. In a previous study, the root extract of *Coleus forskohlii* induced hepatotoxicity in mice by unidentified constituents in the plant rather than by the forskolin itself (Virgona et al., 2013). Similarly, in the present study, forskolin also did not affect fish weight gain and protein content, which are the important variables to evaluate fish growth and nutritional status. These results suggest, forskolin has little influence on Nile tilapia growth, although more studies are still needed to evaluate the health effects of forskolin on fish.

Lipolysis and fatty acid oxidation are two important mechanisms involved in fat reduction. Over stimulation of lipolysis increases the level of FFAs in the serum and causes metabolic perturbation (Koutsari and Jensen, 2006). A large inflow of FFAs to the mitochondria may lead to mitochondrial membrane permeabilization and cell death (Engin, 2017). Lipotoxicity occurs due to unbalanced lipolysis (Bülow et al., 2017) or impaired β-oxidation (Haffar et al., 2015). Therefore, Langin (2006) suggested that the control of molecules that stimulate lipolysis and fatty acid (FA) oxidation release could be a useful approach to decrease fat accumulation. Forskolin seems to be an efficient supplement compared to other weight management compounds like L-carnitine and fenofibrate have been used, which showed different degrees of effectiveness in lipid metabolism. Using L-carnitine, Li et al. (2017) showed enhanced mitochondrial β-oxidation activities and decreased lipid in liver and muscle tissues of treated zebrafish. However, L-carnitine application showed no effects on lipid metabolism in some fish species like African catfish (Torreele et al., 1993), hybrid tilapia (Yang et al., 2009) and hybrid striped bass (Twibell and Brown, 2000) or even caused negative effects on lipid metabolism in red sea bream (Chatzifotis et al., 1995) and rainbow trout (Selcuk et al., 2010). By using fenofibrate, Ning et al. (2016) indicated increased PPARα mRNA expression and decreased hepatic TG in Nile tilapia. Their following work also showed decreased hepatic and plasma TG in Nile tilapia fed with on fat diet

supplemented with fenofibrate (Ning et al., 2017). However, these authors pointed out that dietary fenofibrate did not change mesenteric fat quantity and TG concentrations in muscle and adipose tissues.

Interestingly in the present study, a significant increase in serum FFAs was also obtained when the fish were fed with 1.5 mg/kg forskolin in the in vivo experiments. However, considering the up-regulation of genes such as PPAR α, FABP-1, CPT-1 and ACO, which are all related to fatty acid β-oxidation, forskolin is likely to increase lipolysis to stimulate fatty acid breakdown. Previous studies have also indicated that, forskolin can up-regulate both PPAR isoforms (α, β, γ) in salmon (Pavlikova et al., 2010) and increase FA β-oxidation in vitro and in vivo in mice (Gerhart-Hines et al., 2011). Therefore, forskolin modulates lipid metabolism in fish because the circulation of lipolysis-mediated FFAs has been identified as the ligand of PPARα (Narala et al., 2010; Pace et al., 2008) and the activation of PPARα also triggers FA β-oxidation (Leone et al., 1999; Storch and Thumser, 2000; Wakil and Abu-Elheiga, 2009). These results suggest that, forskolin supplementation can systemically increase the lipid catabolism pathways and efficiently reduce body lipid accumulation in cultured fish.

## 5. Conclusion

Forskolin stimulates Nile tilapia lipolysis by regulating PKA, lipases and FFAs oxidation related genes. Feeding Nile tilapia with forskolin at a dose of 0.5 or 1.5 mg kg body weight per day reduce remarkably the fat accumulation and promote lipid catabolism. This study proposes the use of forskolin to reduce lipid accumulation in cultured fish. Future studies are needed to determine the safety level of forskolin in fish diets.

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**Author contributions**

All authors contributed to the design of the experiment. The in vitro and in vivo experiments were conducted by H.Z. Z-Y.D. and L-Q.C supervised the experiments. Data analyses were performed by H.Z. with help from J.-J.W and SML. The manuscript was drafted by H.Z. SML read and corrected the English language. All authors read and approved the final version of the manuscript for submission.

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**Figure captions**:

**Fig. 1.** Effect of forskolin stimulation on lipolysis in vitro Nile tilapia.

(A) FFAs release, (B) glycerol release and (C) triglyceride content in adipocyte and hepatocyte treated with 50 μM forskolin. Values are mean ± S.E.M from four replicates with each replicate contents ~$10^6$ cells. A single asterisk (*) or double asterisks (**) indicate significant different ($P < .05$) or extremely different ($P < .01$) among treatments.

