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Δ 6 Fatty Acid Desaturase Gene in Estuarine Tapertail Anchovy (*Coilia nasus*): Structure Characterization and mRNA Expression under Different Dietary and Fasting Conditions

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Authors' Contribution
PX conceived and designed the study. JD and GW cloned the gene and detected the Fads6 gene expression. DX and KL performed the fasting experiment. DF analyzed the data. JD, GW and PX wrote the article.

Key words
***Coilia nasus;* Δ6 fatty acid desaturases, gene characterization, mRNA expression, lipid metabolism enzyme, artificial diets.**

ABSTRACT

The enzyme Δ^6-fatty acid desaturase regulates the rate of highly unsaturated fatty acid (HUFA) biosynthesis. We first obtained the cDNA sequences and structures of Fads6 in *Coilia nasus* and investigated the mRNA expression patterns in tissue, and in juveniles under different dietary regulation. The Fads6 cDNA was 2032 bp, specifying a protein of 445 amino acids. A high expression was observed in the brain and intestine, relatively high expression in muscle and liver, and a weak expression was exhibited in the heart, kidney and gill. The effects of three different diets (diet 1-soft pellet; diet 2-slow-sinking pellet; diet 3- Daphnia) were studied on lipid metabolism enzyme activities and gene expression levels in different tissues of juveniles of *C. nasus* for 60 d. The differential expression of Fads6 genes in liver, brain and intestine changed with different diets, respectively. We detected lipid metabolism enzyme-specific activities such as lipase enzyme, lipoprotein lipase enzyme and lipid peroxides in liver, and found that the lipase activity in liver of diet 2 group was significantly ($P<0.05$) higher than the other diets. The mechanism of HUFAs synthesis in *C. nasus* is described in the future.

INTRODUCTION

The estuarine tapertail anchovy, *Coilia nasus*, junior synonym *C. ectenes*, is a commercially important fishery in the Yangtze River estuary and its associated lakes, although production has been declining in recent years. During its early life history, live diets such as rotifers, infusorians, water fleas (*Moina* sp.) and mosquito larvae are mainly consumed. Availability of live diets mostly vary by seasonal changes and environmental impacts. These fluctuations have contributed to the decrease in survival rate of juveniles and slow growth rate in maturing fish. In order to increase growth of juvenile fish to be farmed, artificial diets with improved nutrient utilization and high efficiency are important, However, little information on fatty acid desaturation in *C. nasus* is available. We have cloned the Δ^6-desaturase genes (*Fads6*) in *C. nasus* and investigated the mRNA expression patterns of *Fads*6 under nutritional regulation. At the same time, carnitine palmitoyltransferase -I, carnitine palmitoyltransferase -II, acetyl-coa carboxylase, lipase activity, and lipoprotein lipase in fish tissues were monitored.

Highly unsaturated fatty acids (HUFAs) have been identified as key nutrients for fish, particularly for food in their early life-cycle stages. The Δ^6-desaturase enzyme is involved in the HUFAs biosynthesis and is under nutritional regulation (Francis *et al*., 2009; Vagner *et al*., 2007). Δ^6-Desaturase enzyme is the rate-limiting enzyme involved in HUFAs biosynthesis, which is responsible for the first step of the desaturation/elongation process in HUFAs synthesis. For example, converting18: 3n-3(LNA) and18:2n-6(LA) to18:4n-3and 18:3n-6, respectively. The Δ^6-desaturase enzyme is also involved in the synthesis of 22:6n-3(DHA) from 20:5n-3 (EPA) (Monroig *et al*., 2012). Full-length cDNAs for Δ^6-desaturases have been isolated from some freshwater fish, including Δ^6/Δ^5 desaturase in zebrafish (*Danio rerio)* (Hastings and Guinot, 2001), Δ^6 and Δ5 desaturases in *Salmo salar* (Hastings, 2005), and marine fish such as *Rachycentron canadum*, *Sparus aurata* and *Scophthalmus maximus* (Juárez *et al*., 2008). It is generally thought that freshwater fish have a capacity to convert C18 PUFA to C20/22 HUFA. However, the extent to which fish convert C18 PUFA to C20/22HUFA varies with species, and is associated with their capacity for fatty acid desaturation and elongation (Tocher *et al*., 2003). Several studies reported an increase in the degree of fatty acid unsaturation in different diets in both desaturase and elongase capacities (Agaba *et al*., 2005; Estefanell *et al*., 2011). However, other studies have shown that HUFAs can inhibit the Δ^6-desaturase in freshwater fish as well as in mammals (Panserat *et al*., 2002; Caseras *et al*., 2000).

