

# Title

The miR-15/16 cluster is involved in the regulation of vertebrate LC-PUFA biosynthesis by targeting *ppary* as demonstrated in rabbitfish *Siganus canaliculatus*

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## Abstract

Post-transcriptional regulatory mechanisms play important roles in the regulation of long-chain ( $\geq C_{20}$ ) polyunsaturated fatty acid (LC-PUFA) biosynthesis. Here, we address a potentially important role of the miR-15/16 cluster in the regulation of LC-PUFA biosynthesis in rabbitfish *Siganus canaliculatus*. In rabbitfish, miR-15 and miR-16 were both highly responsive to fatty acids affecting LC-PUFA biosynthesis and displayed a similar expression pattern in a range of rabbitfish tissues. A common potential binding site for miR-15 and miR-16 was predicted in the 3'UTR of peroxisome proliferator-activated receptor gamma (*ppary*), an inhibitor of LC-PUFA biosynthesis in rabbitfish, and luciferase reporter assays revealed that *ppary* was a potential target of miR-15/16 cluster. *In vitro* individual or co-overexpression of miR-15 and miR-16 in rabbitfish hepatocyte line (SCHL) inhibited both mRNA and protein levels of *Ppar $\gamma$* , and increased the mRNA levels of  *$\Delta 6\Delta 5$  fads2*,  *$\Delta 4$  fads2* and *elovl5*, key enzymes of LC-PUFA biosynthesis. Inhibition of *ppary* was more pronounced with co-overexpression of miR-15 and miR-16 than with individual overexpression in SCHL. Knockdown of miR-15/16 cluster gave opposite results, and increased mRNA levels of LC-PUFA biosynthesis enzymes were observed after knockdown of *ppary*. Furthermore, miR-15/16 cluster overexpression significantly increased the contents of 22:6n-3, 20:4n-6 and total LC-PUFA in SCHL with higher 18:4n-3/18:3n-3 and 22:6n-3/22:5n-3 ratio. These suggested that miR-15 and miR-16 as a miRNA cluster together enhanced LC-PUFA biosynthesis by targeting *ppary* in rabbitfish. This is the first report of the participation of miR-15/16 cluster in LC-PUFA biosynthesis in vertebrates.

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49   **Key words**

50   miR-15/16 cluster· *ppary*· *Δ6Δ5 fads2*· *Δ4 fads2*· LC-PUFA biosynthesis· Rabbitfish

51   *Siganus canaliculatus*

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## 1. Introduction

Long-chain ( $\geq C_{20}$ ) polyunsaturated fatty acids (LC-PUFA) including arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are highly bioactive fatty acids with crucial physiological functions in humans and other animals (Janssen and Kiliaan 2014; Calder 2015). It is commonly known that fish, especially marine fish, are major dietary sources of the health promoting n-3 LC-PUFA (e.g. DHA and EPA) for human consumption (Tur et al. 2012; Nordøy and Dyerberg 2015). However, most marine fish do not possess or lack the ability to endogenously convert  $C_{18}$  PUFA such as  $\alpha$ -linolenic acid (ALA; 18:3n-3) to  $C_{20-22}$  LC-PUFA due the lower activity and/or the absence of the complete pathway for LC-PUFA biosynthesis (Tocher 2015).

The rabbitfish is an exception to the above pattern since this herbivorous marine teleost has the ability to biosynthesize LC-PUFA from  $C_{18}$  PUFA. The pathway requires a series of fatty acid desaturations and elongations catalyzed by fatty acyl desaturase (*fads*) and elongation of very-long-chain fatty acids (*elovl*) enzymes, respectively, and key enzymes required for LC-PUFA biosynthesis including  $\Delta 6\Delta 5$  *fads2*,  $\Delta 4$  *fads2*, *elovl4* and *elovl5* have been isolated and functionally characterized rabbitfish (Li et al. 2010; Monroig et al. 2012). Consequently, *S. canaliculatus* serves as a good model for studying the regulatory mechanisms of LC-PUFA biosynthesis in teleosts (Li et al. 2008). Thus, in recent years, considerable research in rabbitfish has demonstrated that *fads* and *elovl* genes were regulated by transcription factors including sterol regulatory element binding protein 1 (*srebp1*), liver X receptor (*lxr*) and hepatic nuclear factor 4

alpha (*hnf4a*), and enhanced LC-PUFA biosynthesis by increasing expression of *Δ4* and *Δ6Δ5 fads2* (Zhang et al. 2016a; Dong et al. 2016, 2018; Wang et al. 2018). In addition, the transcription factor peroxisome proliferator-activated receptor gamma (*ppary*) negatively influenced the biosynthesis of LC-PUFA in rabbitfish by down-regulating *Δ6Δ5 fads2* expression (Li et al. 2019).

Peroxisome proliferator-activated receptors such as PPAR $\gamma$  belong to the steroid hormone receptor superfamily and were so named as they can be activated by peroxisome proliferators (Mangelsdorf et al. 2000). Generally, PPAR can be activated by natural or artificial ligands, then heterodimerize with retinoid X receptor (RXR), and subsequently bind to PPAR response element (PPRE) in target genes and thereby influence transcriptional regulation (Adeghate et al. 2011). In mammals, ligand-activated PPAR $\gamma$  can positively regulate adipocyte differentiation, induce expression of lipoprotein lipase (LPL), and promote the storage of fatty acids (Heikkinen et al. 2007). In addition, PPAR $\gamma$  is a key inducer of differentiation, lipogenesis, and insulin sensitivity in white and brown adipocytes and is involved in lipid deposition in many other cell types (Poulsen et al. 2012). While these data indicate that PPAR $\gamma$  is generally a positive regulatory factor in mammalian lipid metabolism, *ppary* is a negative regulatory factor of LC-PUFA biosynthesis in rabbitfish (Li et al. 2019). *Ppars* gene have been cloned successfully from rabbitfish, and its role in the regulation of LC-PUFA synthesis has been preliminarily explored. It is speculated that *ppary* might be involved in LC-PUFA biosynthesis by regulating the key enzymes expression through the Lxr/Srebp1 pathway *in vitro* (Zhang et al. 2016a; You et al. 2017).

Further unique post-transcriptional regulatory mechanisms have been demonstrated in rabbitfish, with micro-RNAs, miR-17 and miR-146, found to be involved in the regulation of LC-PUFA biosynthesis by directly targeting *fads2* and *elovl5*, respectively (Zhang et al. 2014; Chen et al. 2018). In addition, miR-33 and miR-24 indirectly regulate LC-PUFA biosynthesis in rabbitfish through the Insig1/Srebp1 pathway by targeting *insig1* (Chen et al. 2019; Sun et al. 2019). Previous studies have revealed that miR-15 and miR-16 are highly conserved in animals and play crucial roles in apoptosis and the regulation of lipid metabolism, such as fat deposition and adipocyte differentiation (Dong et al. 2014; Fu et al. 2018; Her et al. 2011). However, nothing is currently known about the functions of miR-15 and miR-16 in the regulation of LC-PUFA biosynthesis in any vertebrate.

Here, we report that miR-15 and miR-16 were highly responsive to ALA, EPA and DHA, and bioinformatic analysis showed a common potential binding site for miR-15 and miR-16 in the 3'UTR of *ppary* in rabbitfish, which prompted further investigation into their possible roles in LC-PUFA biosynthesis. In mammals, miR-15 and miR-16 are generally found as a miRNA cluster (Lagos-Quintana 2001; Janaki et al. 2014), which often forms a polycistron, and are co-transcribed with each other along with nearby protein-coding genes (Baskerville et al. 2005). Compared with an individual miRNA, the regulation mode of miRNA clusters is more complex and its function is more efficient (Poy et al. 2004; Yu et al. 2006). Therefore, whether this was the case with miR-15 and miR-16 in the regulation of LC-PUFA biosynthesis in rabbitfish was worthy of investigation. Consequently, co-overexpression or individual overexpression

of miR-15 and miR-16 in the SCHL rabbitfish hepatic cell line were investigated to determine the potential role of the miR-15/16 cluster in the regulation of LC-PUFA biosynthesis. Furthermore, knockdown of the miR-15/16 cluster and *ppary* were carried out for further verification. The resultant data form the basis for elucidating the mechanism of miR-15/16 cluster involvement in the regulation of LC-PUFA biosynthesis in rabbitfish, and provide us with novel insights into the mechanisms of regulation of LC-PUFA biosynthesis in vertebrates, which may contribute to the optimization and/or enhancement of the LC-PUFA pathway in teleosts.

## **2. Materials and Methods**

### **2.1 Experimental animals and tissue collection**

All procedures performed on fish were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Institutional Animal Care and Use Committee of Shantou University (Guangdong, China). Samples of brain, eyes, heart, kidney, stomach, intestine, spleen, gill, muscle and liver for tissue distribution were collected from six wild rabbitfish obtained from the coast near Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China. Fish ( $196.08 \pm 5.05$  g) were fasted for 24 h and subsequently anesthetized with 0.01% 2-phenoxyethanol (Sigma-Aldrich, USA) prior to tissues being sampled. Immediately upon collection, tissue samples were frozen in liquid nitrogen and subsequently stored at  $-80^{\circ}\text{C}$  prior to analysis.