**Fig. 2.** The in vitro expression of lipolysis and β-oxidation genes in Nile tilapia after stimulation with forskolin.

The mRNA relative expression of lipolysis and β-oxidation related genes in *O. niloticus* (A) adipocytes and (B) hepatocytes treated with 50 μM forskolin. Values are mean ± S.E.M from four replicates. A single asterisk (*) or double asterisks (**) are indicate significant different ($P < .05$) or extremely different ($P < 0.01$) among treatments.

**Fig. 3.** Growth performance, feed efficiency and organ indices of Nile tilapia fed with forskolin for two weeks**.**

(A) Weight gain, (B) feed conversion ratio, (C) hepatosomatic index and (D) mesenteric fat index of *O. niloticus* fed with 0.5 mg/kg body weight (BW) and 1.5 mg/kg BW forskolin. Values are mean ± S.E.M for three replicates, each replicate containing twenty fish. A single asterisk (*) or double asterisks (**) indicate significant different ($P <.05$) or extremely different ($P < .01$) among treatments.

**Fig. 4.** Fish body composition analysis of Nile tilapia fed on forskolin for two weeks. Total lipid of (A) whole fish, (B) liver tissue and (C) muscle, serum (D) glycerol content, (E) FFAs release, total protein of (F) whole fish and (G) muscle after feeding with 0.5 mg/kg BW and 1.5 mg/kg BW forskolin. Values are mean ± S.E.M for three

replicates each replicate containing six fish for whole body analysis and three replicates each replicate containing twenty fish for tissues and serum samples. A single asterisk (*) or double asterisks (**) indicate significant different ($P < .05$) or extremely different ($P < .01$) among treatments.

**Fig. 5.** Adipose and liver tissue histology of Nile tilapia fed with forskolin.

Adipose tissue paraffin sections of *O. niloticus* fed with wheat flour containing (A) DMSO, (B) 0.5 mg/kg BW and (C) 1.5mg/kg BW forskolin. The liver tissue paraffin sections of *O. niloticus* with same treatment are shown in Fig. 5 (D), (E) and (F). Fig. 5 (G) and (H) show the percentage of lipid droplet area of the section area (24162 $\mu m^2$) in adipose and liver tissue, respectively.

**Fig. 6.** The in vivo mRNA expression of lipolysis and β-oxidation genes of Nile tilapia fed with forskolin.

The mRNA relative expression of lipolysis and β-oxidation related genes in *O. niloticus* (A) adipose and (B) liver tissues fed with 0.5 mg/kg BW and 1.5 mg/kg BW forskolin. Values are mean ± S.E.M for twelve fish. A single asterisk (*) or double asterisks (**) are significantly different ($P < 0.05$) or extremely different ($P < 0.01$) among treatments.

**List of tables**

**Table 1**

Primer pair sequences and product size of the genes used for real-time PCR (qPCR)

| Gene | Position | 5'-3' primer sequence | Product size (bp) | GenBank NO. |
|---|---|---|---|---|
| ATGL | Forward | AAAACGTCCTGGTGACCCAGT | 104 | XM_003440346 |
| | Reverse | TAGGAGGAATGATGCCACAGTACA | | |
| HSL | Forward | AACCTGGATGTCCATTTCTGGAAG | 102 | FJ601660 |
| | Reverse | TCGGTTTACCTTGACTTGAGTGGA | | |
| MGL | Forward | ACATCGTCAACGCAGACGGATT | 105 | XM_005478351.1 |
| | Reverse | CACAATGTTCCCCAGCTCCAT | | |
| PKAR I | Forward | AGTTCCCGTTCAGACTCTCGTGAT | 117 | XM_003438740.4 |
| | Reverse | GGCGGCATCCTCCTCTGTGTAA | | |
| PKAR II | Forward | CCTGCCGAGACATCCTACTCTTCA | 99 | XM_005478353.3 |
| | Reverse | CGTGCTCCTGAGGCTTAACAATCA | | |
| PKAC | Forward | TGGGCACGGGCTCATTTGGT | 99 | XM_003449976.4 |
| | Reverse | GCTTGACCACCTTCTGCTTGTTGA | | |
| PPARα | Forward | CTGATAAAGCTTCGGGCTTCCA | 106 | KF871430 |
| | Reverse | CGCTCACACTTATCATACTCCAGCT | | |
| FABP1 | Forward | ACTATCGGACAGGAGGCTGAACTA | 98 | XM_003446092 |
| | Reverse | TTCTTCAGGGTGGTCTTCAGCTT | | |
| CPT1 | Forward | TTTCCAGGCCTCCTTACCCA | 102 | XM_003440552 |
| | Reverse | TTGTACTGCTCATTGTCCAGCAGA | | |
| ACO | Forward | AGTCCCACTGTGAGCTCCATCAA | 108 | KF918710 |
| | Reverse | CAGACCATGGCAGTTTCCAAGA | | |
| EF1α | Forward | ATCAAGAAGATCGGCTACAACCCT | 109 | KJ123689 |
| | Reverse | ATCCCTTGAACCAGCTCATCTTGT | | |