In this study, these results may be helpful for understanding the evolutionary development and

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functional characterization of *Fads*6 genes in the biosynthesis of HUFAs in *C. nasus*, and be beneficial to understanding the molecular mechanism of HUFAs biosynthesis in this fish.

MATERIALS AND METHODS

Experiment fish and sample collection

Juvenile fish were obtained from the Jingjiang section of Yangtze River, the most important area for production of *C. nasus* in China. Tissues of sampled fish were carefully collected and kept at -80°C until analysis. More fish were acclimatized indoors at a freshwater fisheries research center, in recirculating aquaculture ponds (length×width×height: 8m×4m×1m) at a water temperature of 28.5±0.3°C, dissolved oxygen ≥ 6.0mg/l, pH=8.0~8.4. Fish were fed with an ordinary diet for 20 d before starting the experiments. The fish were randomly distributed into 6 cement pools, 500 fish per unit. The experiment was conducted for 20 d by feeding and fasting to juvenile fish in three cement pools respectively. The fish were fed twice daily at 08:00 and 14:00, with the full stomach method of feeding. The feed (Table I) were selected for culturing juveniles of *C. nasus*, the diet added 40% water to dry material made into soft pellets. The diets were dried at 105°C for 24 h before analyzing for protein, lipid, fiber, and ash, as described in AOAC (2005). The values were expressed as ‰ of dry mass. At the end of the experiment, fish were captured that were not fed on that sampling day. Body weight and total length were measured before tissues were carefully collected. The tissues were then kept at -80°C until analysis.

Table I.- Composition of experimental diets (g/1000 g wet weight).

Nutrition levels/mgg^{-1}	Diet 1	Diet 2	Diet 3
Moisture	47.30	5.66	94.63
Dry matter	52.69	94.34	5.37
Crude ash	12.04	13.85	1.24
Crude protein	24.97	59.78	3.42
Crude fat	7.86	23.38	0.71

cDNA cloning for fatty acid desaturases

Total RNA was extracted from liver tissues using the Trizol Reagent Kit (Takara, Kyoto, Japan), and 2 μg of total RNA was reversely transcribed into cDNA using PrimeScript RT-PCR Kit (Takara, Japan). In order to amplify the first fragment of the *Fads*6 cDNA, the sequences of Fads6 proteins from *Oncorhynchus mykiss* (AF301910.1), *Muraenesox cinereus* (HQ727979.1), *Rachycentron canadum* (FJ440238.1), *Solea senegalensis* (JN673546.1), *Salmo salar* (AY458652), D*anio rerio* (AF309556.1), *Labeo rohita* (EF634246.2), *Cyprinus carpio* (AF309557.1), *Homo sapiens* (AF126799), and *Macaca mulatta* (NM001194717.1) were aligned using ClustalX 1.83 multiple-alignment software (http://www.clustal.org/). Subsequently, the primers were designed based on the conserved nucleotides of the sequences (Table II). PCR amplifications were performed with an initial denaturation at 94°C for 3 min, then followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60s, followed by a final extension at 72°C for 8 min. The gel-purified PCR products were cloned into the pMD18-TVector (Takara, Japan), and the positive clones were sequenced in BiosuneInc (Shanghai, China). The PCR fragment was sequenced at the DNA Sequencing Service of IBMCP-UPV (Valencia, Spain), and gene-specific primers were designed for 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR (Applied Biosystems, Warrington, UK) to get a full-length cDNA. The details of all primers used for RACE PCR are given in Table II.

Table II.- Primers for genomic and determining mRNA content of *Fads*6 in *Coilia nasus*.