## 2.2 Cell culture

The rabbitfish *S. canaliculatus* hepatocyte line (SCHL) was previously established in our laboratory (Liu et al. 2017). Cells were cultured at 28 °C in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12, Gibco, Life Technologies, USA) containing 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES, Sigma-Aldrich, USA), 10 % fetal bovine serum (FBS, Gibco, Life Technologies, USA), 0.5 % rainbow trout *Oncorhynchus mykiss* serum (Caisson Labs), penicillin (100 U ml<sup>-1</sup>, Sigma-Aldrich, USA) and streptomycin (100 U ml<sup>-1</sup>, Sigma-Aldrich, USA). Human embryonic kidney cells (HEK 293T, Chinese Type Culture Collection, Shanghai, China) were grown in High Glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, USA) supplemented with 10 % FBS (Sijiqing Biological Engineering Material Company, China) and maintained at 37 °C with 5 % CO<sub>2</sub>.

## 2.3 Molecular cloning of miR-15/16 cluster and sequence analysis in rabbitfish

In human, miR-15 and miR-16 are clustered within 0.5 kb at 13q14 (Lagos-Quintana 2001). In order to obtain sequence information of the miR-15/16 cluster, part of the miR-15/16 cluster gene was first cloned by PCR (LA Taq, Takara, Beijing, China) using primers (1516-part-F and 1516-part-R) designed in mature sequences of miR-15 (LM379588.1) and miR-16 (LM379591.1) of zebrafish. Genomic DNA (gDNA) prepared from rabbitfish liver (DNeasy® blood & tissue kit, Qiagen, Hilden, Germany) was used as PCR template. By alignment with rabbitfish genome sequence (BGI, Shenzhen, China), a 530 bp coincident sequence between the obtained gene sequence



and the genome sequence was found. According to the genome sequence, 1058 bp upstream (1516-ups-F and 1516-ups-R) and 978 bp downstream sequences (1516-down-F and 1516-down-R) were obtained. The secondary structure of miR-15 and miR-16 was determined by RNAfold online (<http://rna.tbi.univie.ac.at/>). Phylogenetic trees were constructed on the basis of nucleotide sequence alignments between rabbitfish sca-pre-miR-15 or sca-pre-miR-16 and their orthologs using the Neighbour Joining method with MEGA 6.0.

#### **2.4 RNA isolation and quantitative real-time PCR (qPCR)**

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and concentration and quality of total RNA confirmed by spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). cDNA was synthesized from 1 µg total RNA using the miScript II RT Kit (Qiagen, Hilden, Germany). The expression of miR-15 and miR-16 were determined by quantitative PCR (qPCR) using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) with miR-15 and miR-16 specific primers (qPCR-miR-15, qPCR-miR-16) and universal primer. For qPCR measurement of *ppary* (JF502072.1), *Δ6Δ5 fads2* (EF424276.2), *Δ4 fads2* (GU594278.1) and *elovl5* (GU597350.1) mRNA expression levels, LightCycler® 480 SYBR Green I Master (Roche, Germany) was used with rabbitfish gene-specific primers (Table 1). The relative mRNA levels of each sample was normalized to 18s rRNA (AB276993) and calculated by the comparative threshold cycle method (Livak and Schmittgen 2012). All reactions were run in LightCycler® 480 thermocycler (Roche, Germany) using

qPCR programs according to the manufacturer's specifications.

## 2.5 Plasmid construction

For construction of dual luciferase reporter vectors, DNA fragments were inserted into pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) by digestion with *SacI* and *XbaI*. The recombinant vectors were: i) pmirGLO-*ppary*-3'UTR; A partial DNA fragment including the binding site of miR-15 and miR-16 in rabbitfish *ppary* 3'UTR was amplified by *ppary*-3'UTR-F/R primers and then inserted into pmirGLO vector; ii) pmirGLO-*ppary*-3'UTR-MU; A 43 nt oligonucleotide of the *ppary* 3'UTR containing a mutated binding site for miR-15 and miR-16 was synthesized (Sangon Biotech, Shanghai, China) using mutation primers named *ppary*-3'UTR-Mu-F/R, such that the predicted binding site of miR-15 and miR-16 in the *ppary* 3'UTR 5'-TGCTGCT-3' was mutated to 5'-GGTTACG-3' to prevent complementarity of miR-15/16 and then annealed and ligated into the pmirGLO vector. The PCR reactions were performed using high-fidelity *pfu* DNA polymerase (Tiangen Biotech, Beijing, China) and the insert fragments of recombinant plasmids were sequenced (Sangon Biotech).

## 2.6 Rabbitfish SCHL cells incubation with PUFA

Polyunsaturated fatty acid (Cayman Chemical Co., Ann Arbor, USA) / bovine serum albumin (BSA, fatty acid free, Cayman, USA) complexes of ALA, EPA and DHA at 10 mM concentration were prepared according to Ou et al. (2001) and stored at

–20 °C. SCHL cells were seeded into six-well plates at a density of  $5 \times 10^5$  cells per well in DMEM/F12 supplemented with 5 % FBS and 0.2 % rainbow trout serum. After 24 h, cells were incubated for 1 h in serum-free DMEM/F12 prior to incubation with 100  $\mu$ M ALA-BSA, EPA-BSA or DHA-BSA complexes in triplicate wells in serum-free medium. Each assay was incubated with equal amounts of BSA. After 24 h incubation, cells were lysed and harvested for total RNA extraction.

## **2.7 Transfection of SCHL cells with miRNA mimic or inhibitor**

miRNA mimics or inhibitors were transfected into SCHL cells to achieve up-regulation or down-regulation of miRNA expression, respectively. The miRNA mimics (dsRNA oligonucleotides), miRNA inhibitors (single-stranded oligonucleotides) and NC oligonucleotides (negative control) were obtained from Genepharma (Shanghai, China). SCHL cells were seeded into 6-well plates or 100 mm vessels, grown for 24 h to 80 % confluence in DMEM/F12 supplemented with 10 % FBS and 0.5 % rainbow trout serum, and triplicate wells transfected with 50 or 150 nM of each oligonucleotide using Lipofectamine<sup>®</sup> 2000 Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After transfection for 24 h or 48 h, cells were harvested for qPCR analysis and Western blotting.

## **2.8 Dual-luciferase experiment to confirm the interaction between miR-15/16 and *ppary***

To determine whether *ppary* was a direct target gene of miR-15 and miR-16, a dual

luciferase assay was performed using human embryonic kidney cells (HEK 293T; Chinese Type Culture Collection, Shanghai, China) seeded in 96-well cell culture plates. Cells were grown for 24 h to 80 % confluence and then co-transfected with either miRNA mimics (50 nM) or NC (50 nM) with different recombinant dual luciferase reporter vectors (50 ng) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). Firefly and Renilla luciferase activities were quantified after 48 h transfection using a microplate reader (Infinite M200 Pro, Tecan, Switzerland) with firefly luciferase activity normalized to Renilla luciferase activity. Eight replicate wells were used for each treatment.

## **2.9 Western blotting**

For miR-15/16 cluster target identification at the protein level, Western blotting was used to detect the protein expression level of Ppar $\gamma$ . Total protein was extracted at 48 h post-transfection using cell total protein extraction kit (Sangon Biotech) and concentrations quantified with non-interference protein assay kit (Sangon Biotech). Aliquots of protein (20~40 $\mu$ g) were loaded and separated on a 10 % sodium dodecyl sulphate-polyacrylamide gel (SDS/PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) with a semidry transfer cell (Bio-Rad Trans Blot SD, USA). After incubating in blocking buffer (Tris-Buffered Saline Tween (TBST) containing 5% dried skimmed milk powder) for 2 h at room temperature, membranes were incubated at 4 °C overnight with rabbit polyclonal antibody against human PPAR $\gamma$  (1:500; predicted molecular weight: ~54 kDa) (Wanleibio, Shenyang, China), and

mouse monoclonal antibody against  $\beta$ -actin (1:2000; ~42 kDa) (Immunoway, USA). After three washes with TBST, membranes were incubated with HRP goat anti-rabbit/mouse IgG (Abcam, USA) secondary antibodies at a ratio of 1:5000. Membranes were washed three times with TBST, and immunoreactive bands were visualized using the Amersham Imager 600 (GE Healthcare, USA) and the intensity of each band analyzed with Image J software (version 1.8.0, NIH, Bethesda, MD, USA). The optical density of each sample was normalized by  $\beta$ -actin for statistical analysis, and three independent experiments were conducted.

## 2.10 RNA interference

Silencing of *ppary* expression was performed using small interfering RNA (siRNA) duplexes (Genepharma, Shanghai, China) with the following sequences: si-*ppary* sense, 5'-CCUCCCAAACAGUCAGAUUdTdT-3'; si-*ppary* antisense, 5'-AAUCUGACUGUUUGGGAGGdTdT-3'. The SCHL cells were seeded into 6-well plates, grown for 24 h to 80 % confluence and subsequently transfected with 50 nM of *ppary*-specific siRNA (siRNA-*ppary*) or negative control using Lipofectamine<sup>®</sup> 2000 Reagent. The cells were harvested for qPCR analysis at 24 h post-transfection.