ATGL, adipose triglyceride lipase. HSL, hormone-sensitive lipase. MGL, monoacylglycerol lipase. PKARI, protein kinase cAMP-dependent type I regulatory subunit alpha. PKARII, protein kinase cAMP-dependent type II-alpha regulatory subunit. PKAC, protein kinase cAMP-activated catalytic subunit. PPARα, peroxisome proliferator activated receptor alpha. FABP1, fatty acid binding protein 1. CPT1, carnitine O-palmitoyltransferase 1. ACO, acyl-CoA oxidase. EF1α, elongation factor 1α.

**Table 2**

Feed formulation (g/kg) and proximate composition of high fat diet (HFD) fed to *Oreochromis niloticus*

| Ingredients | |
|---|---|
| Casein | 304 |
| Gelatin | 76 |
| Soybean oil | 75 |
| Flaxseed oil | 75 |
| Corn starch | 300 |
| Vitamin pre-mix[a] | 14.4 |
| Mineral pre-mix[b] | 22.4 |
| Carboxymethyl cellulose | 50 |
| Cellulose | 68.8 |
| Choline chloride | 5 |
| Butylated hydroxytoluene | 0.25 |
| Total | 1000 |
| *Analyzed proximate composition* (%) | |
| Moisture | 10.6 |
| Total protein | 36.27 |
| Total lipid | 15.31 |
| Ash | 3.8 |
| Wheat flour component (100 g) | |
| Protein (g) | 12.2 |
| Lipid (g) | 1.5 |
| Carbohydrate (g) | 70 |

[a] Vitamin premix (mg or IU/kg): 500,000 I.U. (international units) Vitamin A, 50,000 I.U. Vitamin D3, 2500 mg Vitamin E, 1000 mg VitaminK3, 5000 mg Vitamin B1, 5000 mg Vitamin B2, 5000 mg Vitamin B6, 5000 μg Vitamin B12, 25,000 mg Inositol, 10,000 mg Pantothenic acid, 100,000 mg Choline, 25,000 mg Niacin, 1000 mg Folic acid, 250 mg Biotin, 10,000 mg Vitamin C. [b]Mineral premix, (g/kg): 314.0 g $CaCO_3$; 469.3 $KH_2PO_4$; 147.4 g $MgSO_4 \cdot 7H_2O$; 49.8 g NaCl; 10.9 g Fe (II) gluconate; 3.12 g $MnSO_4 \cdot H_2O$; 4.67 g $ZnSO_4 \cdot 7H_2O$; 0.62 g $CuSO_4 \cdot 5H_2O$; 0.16 g KJ; 0.08 g $CoCl_2 \cdot 6H_2O$; 0.06 g $NH_4$ molybdate; 0.02 g $NaSeO_3$.

**Supplementary**

**Fig. 1S.** Effect of different doses of forskolin stimulation on lipolysis in vitro Nile tilapia.

FFAs release in adipocyte and hepatocyte treated with 50 and 100 μM forskolin. Values are mean ± S.E.M from four replicates with each replicate contents ~$10^6$ cells. A single asterisk (*) or double asterisks (**) indicate significant different ($P < .05$) or extremely different ($P < .01$) among treatments.

1. Forskolin supplementation in the diet reduced fat accumulation in Nile tilapia.
2. Lipolysis and β−oxidation in adipocytes and hepatocytes were stimulated by 50 μM forskolin in vitro.
3. The oral doses of 0.5 and 1.5 mg/kg forskolin reduced fat in the liver and whole fish.
4. Forskolin up-regulated the expression of genes relevant to lipolysis and β−oxidation.

Conflict of interest

The authors declare no competing financial interest

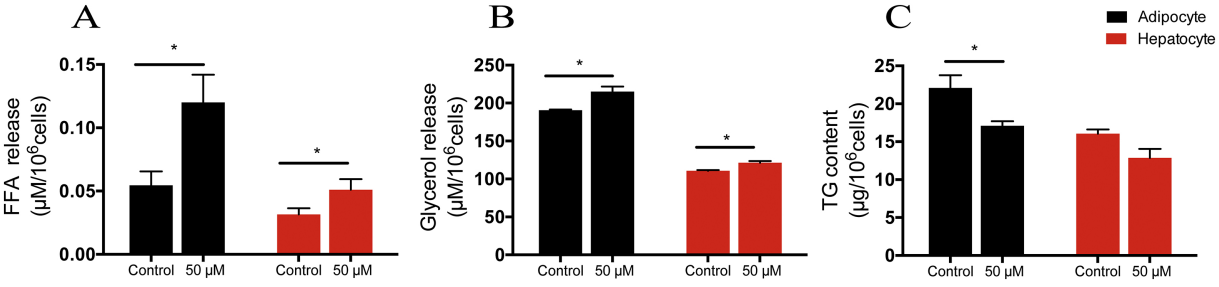
A
B
C
Adipocyte
Hepatocyte
FFA release (μM/10^6cells)
Glycerol release (μM/10^6cells)
TG content (μg/10^6cells)
0.15
0.10
0.05
0.00
250
200
150
100
50
0
25
20
15
10
5
0
*
Control
50 μM
Control
50 μM