Primer sequence (5′ → 3′)
For gene cloning
F1: 5'-CTGGTGGAA(C/T)CA(A/T)CG (A/T/G)CA(T/C) TTCCAGC -3'
R1:5'-AGTTGAG(G/A)TGTCC(G/A) CT(G/A)AACCAGTCG -3'
3'F1:5'-GCAGCTTCGTCAGGTTCCTGGAGAG-3'
3'F2: 5'-GACTGGCTGACCATGCAGTTG AAGG-3'
3'RACE Outer Primer : 5'-TACCGTCGTTCCACTAGTGATTT-3'
3' RACE inter Primer:
5'-CGCGGATCCTCCACTAGTGATTTCACTA TAGG-3'
5' R1: 5'-ATACTCCACAGGCTGGGTCT-3'
5'R2: 5'-CGGGTCCTTGCTGAACACAT-3'
5' RACE Outer Primer :
5'-CATGGCTACATGCTGACAGCCTA -3'
5' RACE inter Primer:
5'-CGCGGACCACAGCCTACTGATGATCAGTCGATG -3'
RT-qPCR
FAD-F: 5'-GCAGACCCAGCCTGTGGAGTATG-3'
FAD-R:5'-CATCGACC AGGCCATGTCCAC-3'
β-actinF: 5'-AACGGATCCG GTATGTGCAAAGC-3'
β-actin R:5'-GGGTCAGGAT ACCTCTCTTGCTCTG -3'

Fasting experiment

Fish weighing 18.32±5.0 g (mean±SE) were divided into two groups of 400 fish each and were either fed or fasted for 20 days. At 0, 2, 5, 10, 15, 20 day after experiment, 9 fish from every pond were weighed and processed immediately. RT-qPCR was employed to detect the *Fads*6 expression profiles as below.

Real time quantitative PCR (RT-qPCR) in juvenile fish

Total RNA was extracted from different tissues as above, and 2 µg of total RNA was reversely transcribed into cDNA using PrimeScript RT-PCR Kit (Takara, Japan). Expression of the genes was measured by real-time quantitative PCR (RT-qPCR) (SYBR Green II) on a ABI 7500 sequence detection system (Takara, Japan), using *β-actin* as a reference gene. Primers for gene expression analysis (Table II) were designed based on the full-length of *Fads6* cDNAs. Each sample was run in triplicate in order to reduce the difference, and PCR reaction without template was used as a negative control. The relative mRNA expression level of *Fads6* in each sample was normalized with *β-actin* expression and calculated with the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) in triplicate observations. Differences in the expression of *Fad* gene and digestive enzyme activities were analyzed by one- way ANOVA followed by Tukey's multiple comparison. All statistical analyses were computed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA). Significant differences between means were ranked using a least significant difference test (LSD) at 95% significance level .

RESULTS

Coilia nasus Fads6 *gene structure*

The full-length of *Fads*6 cDNAs was 2032 bp, including an ORF (open reading frame) of 1338 bp specifying a protein of 445 amino acids, which contains three histidine-rich regions and two putative trans-membrane domains (Fig. 1). Phylogenetic analysis of the amino acid sequences, deduced from the *Fads*6 cDNA and those from other fish including several marine fish, was performed by constructing a tree using the neighbour-joining method (Saitou and Nei, 1987; Haikui *et al*., 2014), with confidence in the resulting tree branch topology measured by bootstrapping through 10,000 iterations (Fig. 2). The *Fads*6 was 79% identical to that of *Muraenesox cinereus*, 75% identical to *Rachycentron canadum*, 74% identical to *Salmo salar*, 69% identical to *Pangasianodon hypophthalmus*, and 67% identical to *Mus musculus.*

Fads6 *gene expression in different tissues*

The tissue-specific expressions of *Fads*6 mRNAs were detected in *C. nasus*. A high expression was observed in the brain and intestine, relatively high expression in muscle and liver, and a weak expression in heart, kidney and gill.