## 2.11 Fatty acids isolation and GC analysis

SCHL cells were seeded into 100 mm plates at a density of  $2 \times 10^6$  cells per plate or six-well plates at a density of  $5 \times 10^5$  cells per well, grown for 24 h to 80 % confluence, and triplicate wells transfected with 150 or 50 nM miRNA mimics or NC using

Lipofectamine<sup>®</sup> 2000 Reagent (Invitrogen, Carlsbad, CA, USA). After 48 h post-transfection, SCHL cells were harvested for qPCR and fatty acid composition analysis. Fatty acid composition of cell total lipid was analyzed by gas chromatography (GC) after chloroform/methanol extraction, saponification and methylation with boron trifluoride (Sigma-Aldrich, USA) as described previously (Li et al. 2010; Chen et al. 2016). For identification, the retention times of the fatty acid methyl esters were compared to those of standards (Sigma-Aldrich, USA), with quantification of each fatty acid in a certain number of cells ( $10^7$  cells) being estimated using the signal of the internal standard C21:0 (heptadecanoic acid) (Sigma-Aldrich). Fatty acid contents were expressed as a percentage of total fatty acids (Table 2).

## 2.12 Statistical analysis

All data were presented as means  $\pm$  SEM with n value as stated. Significance of differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's t-test at a significance level of  $P < 0.05$  using SPSS 19.0 software (SPSS Inc, Chicago, IL).

## 3. Results

### 3.1 The sequence, structure and tissue distribution of rabbitfish miR-15 and miR-16

Based on rabbitfish genomic data, the gene sequence of the miR-15/16 cluster was cloned, and mature miR-15 and miR-16 encoding capability was found to be clustered

within 0.5 kb (Fig. 1). In rabbitfish, miR-16 is located upstream of miR-15 (Fig. 1), which was consistent with that of human (Lagos-Quintana 2001). Through multiple alignment with its orthologs in other species, the precursor and mature sequences of miR-15 and miR-16 were identified, and a 7 nt common “seed sequence” (AGCAGCA), which is pivotal for target recognition of miRNA, was identified at the 5' end of the rabbitfish miR-15 and miR-16 (Supplementary Fig. S1 and S2). The rabbitfish sca-pre-miR-15 (58 nt) and sca-pre-miR-16 (80 nt) contained the typical stable stem-loop secondary structure necessary for mature miRNA processing (Supplementary Fig. S3). Phylogenetic analysis showed that sca-pre-miR-15 and sca-pre-miR-16 clustered together with those of other fish species and have close homologous relationships with zebrafish and Atlantic salmon (Fig. 2).

To determine whether miR-15 and miR-16 co-transcribed with each other in rabbitfish, the abundance of miR-15 and miR-16 mRNA were determined in selected tissues. The results showed that miR-15 and miR-16 were widely expressed in the examined tissues with highest expression level in brain, and intermediate levels in stomach, intestine, gill, kidney, spleen, eye and heart, and low expression in muscle and liver (Fig. 3).

### **3.2 miR-15 and miR-16 show similar responses to PUFA in rabbitfish SCHL cells *in vitro***

In order to study the role of the miR-15/16 cluster in LC-PUFA biosynthesis, SCHL cells were incubated with 100μM ALA, EPA and DHA. *In vitro*, the expression levels

of both miR-15 and miR-16 were higher in SCHL cells supplemented with ALA, with the expression of miR-15 significantly higher compared to BSA controls (Fig. 4a). The expression levels of both miR-15 and miR-16 were lower in SCHL cells supplemented with EPA or DHA, with the expression of miR-16 significantly lower compared to BSA controls (Fig. 4b and 4c).

### 3.3 Rabbitfish *ppary* is a target of miR-15 and miR-16

Bioinformatic analysis showed that potential binding sites of miR-15 and miR-16 were present in the 3'UTR of *ppary* in rabbitfish (Fig. 5a). Based on this finding, dual luciferase assays were used to verify the interaction between the miR-15/16 cluster and *ppary*. Results from the qPCR analysis revealed that HEK 293T cells transfected with miR-15 and miR-16 mimics had levels of rabbitfish miR-15 and miR-16 expression 16-fold and 11-fold higher than NC, respectively ( $P < 0.01$ ) (Fig. 5b). If the heterologous expression of miRNA interacts with the inserted target fragment, the luciferase activity will be reduced. As shown in Fig. 5c, heterologous expression of miR-15, miR-16 and miR-15/16 mimics effectively reduced luciferase activities when co-transfected with pmirGLO-*ppary*-3'UTR reporter plasmid into HEK 293T cells (Fig. 5c, lanes 5-8). In particular, the luciferase activity was lowest with co-transfection of the miR-15/16 mimic (Fig. 5c, lane 8). However, when mutation was introduced into the predicted miR-15 and miR-16 binding sites in the 3'UTR of *ppary* mRNA, the inhibition was eliminated (Fig. 5c, lanes 9-12). Additionally, the Ppary protein levels in SCHL cells transfected with miR-15 mimic, miR-16 mimic and miR-15/16 mimic were lower than



that of cells transfected with NC (Fig. 6). In particular, the inhibition effect of miR-15/16 mimic on Ppar $\gamma$  protein expression was stronger than that of individual miR-15 mimic and miR-16 mimic. In summary, these data demonstrate that *ppar $\gamma$*  may be a common target gene of miR-15 and miR-16.

### **3.4 miR-15/16 cluster up-regulates expression of *$\Delta 6\Delta 5$ fads*, *$\Delta 4$ fads* and *elovl5* by targeting *ppar $\gamma$* in rabbitfish hepatocytes**

The potential role of the miR-15/16 cluster in the regulation of LC-PUFA biosynthesis was further investigated by overexpression and knockdown of miR-15/16 cluster. The qPCR analysis showed that the expression of target gene *ppar $\gamma$*  was reduced, whereas the expression levels of genes of key enzymes involved in LC-PUFA synthesis, i.e.  *$\Delta 6\Delta 5$  fads2*, *elovl5*,  *$\Delta 4$  fads2*, were increased by overexpression of the miR-15/16 cluster in SCHL cells (Fig. 7a). In contrast, the expression levels of  *$\Delta 6\Delta 5$  fads2*, *elovl5* and  *$\Delta 4$  fads2* mRNAs were significantly reduced, and the expression of *ppar $\gamma$*  increased, by miR-15/16 cluster knockdown in SCHL cells (Fig. 7b). In consequence, miR-15/16 cluster can promote LC-PUFA biosynthesis in rabbitfish hepatocytes.

To investigate whether the miR-15/16 cluster was involved in LC-PUFA biosynthesis by targeting *ppar $\gamma$* , the expression of *ppar $\gamma$*  was silenced by RNA interference technology. After silencing, the expression of *ppar $\gamma$*  was reduced significantly, whereas the expression levels of key enzyme genes involved in LC-PUFA synthesis, i.e.  *$\Delta 6\Delta 5$  fads2*, *elovl5* and  *$\Delta 4$  fads2*, were increased with the expression of  *$\Delta 6\Delta 5$  fads2*, and  *$\Delta 4$  fads2* being significantly higher (Fig. 8).

### 3.5 Up-regulation of the miR-15/16 cluster promoted biosynthesis of LC-PUFA in rabbitfish hepatocytes

Whether decreasing the endogenous level of *ppary* by overexpression of miR-15/16 cluster affected LC-PUFA biosynthesis was assessed in SCHL cells *in vitro*. Co-overexpression or individual overexpression of miR-15 and miR-16 in SCHL cells resulted in 16-fold and 13-fold higher levels of miR-15 in cells receiving miR-15 mimic and miR-15/16 mimic, respectively, as well as 11-fold and 8-fold higher levels of miR-16 in cells receiving miR-16 mimic and miR-15/16 mimic, respectively ( $P < 0.01$ ), at 48 h post transfection compared to NC (Fig. 9a). In addition to lower levels of *ppary* mRNA by overexpression of miR-15/16 cluster in SCHL cells (Fig. 9b). Fatty acid analysis showed that miR-15 overexpression resulted in higher conversion of ALA to C18:4n-3 and C22:5n-3 to DHA. Overexpression of miR-16 and co-overexpression of miR-15/16 cluster both resulted in higher conversion of ALA to C18:4n-3, EPA to C22:5n-3 and C22:5n-3 to DHA (Fig. 9b). Compared with the NC group, the contents of DHA and ARA, products of LC-PUFA biosynthesis, and total LC-PUFA accumulation in SCHL cells increased either by co-overexpression or individual overexpression of miR-15 and miR-16, and among them, the accumulation level of LC-PUFA was highest with co-overexpression of miR-15 and miR-16 (Table 2).

## 4. Discussion

Post-transcriptional regulatory mechanisms have been shown to play important

roles in the regulation of LC-PUFA biosynthesis in rabbitfish (Zhang et al. 2014, 2016b; Chen et al. 2018, 2019; Sun et al. 2019). However, the mechanisms of post-transcriptional regulation of LC-PUFA biosynthesis by miRNAs remains largely unclear, and nothing is known on the regulation of LC-PUFA biosynthesis by miRNA clusters. Here, we investigated a potentially important role of miR-15/16 cluster in the regulation of LC-PUFA biosynthesis in rabbitfish.