Figure 1

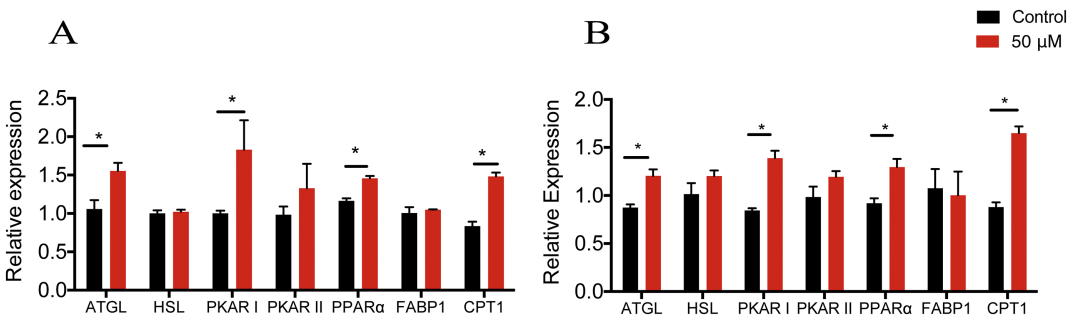
A
B
Control
50 μM
Relative expression
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
ATGL
HSL
PKAR I
PKAR II
PPARα
FABP1
CPT1

Figure 2

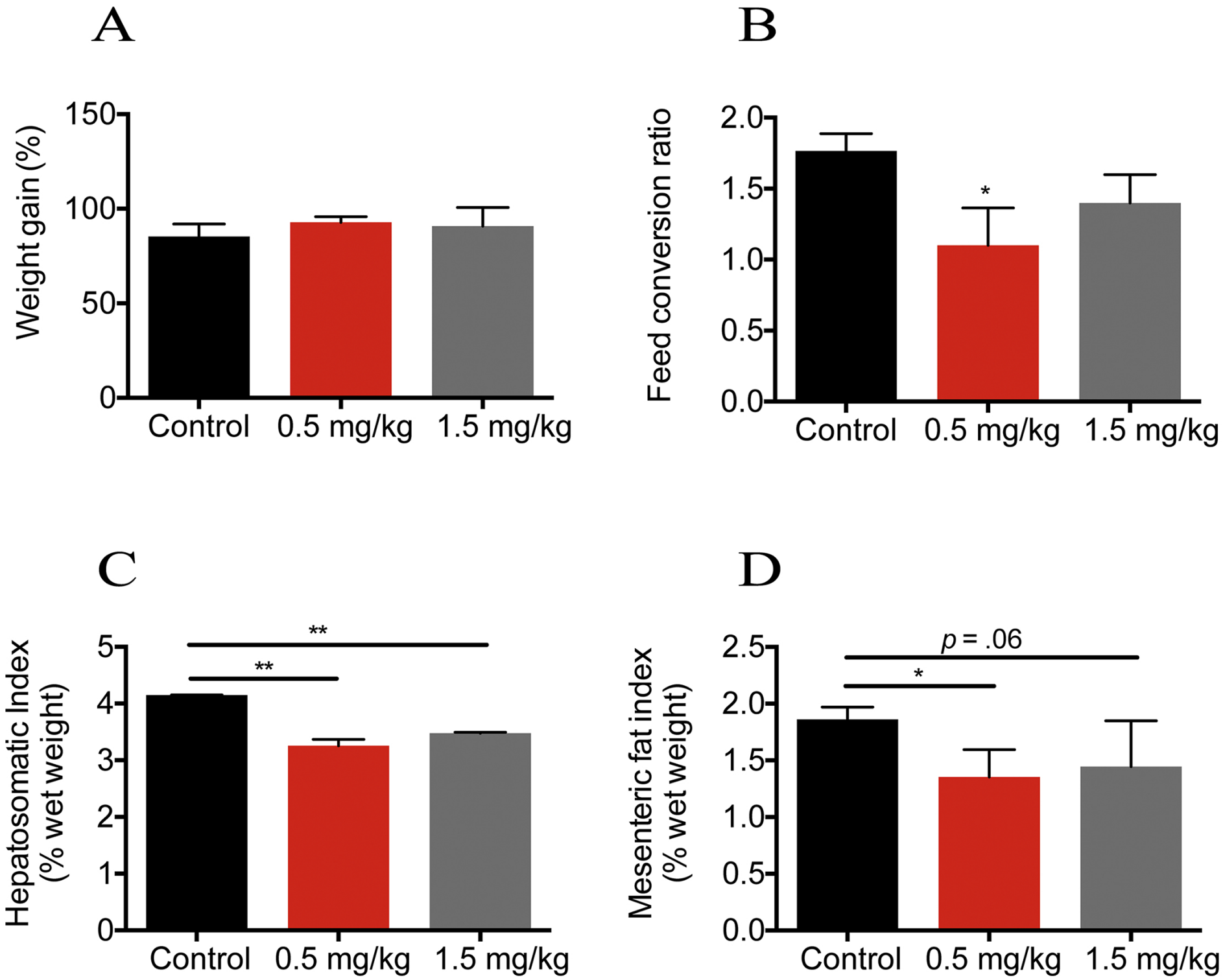
A
Weight gain (%)
150
100
50
0
Control
0.5 mg/kg
1.5 mg/kg
B
Feed conversion ratio
2.0
1.5
1.0
0.5
0.0
*
Control
0.5 mg/kg
1.5 mg/kg
C
Hepatosomatic Index
(% wet weight)
5
4
3
2
1
0
**
**
Control
0.5 mg/kg
1.5 mg/kg
D
Mesenteric fat index
(% wet weight)
2.5
2.0
1.5
1.0
0.5
0.0
p = .06
*
Control
0.5 mg/kg
1.5 mg/kg