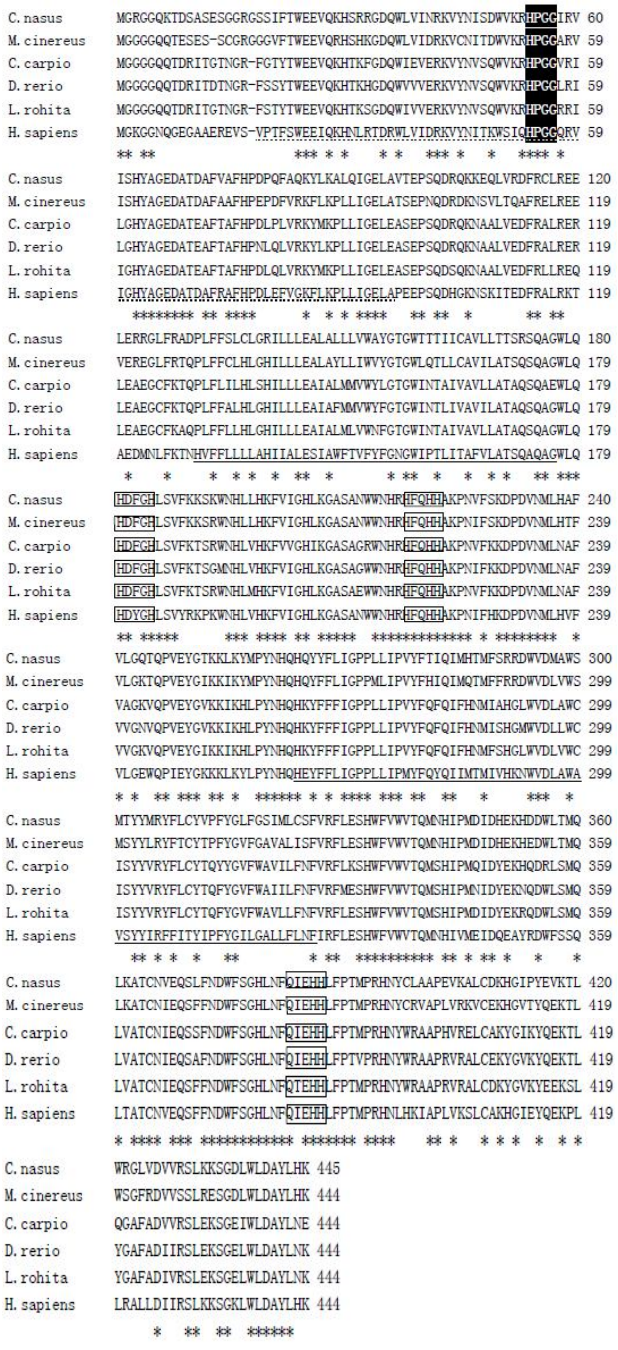

Fig. 1. Alignment of amino acid sequences of *Fads*6 in *Coilia nasus* with that of Δ^6 desaturase from other fish. *M. cinereus*: HQ727979.1, *C. carpio*: AF309557.1, *D. rerio*: AF309556.1, *L. rohita*: EF634246.2, *H. sapiens*: AF126799. The heme-binding motif is dotted underline, the cytochrome b5-like domain is shade, three histidine-rich regions are framed, and two putative

trans-membrane domains are underlined.

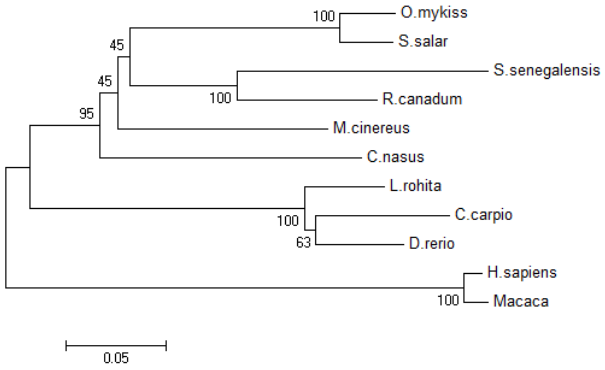

Fig. 2. The phylogenetic tree of *Fads*6. The Phylogenetic tree was constructed using the Neighbor Joining method with MEGA3. 0. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with the tree topology presented after 1,000 iterations. *O.mykiss*: AF301910.1, *M. cinereus*: HQ727979.1, *R.canadum* : FJ440238.1, *S.senegalensis*: JN673546.1, *S. salar*: AY458652, *D. rerio*: AF309556.1, *L.rohita*: EF634246.2, *C.carpio*: AF309557.1, *H.sapiens* : AF126799, *M. mulatta*: NM001194717.1

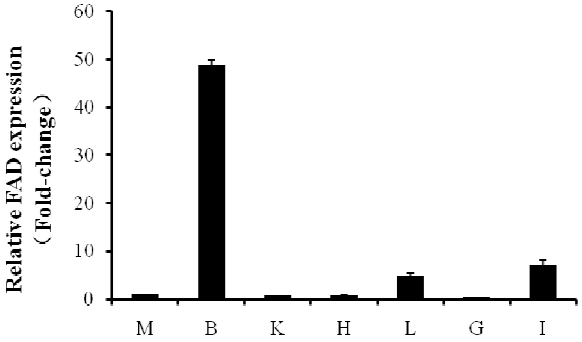

Fig. 3. Expression level of delta 6 desaturase in tissues of *Coilia nasus*. A high expression was observed in the brain(B) , intestine(I) , muscle(M) and liver(L); a weak expression was encountered in the heart(H), kidney(K) and gill(G). The expression in the brain and intestine were significantly (*P*<0.05) higher than in other tissues.