Previous studies have revealed that miR-15 and miR-16 are highly conserved in animals and play crucial roles in the regulation of lipid metabolism including fat deposition and adipocyte differentiation (Dong et al. 2014; Fu et al. 2018; Her et al. 2011). In humans, miR-15 and miR-16 belong to a common precursor family and are highly conserved, and clustered within 0.5 kb at 13q14 (Lagos-Quintana 2001). In the present study, we cloned the sequence of the miR-15/16 cluster in rabbitfish, and found that miR-15 and miR-16 were clustered on the same chromosome within 0.5 kb. Multiple alignment with its orthologs in other species showed the mature sequences of miR-15 (22 nt) and miR-16 (23 nt) are highly conserved in rabbitfish. Generally, miRNAs depend on the "seed sequence" to identify and partially combine with the 3'UTR of target genes, thereby inducing target mRNA degradation or inhibiting protein translation. The seed sequences of rabbitfish miR-15 and miR-16 showed high identity to those of other species, which suggested that miR-15 and miR-16 may perform similar functions in rabbitfish. In addition, miRNA clusters often form a polycistron, and co-transcribe with each other along with nearby protein-coding genes in mammals (Baskerville et al. 2005). In the present study, miR-15 and miR-16 displayed a similar

expression pattern in different rabbitfish tissues, as well as in hepatocytes incubated with different PUFA that influence LC-PUFA biosynthesis. These above evidences indicated that miR-15 and miR-16 as a miRNA cluster may be co-transcribed with each other and have a combined effect on LC-PUFA biosynthesis in rabbitfish.

To further understand how the miR-15/16 cluster was involved in the regulation of LC-PUFA biosynthesis in rabbitfish, bioinformatic analysis showed that common potential binding sites of miR-15 and miR-16 were predicted in the 3'UTR of *ppary*, which is the transcription factor that plays a role in the negative regulation of LC-PUFA biosynthesis in rabbitfish (Li et al. 2019). Dual luciferase assays revealed that *ppary* may be a direct target gene of miR-15 and miR-16. *In vitro*, overexpression of miR-15 and miR-16 significantly decreased both the mRNA and protein abundance of Ppar $\gamma$  in SCHL, but suggested that the negative regulation of miR-15 and miR-16 predominantly occurred at the translational level since the effect on protein level was greater than on mRNA level. It was demonstrated previously that, compared with an individual miRNA, regulation by miRNA clusters was more complex and functionally more efficient (Poy et al. 2004; Yu et al. 2006). A similar situation was also found in rabbitfish as the decrease of Ppar $\gamma$  expression in SCHL cells was more significant with co-overexpression of miR-15 and miR-16 than that with overexpression of miR-15 or miR-16 individually, suggesting that *ppary* may be a common direct target gene of both miR-15 and miR-16. Moreover, co-overexpression or individual overexpression of miR-15 and miR-16 in SCHL cells increased the mRNA levels of  *$\Delta 6\Delta 5$  fads2*,  *$\Delta 4$  fads2* and *elovl5*. Consistent with this, inhibition of miR-15 and/or miR-16 resulted in the opposite

effect, decreased expression of *Δ6Δ5 fads2*, *Δ4 fads2* and *elovl5*. However, individual overexpression of miR-15 or miR-16 had a stronger regulatory effect on the mRNA levels of the LC-PUFA biosynthesis enzymes than that of co-overexpression of miR-15 and miR-16. Elovl and Fad enzymes are considered as the key enzymes for the biosynthesis of LC-PUFA and the enzymes activities will ultimately affect the LC-PUFA biosynthetic capability (Tocher et al. 2003). Previous studies demonstrated that Elovl and Fad enzymes were regulated by multiple transcriptional factors in vertebrates, such as Srebp1, Hnf4α, Lxra and Pparγ, and negative feedback mechanisms may be present in the overexpression system (Chen et al. 2019; Sun et al. 2019; Zhang et al. 2016a). This may be one reason why the effect on expression of these key enzyme genes was lower in cells co-transfected with both miR-15 and miR-16 than in cells transfected with miR-15 or miR-16 individually. In SCHL, PPAR agonists 2-bromopalmitate (2-Bro) and fenofibrate (FF) increased the expression of *pparγ*, and induced the expression changes of *Δ6Δ5 fads2*, *Δ4 fads2* and *elovl5*, which indicated that *pparγ* might be involved in LC-PUFA biosynthesis by regulating the key enzymes expression in rabbitfish (You et al. 2017). In the present study, silencing the expression of *pparγ* and along with that the levels of *Δ6Δ5 fads2*, *Δ4 fads2* and *elovl5* mRNAs were significantly increased. Based on the above data, we therefore speculated that miR-15 and miR-16 may participate together in the regulation of LC-PUFA biosynthesis in rabbitfish by targeting *pparγ*.

In order to investigate the potential role of the miR-15/16 cluster in the regulation of LC-PUFA biosynthesis in rabbitfish, we investigated whether decreasing *pparγ* by

overexpression of miR-15/16 affected LC-PUFA biosynthesis in SCHL cells. As stated above, down-regulation of *ppary* by miR-15 and miR-16 overexpression increased the contents of DHA and ARA, products of LC-PUFA biosynthesis, and also total LC-PUFA accumulation in SCHL cells. In rabbitfish, functional characterization showed that  $\Delta 6/\Delta 5$  Fads2 could efficiently convert 18:3n-3 and 18:2n-6 to 18:4n-3 and 18:3n-6, respectively (Li et al. 2010). Here, we observed that overexpression of miR-15 and miR-16 caused an increase in 18:4n-3/18:3n-3, which indicates an increase in  $\Delta 6\Delta 5$  Fads2 enzymatic activity. This was consistent with the results of our previous study in which knock-down of *ppary* in SCHL increased conversion of 18:3n-3 to 18:4n-3 and 18:2n-6 to 18:3n-6, while overexpression of *ppary* led to lower conversions, and ultimately to significantly lower ARA, EPA and DHA production (Li et al. 2019). In some basal vertebrate lineages, such as teleosts, the production of DHA from EPA can occur directly via a  $\Delta 4$  desaturase that produces DHA from the EPA elongation product, 22:5n-3 (Li et al. 2010; Castro et al. 2016). We showed that overexpression of miR-15 and miR-16 caused an increase in ratio of 22:6n-3/22:5n-3 in rabbitfish hepatocyte cells, which indicates an increase in  $\Delta 4$  Fads2 enzymatic activity. These were consistent with the above results of mRNA expression in SCHL. However, individual overexpression of miR-15 or miR-16 had a stronger regulatory effect on the conversion ratio levels of the LC-PUFA biosynthesis enzymes than that of co-overexpression of miR-15 and miR-16. In the above discussion, it has been mentioned that Elovl and Fad enzymes were regulated by multiple transcriptional factors in vertebrates, such as Srebp1, Hnf4 $\alpha$ , Lxr $\alpha$  and Ppar $\gamma$ , and negative feedback mechanisms may be present in the overexpression

system, which might led to the above results (Chen et al. 2019; Sun et al. 2019; Zhang et al. 2016a). As expected, co-overexpression of miR-15 and miR-16 resulted in the highest accumulation of LC-PUFA, which suggested that there might be cooperativity among the miR-15/16 cluster to promote LC-PUFA biosynthesis in rabbitfish. Taken together, these results suggested that the miR-15/16 cluster could promote LC-PUFA biosynthesis by negatively regulating *ppary* activation, and subsequently, promoted the expression of *ppary* target genes required for LC-PUFA biosynthesis.

In summary, we confirmed that miR-15 and miR-16 are present in a miRNA cluster and together enhanced LC-PUFA biosynthesis by targeting *ppary* in rabbitfish. To our knowledge, this is the first report of the cooperativity between miR-15 and miR-16 in the regulation of LC-PUFA biosynthesis in a vertebrate. How exactly miR-15 and miR-16 are co-transcribed and combine to function together requires further investigation.