Figure 3

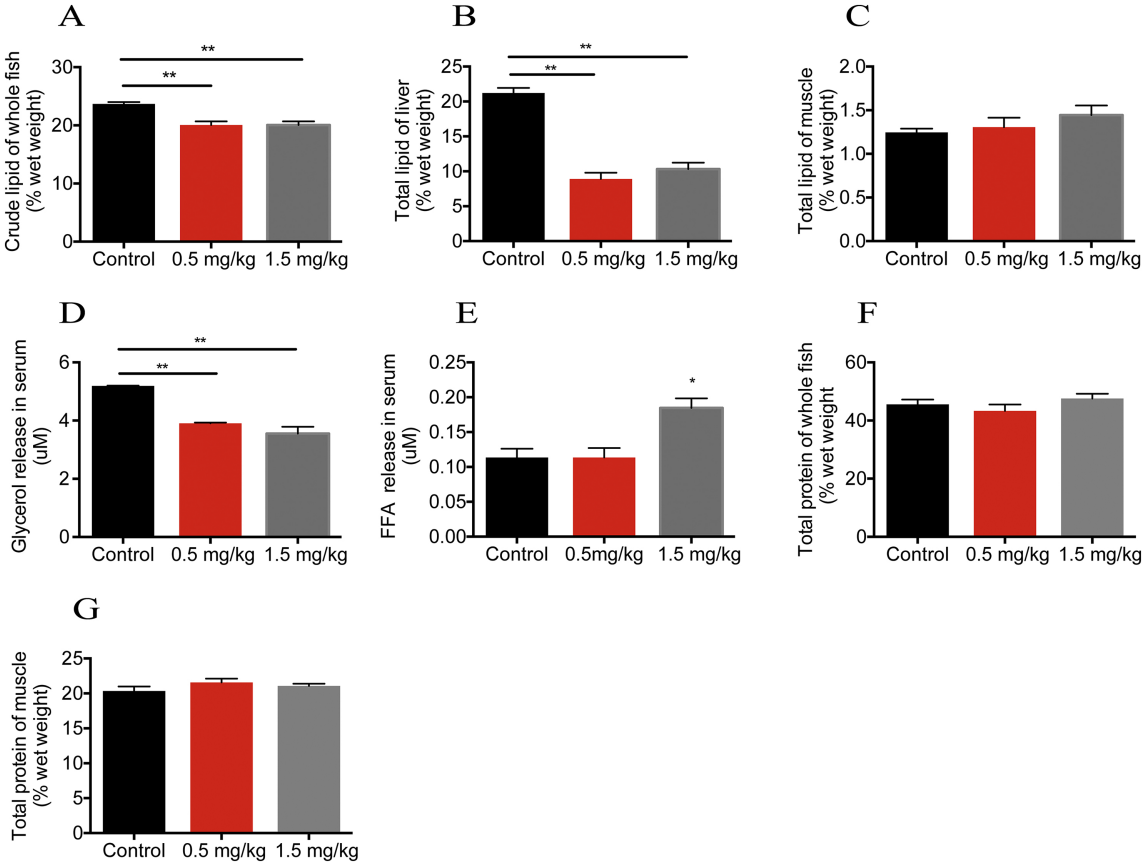
A
Crude lipid of whole fish (% wet weight)
30
20
10
0
**
**
Control
0.5 mg/kg
1.5 mg/kg
B
Total lipid of liver (% wet weight)
25
20
15
10
5
0
**
**
Control
0.5 mg/kg
1.5 mg/kg
C
Total lipid of muscle (% wet weight)
2.0
1.5
1.0
0.5
0.0
Control
0.5 mg/kg
1.5 mg/kg
D
Glycerol release in serum (uM)
6
4
2
0
**
**
Control
0.5 mg/kg
1.5 mg/kg
E
FFA release in serum (uM)
0.25
0.20
0.15
0.10
0.05
0.00
*
Control
0.5mg/kg
1.5 mg/kg
F
Total protein of whole fish (% wet weight
60
40
20
0
Control
0.5 mg/kg
1.5 mg/kg
G
Total protein of muscle (% wet weight)
25
20
15
10
5
0
Control
0.5 mg/kg
1.5 mg/kg