Expression of Fads6 *with different diets*

Fads6 expression levels were higher in brain than in liver in diets 1 and 3 (P<0.05) and higher in liver than in brain in diet 2 (P<0.05). Furthermore, the expression levels in the muscle, heart, and intestine in diets 2 and 3 were significantly higher (P<0.05) than in diet 1. The expression levels in the brain of diet 3 were significantly higher (P<0.01) than those in diets 1 or 2, whereas the expression levels in the liver of diet 2 were significantly higher (P<0.05) than those in diets 1 or 3. There were no significant differences (P>0.05) in Fads6 expression levels in the kidney and gill among the three diets (Fig.4).

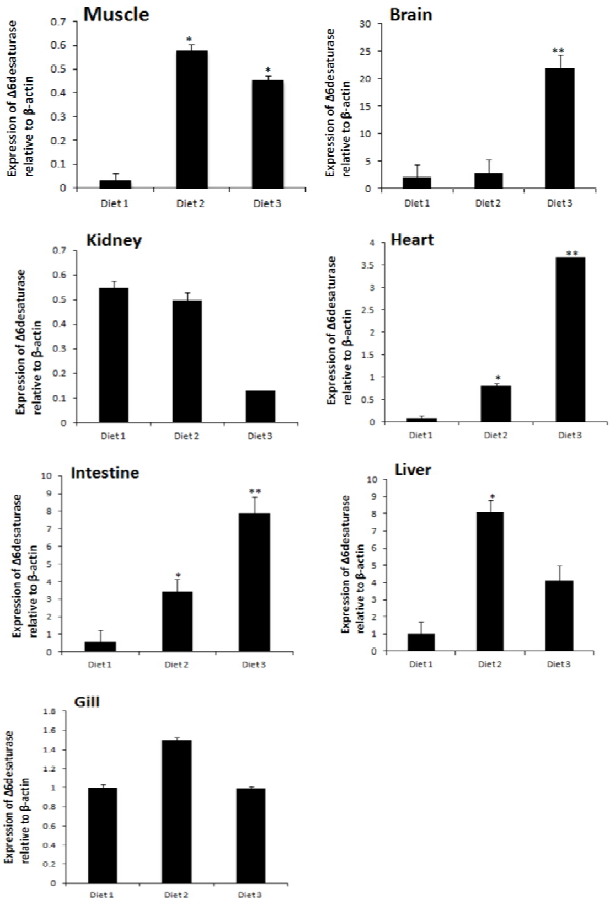

Fig. 4 .Relative expression levels of *Fads*6 in different tissues of *Coilia nasus* fed three different diets. Values are means ± SEM (n = 9). Asterisks represent significant differences within similar genes. * *P*< 0.05 and ** *P*<0.01. The expression in the muscle, heart and intestine in diet 2 and diet 3 groups were significantly (*P*<0.05) higher than in diet1 group tissues. The expression in the liver of diet 2 group was significant (*P*<0.05) higher than in the others.

Enzyme-specific activities

There were no significant differences (P > 0.05) in the lipid peroxide (LPO) levels among the diets (Table III). Nevertheless, hepatic lipase activity in diet 2 was significantly higher (P < 0.05) than that of the other two

diets. On the other hand, fish fed diet 3 had significantly lower ($P < 0.05$) levels of lipoprotein lipase, carnitine palmitoyltransferase-I (CPT-I), carnitine palmitoyl-transferase-II (CPT-II) and acetyl-Coa carboxylase (ACC) than those fed diets 1 or 2.

Table III.- Effect of different feed on the fat metabolism enzymes of juveniles of *Coilia nasus.*

index	Diet 1	Diet 2	Diet 3
LPS (U/gprot)	14.84±3.46[a]	21.26±1.35[b]	19.92±0.19[b]
LPO(μmol/gprot)	3.79±1.12	5.61±1.48	4.00±0.03
LPL(U/mgprot)	2.18±0.69[ab]	4.07±0.96[a]	1.73±0.01[b]
CPT-Ⅰ (pg/ml)	96.33±1.78[a]	90.01±2.19[b]	83.71±0.88[c]
CPT-Ⅱ (pg/ml)	145.71±1.22[a]	145.19±1.51[a]	131.29±1.97[b]
ACC (U/ml)	18.59±0.60[a]	18.69±0.31[a]	16.01±0.17[b]

Expression of Fads6 *genes under fasting*

In the fasting experiment, fed fish gained an average of 6.45 g during the 20-day experimental period, whereas the fasted fish lost 1.80 g. This accounted for an 8.25 g difference in body weight between fed and fasted fish (Fig. 4). The *Fads6* mRNA revealed 7.6- to 19.6-fold increase in brain (Fig. 5a), on the contrary, it showed 7.9- to 380.9-fold increase in brain (Fig. 5b).