## Acknowledgements

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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**Table 1** Primers or oligonucleotides used for gene clone, qPCR or vector reconstruction

Aim	Gene/Vector name	Primers/Oligonucleotides	Nucleotide sequence
<b>miR-15/16 cluster gene clone</b>	partial sequence	1516-part-F	TAGCAGCACGTAAATATTGGAG
		1516-part-R	CACAAACCATTCTGTGCTGCTA
	upstream sequence	1516-ups-F	AAATACGTTCACTGGGCA
		1516-ups-R	GGGTAGGAATCTGGTCTTCTA
	downstream sequence	1516-down-F	CTTCCTTCTCTGCCCTCATC
		1516-down-R	GTTTCAGGACTCGCTTCTATGT
<b>Construction of reporter vectors</b>	pmirGLO-ppary-3'UTR	ppary-3'UTR-F	CCCGGGTCTAGAAAGGTGGACATGTGCTT ACATC
		ppary-3'UTR-R	CCCGGGGAGCTCCTGTCTGGCTACTTTCT TTATTCATC
	pmirGLO-ppary-3'UTR-MU	ppary-3'UTR-MU-F	CCCGGGTCTAGACTTGTGAAATTTGACAA GAAAAAGGTTACGC
		ppary-3'UTR-MU-R	CCCGGGGAGCTCCTGTCTGGCTACTTTCT TTATTCATC
	miR-15	qPCR-miR-15	TAGCAGCACAGAAUGGTTTGTG
		qPCR-miR-16	TAGCAGCACGTAAATATTGGAG
<b>Q-PCR</b>	miR-16	$\Delta 6\Delta 5 fads2$ -F	TCACTGGAACCTGCCCACAT
		$\Delta 6\Delta 5 fads2$ -R	TTCATTCTCAGACAGTGCAAACAG
	<i>elovl5</i>	<i>elovl5</i> -F	GCACTCACCGTTGTGTATCT
		<i>elovl5</i> -R	GCAGAGCCAAGCTCATAGAA
	<i>ppary</i>	<i>ppary</i> -F	CTGCTGGCTGAGTTCTCGTCT
		<i>ppary</i> -R	ATGACAAAAGGCGCGTTATCTC
	18S	18S-F	CGCCGAGAAGACGATCAAAC
		18S-R	TGATCCTTCCGCAGGTTAC

615 Notes: The underscore indicates the restriction site in primers

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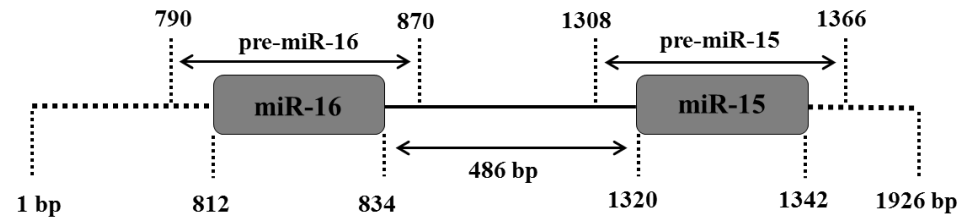
**Table 2** Fatty acid composition in rabbitfish hepatocytes transfected with miR-15/16 mimic and negative control (NC) ( $\mu\text{g}/10^7$  cells)

Fatty acids composition	Treatments			
	NC	miR-15 mimic	miR-16 mimic	miR-15/16 mimic
C14:0	1.46 $\pm$ 0.16	1.78 $\pm$ 0.20	1.22 $\pm$ 0.17	1.93 $\pm$ 0.30
C16:0	27.57 $\pm$ 2.91	35.19 $\pm$ 5.92	29.46 $\pm$ 7.29	35.16 $\pm$ 5.77
C18:0	15.06 $\pm$ 1.65	18.94 $\pm$ 3.22	17.28 $\pm$ 2.74	19.98 $\pm$ 2.61
C18:1n-9	17.48 $\pm$ 1.76 <sup>a</sup>	18.92 $\pm$ 1.68 <sup>ab</sup>	22.23 $\pm$ 0.12 <sup>b</sup>	20.51 $\pm$ 1.19 <sup>ab</sup>
C18:2n-6	3.45 $\pm$ 0.29	3.77 $\pm$ 0.39	3.37 $\pm$ 0.30	4.16 $\pm$ 0.39
C18:3n-6	0.72 $\pm$ 0.07	0.54 $\pm$ 0.12	0.44 $\pm$ 0.08	0.63 $\pm$ 0.09
C18:3n-3	0.60 $\pm$ 0.07 <sup>a</sup>	0.43 $\pm$ 0.08 <sup>ab</sup>	0.30 $\pm$ 0.03 <sup>b</sup>	0.46 $\pm$ 0.09 <sup>ab</sup>
C18:4n-3	1.59 $\pm$ 0.37	1.27 $\pm$ 0.29	1.00 $\pm$ 0.04	1.47 $\pm$ 0.21
C20:2n-6	1.02 $\pm$ 0.18 <sup>a</sup>	0.62 $\pm$ 0.14 <sup>ab</sup>	0.47 $\pm$ 0.04 <sup>b</sup>	0.63 $\pm$ 0.15 <sup>ab</sup>
C20:3n-6	1.28 $\pm$ 0.09	1.23 $\pm$ 0.10	1.32 $\pm$ 0.08	1.37 $\pm$ 0.09
C20:4n-6(ARA)	5.99 $\pm$ 0.40	6.52 $\pm$ 0.65	7.20 $\pm$ 0.16	7.11 $\pm$ 0.39
C20:3n-3	0.58 $\pm$ 0.10	0.35 $\pm$ 0.03	0.29 $\pm$ 0.01	0.58 $\pm$ 0.17
C20:5n-3 (EPA)	4.93 $\pm$ 0.68 <sup>ab</sup>	5.82 $\pm$ 0.56 <sup>a</sup>	3.69 $\pm$ 0.32 <sup>b</sup>	4.14 $\pm$ 0.45 <sup>ab</sup>
C22:5n-3	1.49 $\pm$ 0.27	1.53 $\pm$ 0.35	1.72 $\pm$ 0.37	1.66 $\pm$ 0.36
C22:6n-3(DHA)	9.91 $\pm$ 1.09 <sup>a</sup>	10.70 $\pm$ 0.97 <sup>ab</sup>	13.52 $\pm$ 0.20 <sup>b</sup>	13.27 $\pm$ 0.83 <sup>b</sup>
$\Sigma$ SFA	44.10 $\pm$ 4.59	55.92 $\pm$ 9.18	47.95 $\pm$ 10.62	57.07 $\pm$ 8.50
$\Sigma$ MUFA	17.48 $\pm$ 1.76 <sup>a</sup>	18.92 $\pm$ 1.68 <sup>ab</sup>	22.23 $\pm$ 0.12 <sup>b</sup>	20.51 $\pm$ 1.19 <sup>ab</sup>
$\Sigma$ LC-PUFA	24.89 $\pm$ 1.29	27.19 $\pm$ 1.39	29.02 $\pm$ 0.85	29.62 $\pm$ 0.99
C18:4n-3/C18:3n-3	2.61 $\pm$ 0.48	2.90 $\pm$ 0.24	3.45 $\pm$ 0.42	3.26 $\pm$ 0.21
C22:5n-3/C20:5n-3	0.32 $\pm$ 0.09	0.28 $\pm$ 0.07	0.45 $\pm$ 0.10	0.38 $\pm$ 0.08
C22:6n-3/C22:5n-3	6.90 $\pm$ 0.93	7.53 $\pm$ 1.36	8.93 $\pm$ 1.51	10.40 $\pm$ 1.57

Notes: Data are means  $\pm$  SEM (n = 3). Different superscript letters within a row represent significant differences ( $P < 0.05$ ; t-test). SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid

**Figures**

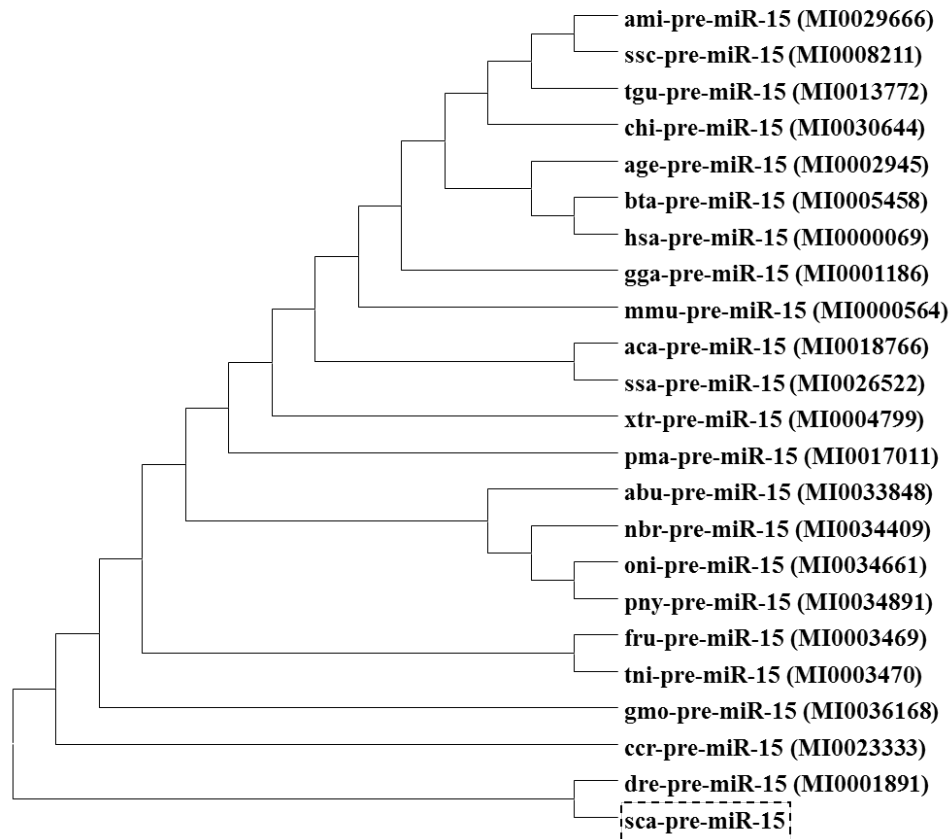
**Fig. 1**



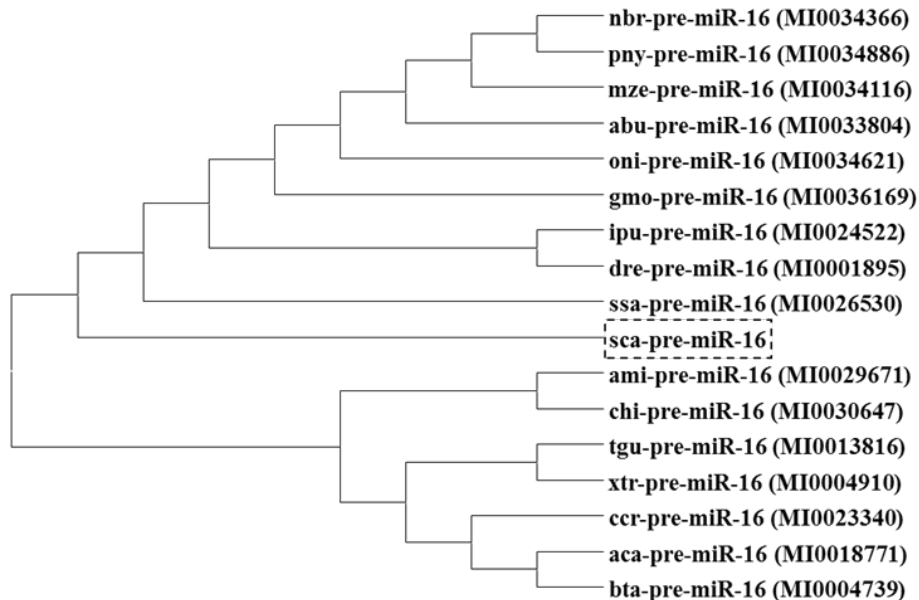
**Fig. 1** The genomic structure of the rabbitfish miR-15/16 cluster. A 1926 bp gene fragment encompassing the miR-15/16 cluster was obtained. The two mature miRNAs of miR-15/16 cluster are predicted (gray boxes). Sequences between mature miRNAs including non-coding regions are indicated by black lines and the nucleotide sequence at both ends are indicated by broken line. The sequence encoding pre-miR-15 and pre-miR-16 have been noted by horizontal arrows. The vertical dotted lines with numbers indicate the positions of each element at the 1926 bp gene fragment

**Fig. 2**

**(a)**



**(b)**



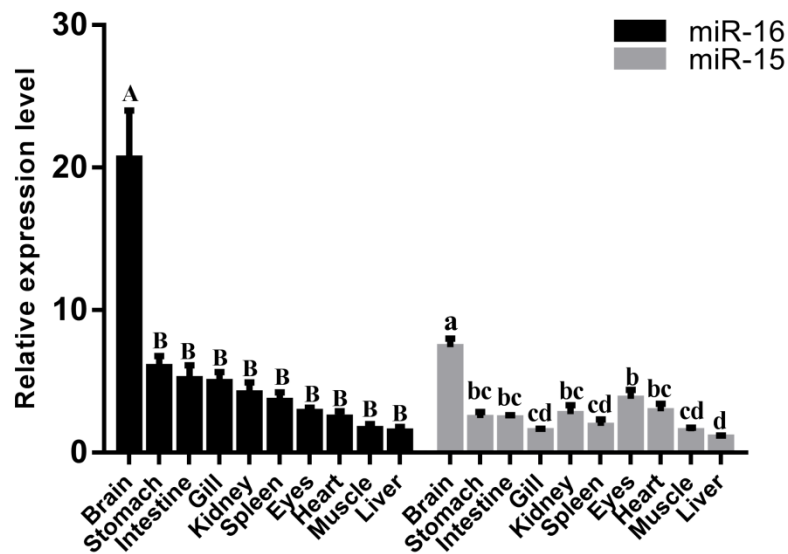
**Fig. 2** Phylogenetic trees were constructed to compare the putative rabbitfish pre-miR-15 (sca-pre-miR-15) (a) and pre-miR-16 (sca-pre-miR-16) (b) nucleotide sequences with their orthologs in American alligator (*Alligator mississippiensis*, ami), Wild boar (*Sus scrofa*, ssc), Zebra finch



643 (*Taeniopygia guttata*, tgu), Goat (*Capra hircus*, chi), Ateles geoffroy (*Ateles geoffroyi*, age), Cattle  
 644 (*Bos taurus*, bta), Human (*Homo sapiens*, has), Junglefowl (*Gallus gallus*, gga), Mouse (*Mus*  
 645 *musculus*, mmu), Green lizard (*Anolis carolinensis*, aca), *Xenopus laevis* (*Xenopus tropicalis*, xtr),  
 646 Lamprey (*Petromyzon marinus*, pma), Green angel wood (*Astatotilapia burtoni*, abu),  
 647 Neolamprologus bricharde (*Neolamprologus brichardi*, nbr), Tilapia (*Oreochromis niloticus*, oni),  
 648 Cichlidae (*Pundamilia nyererei*, pny), Spotted green pufferfish (*Tetraodon nigroviridis*, tni) Atlantic  
 649 salmon (*Salmo salar*, ssa), Carp (*Cyprinus carpio*, ccr), Takifugu rubripes (*Fugu rubripes*, fru),  
 650 Atlantic cod (*Gadus morhua*, gmo), Channel Catfish (*Ictalurus punctatus*, ipu), Zebrafish (*Danio*  
 651 *rerio*, dre), Metriaclima zebra (*Metriaclima zebra*, mze). miRBase accession number is in  
 652 parentheses  
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655 **Fig. 3**



656

657 **Fig. 3** Relative tissue distribution profile of miR-15 and miR-16 in rabbitfish determined by qPCR.

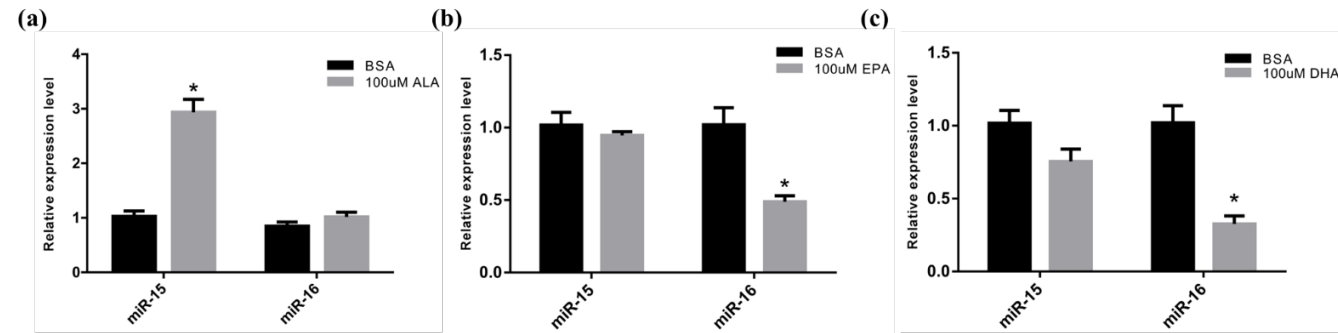
658 Values are means  $\pm$  SEM ( $n = 6$ ) as fold change from the brain. Bars not sharing a common

659 superscript letter indicates significant differences among the analysed tissues ( $P < 0.05$ ; ANOVA,

660 Tukey's test)

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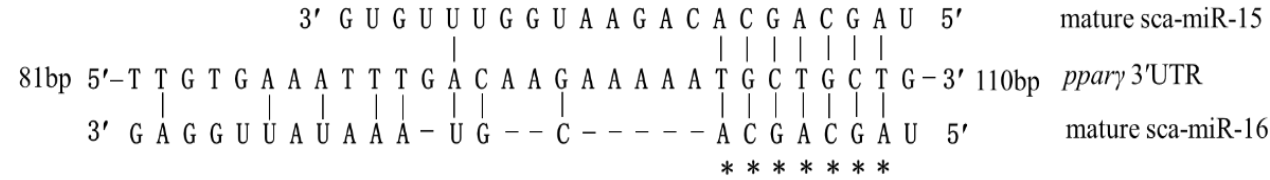
Fig. 4