Figure 4

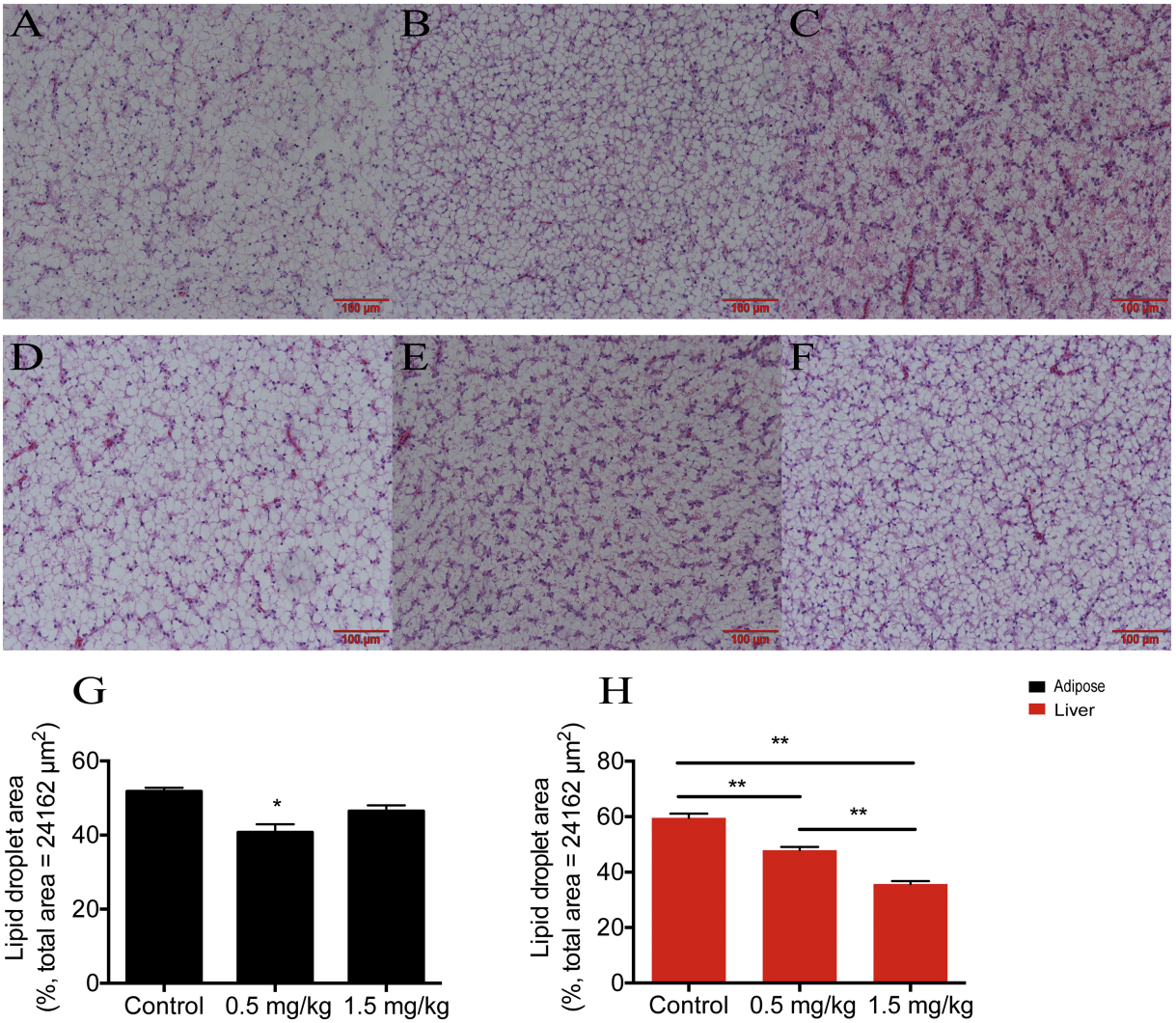
A
B
C
D
E
F
100 µm
G
Lipid droplet area
(%, total area = 24162 µm²)
60
40
20
0
*
Control
0.5 mg/kg
1.5 mg/kg
H
Adipose
Liver
80
60
40
20
0
**
**
**
Control
0.5 mg/kg
1.5 mg/kg

Figure 5

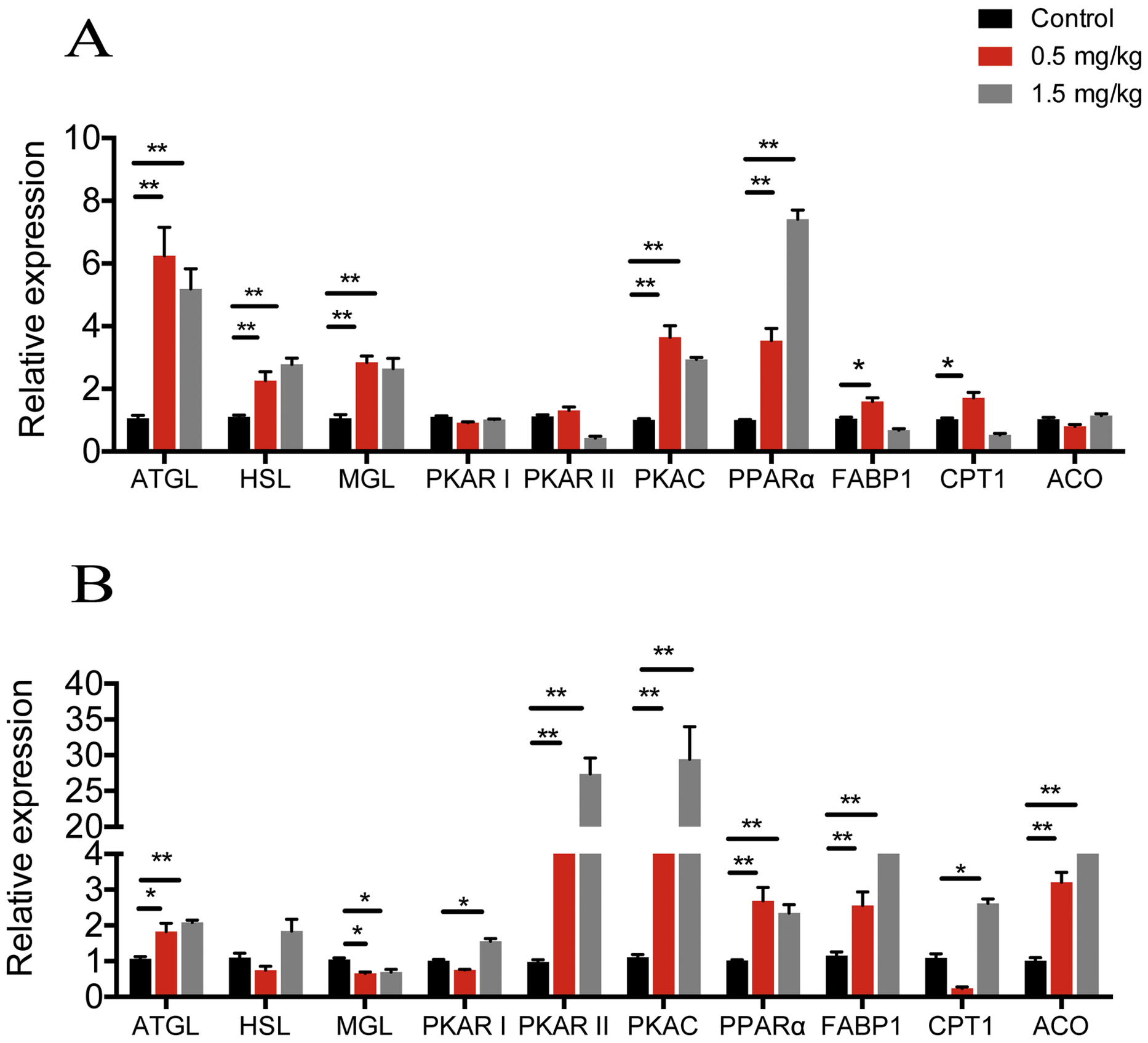
A
Control
0.5 mg/kg
1.5 mg/kg
Relative expression
10
8
6
4
2
0
ATGL
HSL
MGL
PKAR I
PKAR II
PKAC
PPARα
FABP1
CPT1
ACO
B
Relative expression
40
35
30
25
20
4
3
2
1
0
ATGL
HSL
MGL
PKAR I
PKAR II
PKAC
PPARα
FABP1
CPT1
ACO