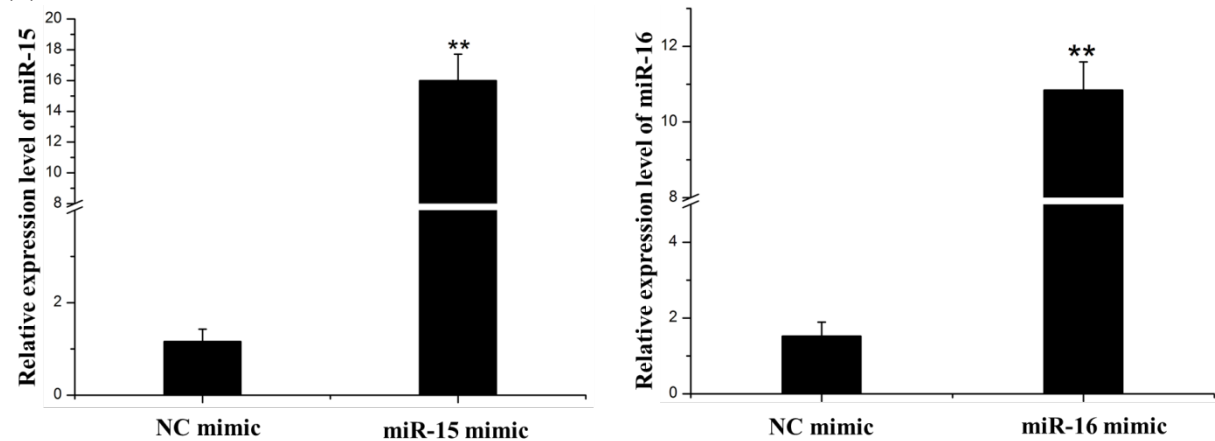
**Fig. 4** Expression of miR-15 and miR-16 in SCHL cells treated with PUFA. Rabbitfish SCHL cells were incubated with ALA(a), EPA(b) and DHA(c) at 100  $\mu$ M, or 0.1 % BSA (control) for 24 h, the relative levels of miR-15 and miR-16 mRNA were assessed by qPCR. Results were presented as the fold change from control in means  $\pm$  SEM from three independent experiments performed in triplicate. \* $P < 0.05$ , student t-test

Fig. 5

(a)



(b)



(c)

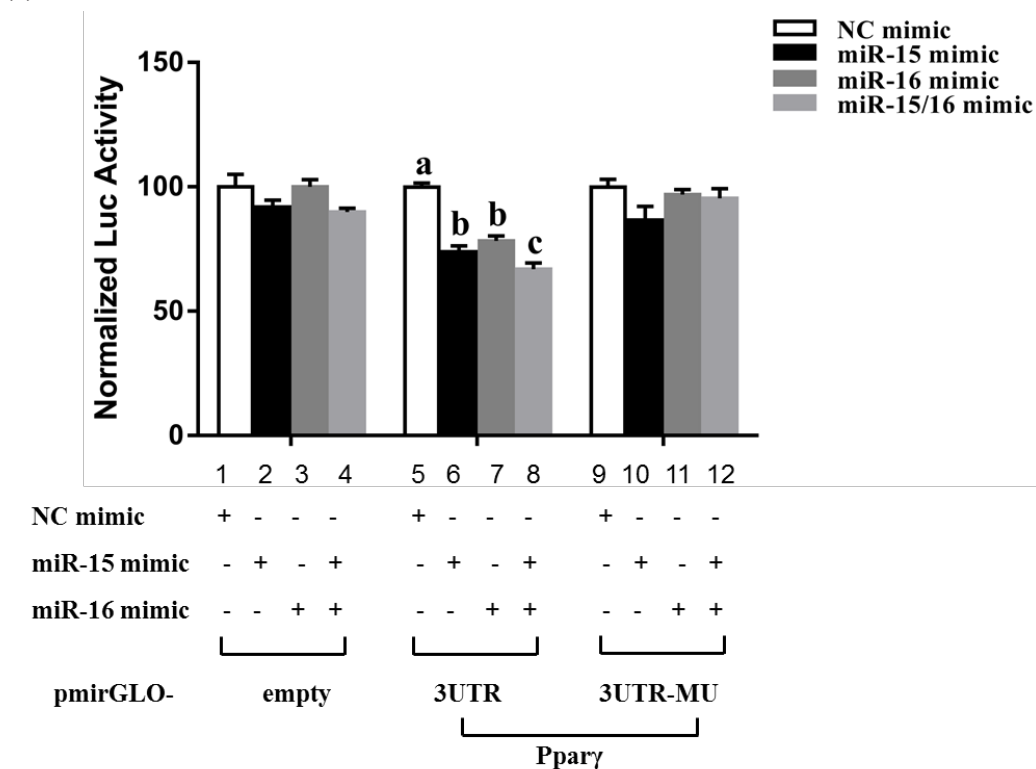
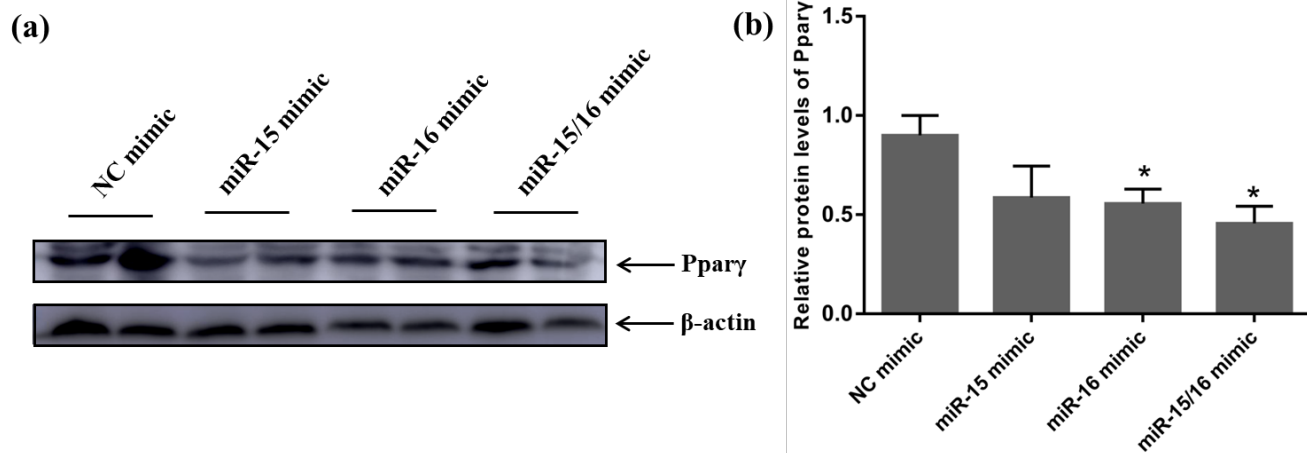


Fig. 5 miR-15/16 cluster target the 3'UTR of *ppary*. (a) Scheme of miR-15 and miR-16 base pairing

the 3'UTR of the rabbitfish *ppary*. (b) Rabbitfish miR-15 and miR-16 is over-expressed in HEK 293T cells by transfecting with miRNA mimics. (c) Luciferase activity in HEK 293T cells co-transfected with miRNA mimic or miRNA-NC with different recombinant dual luciferase reporter vectors: pmirGLO-empty as negative control (lanes 1-4); pmirGLO- PPAR $\gamma$ -3UTR containing 3'UTR of *ppary* (lanes 5-8); pmirGLO- PPAR $\gamma$  -3UTR-MU with 4 nt site-directed mutation in 3'UTR of *ppary* (lanes 9-12). The Renilla luciferase activity was used to normalize that of firefly luciferase. Data are shown as means  $\pm$  SEM (n = 8) and different superscript letters represent significant differences (P < 0.05; ANOVA, Tukey's test)

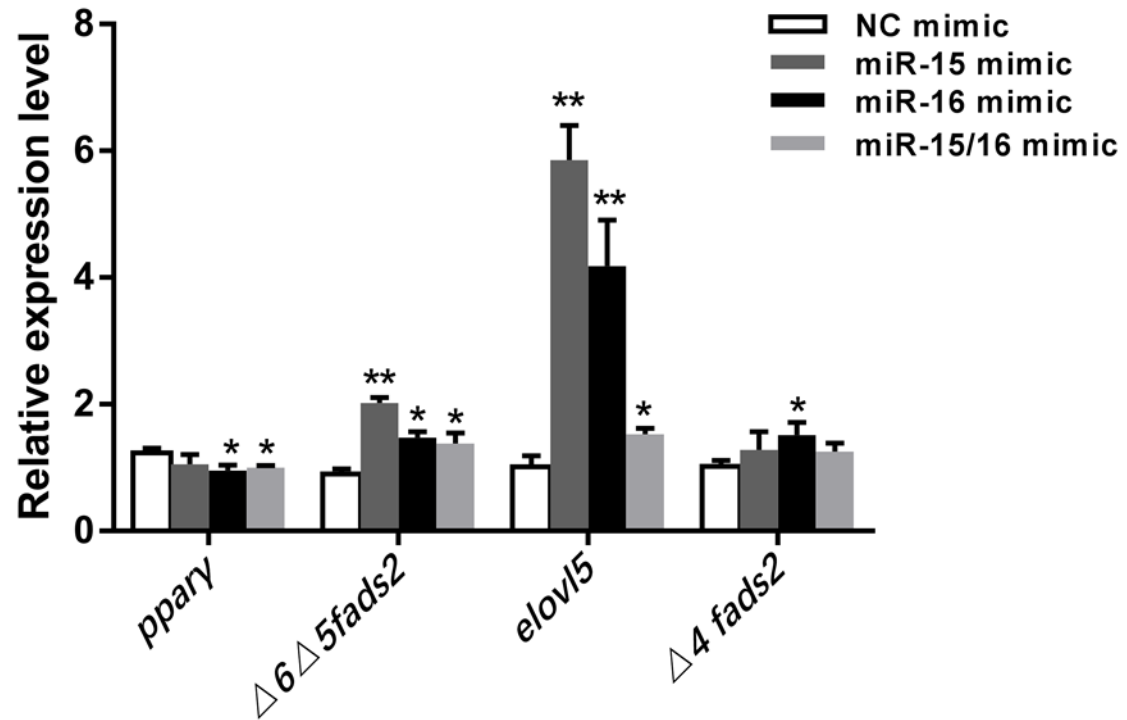
**Fig. 6**



**Fig. 6** miR-15/16 cluster decrease the abundance of Pparγ at protein level. (a) Rabbitfish SCHL cells were transfected with miR-15/16 mimic or NC mimic. After 48 h, aliquots of protein from cells were subjected to 10% SDS-PAGE gels and immunoblot analysis of the protein levels of Pparγ (~54 kDa) as above. (b) The relative protein levels of Pparγ, the Image J software v1.8.0 was used to quantify the intensity of the Western blotting bands. Data are means ± SEM of triplicate treatments as fold change from the control (\*  $P < 0.05$ , \*\* $P < 0.01$ )