Figure 6

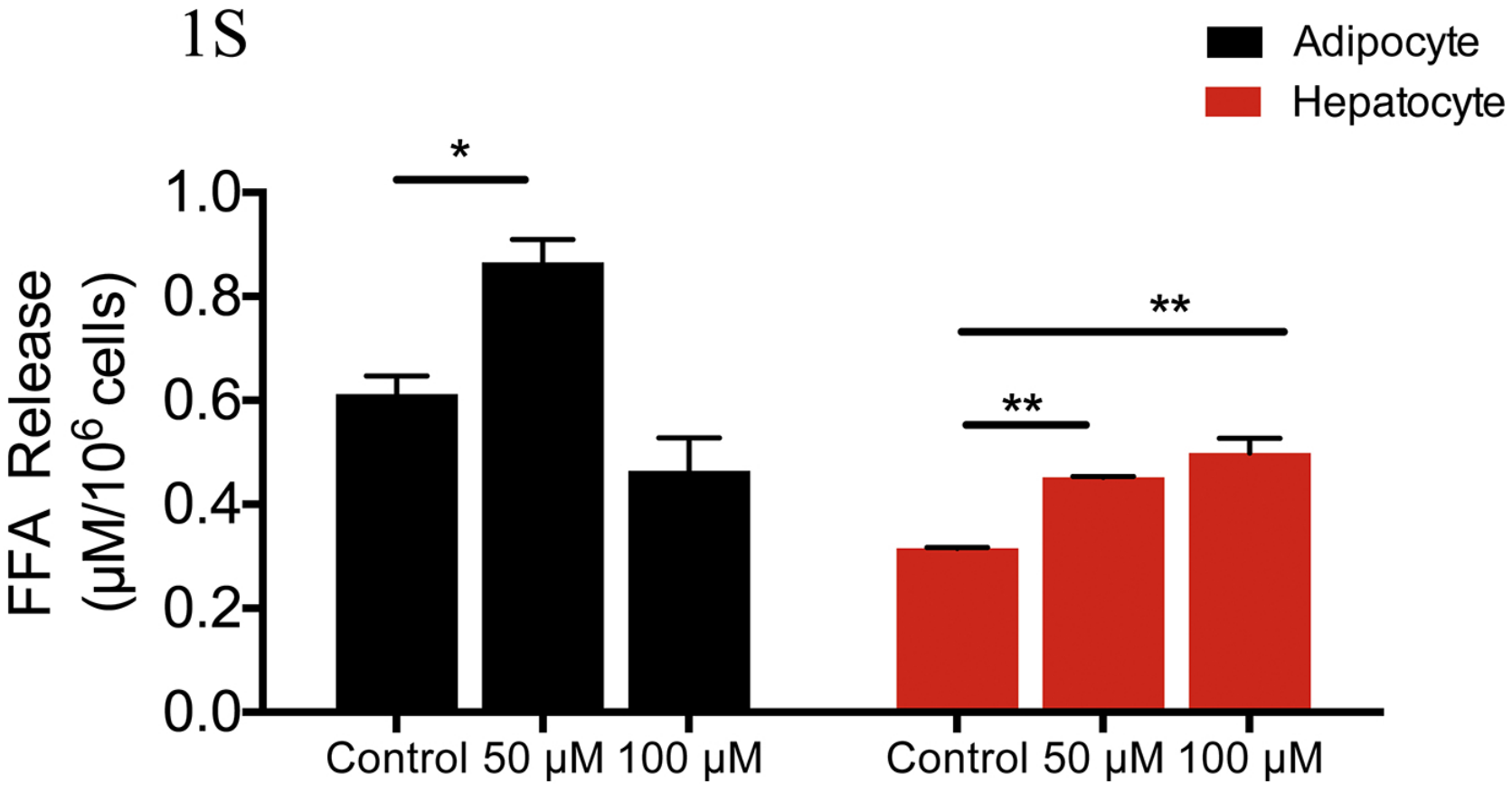
1S
Adipocyte
Hepatocyte
FFA Release
(μM/10^6 cells)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
**
Control 50 μM 100 μM
Control 50 μM 100 μM

Figure 7