Fig. 7

(a)



(b)

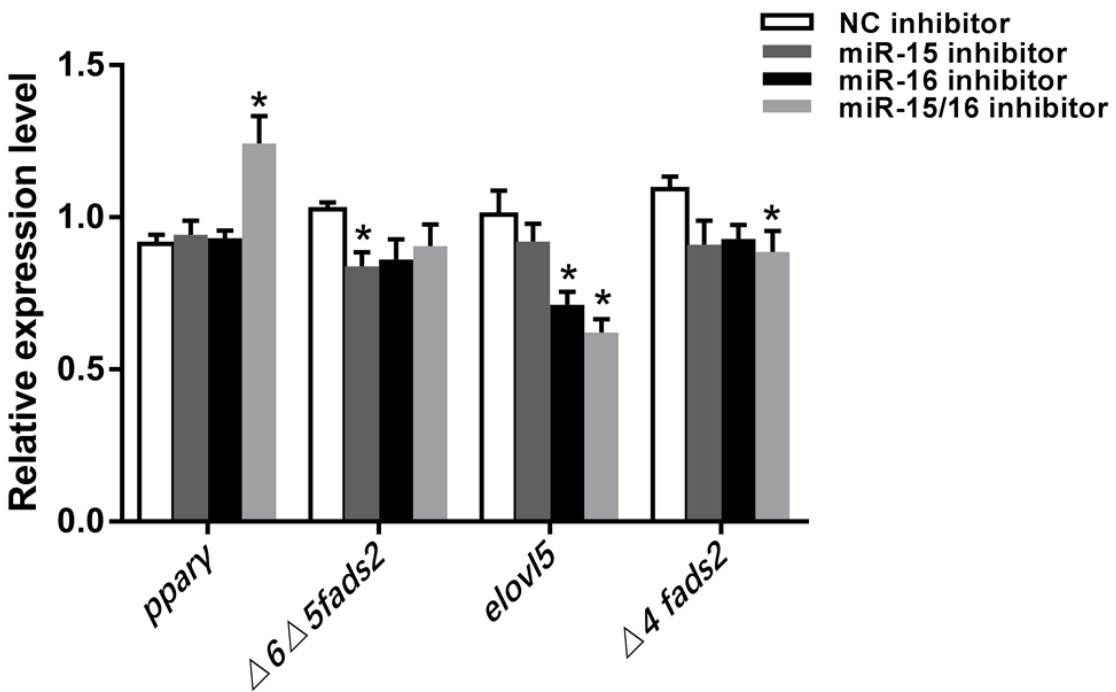


Fig. 7 The promotion role of miR-15/16 cluster on LC-PUFA biosynthesis is mediated by *pparγ*. (a)

Effects of miR-15 and miR-16 overexpression on the mRNA level of *pparγ*,  $\Delta 6\Delta 5fads2$ , *elovl5* and

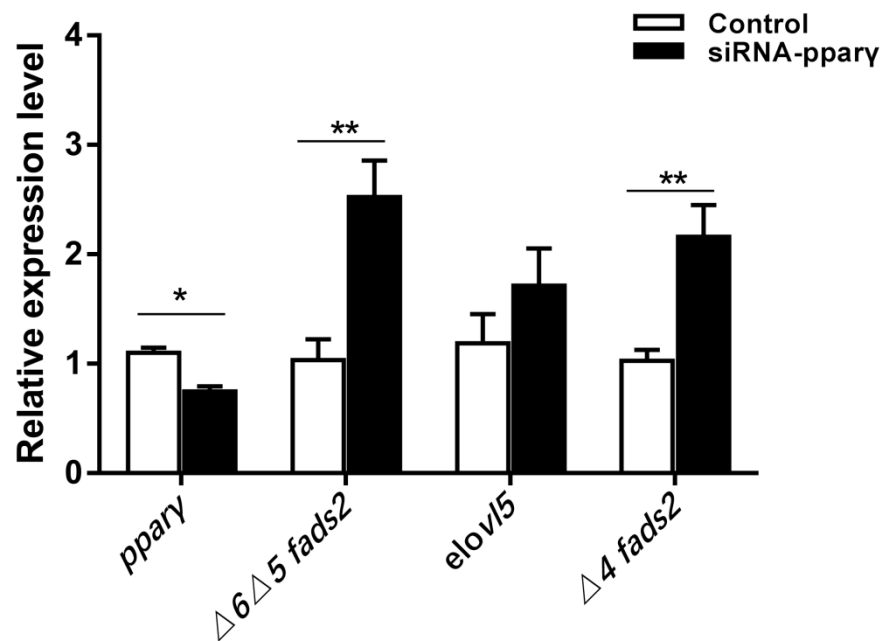
706  $\Delta 4 fads2$  in SCHL cells. (b) Effects of miR-15 and miR-16 inhibition on the mRNA level of *ppary*,  
707  $\Delta 6\Delta 5 fads2$ , *elovl5* and  $\Delta 4 fads2$  in SCHL cells. Data are means  $\pm$  SEM (n = 6). Asterisks represent  
708 significant differences (\*  $P < 0.05$ , \*\* $P < 0.01$ ; ANOVA, Tukey's test)

709

710



711 Fig. 8

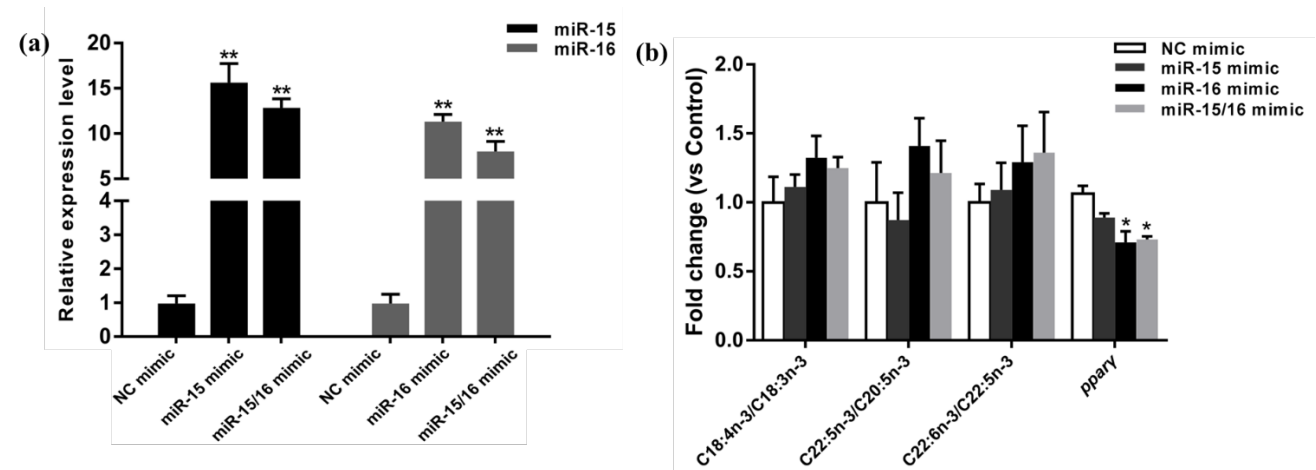


712

713 **Fig. 8** Effects of *pparγ* inhibition on the mRNA level of *pparγ*,  $\Delta 6 \Delta 5$  *fads2*, *elovl5* and  $\Delta 4$  *fads2* in  
714 SCHL cells. Data are means  $\pm$  SEM (n = 6). Asterisks represent significant differences (\*  $P < 0.05$ ,  
715 \*\* $P < 0.01$ ; ANOVA, Tukey's test)

716

Fig. 9



**Fig. 9** Up-regulation of miR-15/16 cluster promoting LC-PUFA biosynthesis through inhibiting *pparγ* in rabbitfish hepatocytes. (a) The expression of miR-15 and miR-16 mRNA was determined by qPCR as described above. (b) The evaluation of  $\Delta 6\Delta 5$  Fads2, Elovl5 and  $\Delta 4$  Fads2 activity by desaturation and elongation indexes were performed in miR-15/16 cluster overexpressed cells and the control cells. Additionally, the expression of *pparγ* was also analyzed by qPCR as described above. Data are means  $\pm$  SEM (n = 6). Asterisks represent significant differences (\*  $P < 0.05$ , \*\* $P < 0.01$ ; ANOVA, Tukey's test)