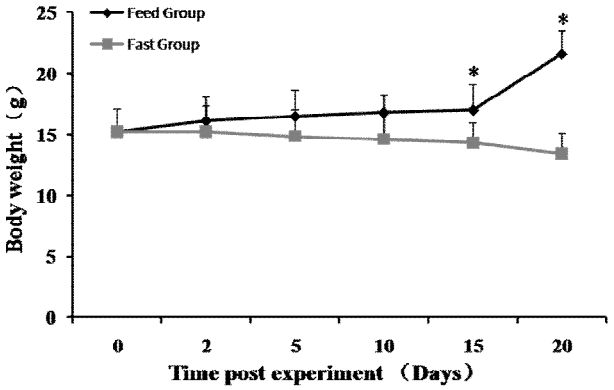

Fig. 5. Effects of fasting on body weight in *Coilia nasus*. *, $P < 0.05$, differences between means (± SE; n = 5) were determined by t-test.

DISCUSSION

Fat content in diets affects lipid enzymes activities in C. nasus

C. nasus is a migratory fish, living in the ocean for growth, development and maturation. The species migrates from coastal waters to freshwater areas every year during the spawning season (Shi and Gong, 2003; Haacke *et al*., 2001). They ingest a diversity of live foods such as rotifers, infusorians and mosquito larvae, which facilitates their adaptation to inland environments. Our results explored how *C. nasus* adjusts to the type of food intake in these complex environments, and how the enzymes and genes respond to this change (Tang *et al*., 2001; Whitehead, 1985; Kirchaner *et al*., 2008).

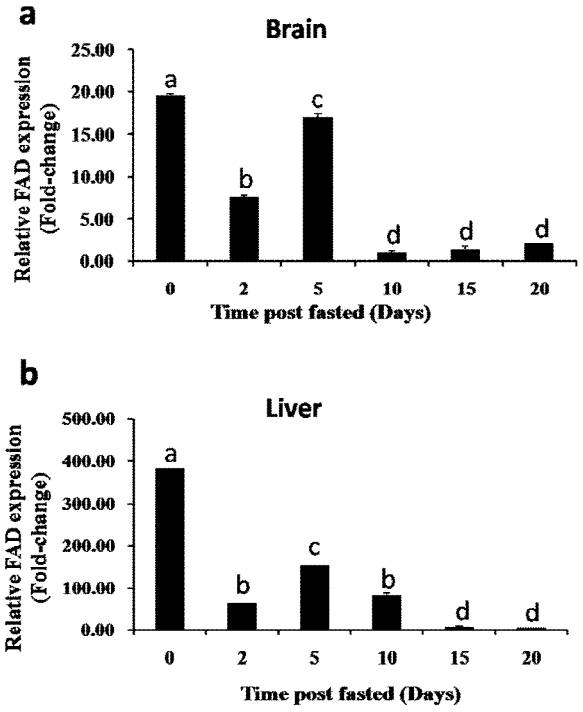

Fig.6. *FAD* gene in brain and liver responsed to fasting. Bars are the mean for each replicate ± SD. Replicates or treatments with different letters are significantly different at $P < 0.05$. (a). Expression profiles of brain *FAD* in fasting. (b). Expression profiles of liver *FAD* in fasting.

Diet is an important factor for growth and development, and affects energy metabolism and tissue structures of the body (Hidalgo *et al*., 1999; Lee *et al*., 2003). Crude fat content in *C. nasus* is 25%, implying that fat content is extremely important in the food of this species. Studies have shown that fat content in the diet of fish has a significant impact on immunity, enzyme activity, lipid metabolism, and other factors (Rbeiro *et al*., 1999; Grisdale *et al*., 2002; Xu *et al*., 2011). There are many lipid enzymes involved in fat metabolism. CPT-I and CPT-II are important enzymes for composing the carnitine transport system, which are the speed limit in

the lipolytic enzyme, while ACC can promote synthesis of fatty acids (Kerner and Hoppel, 2000; Scanu, 1966). Lipid enzymes play an important role in fish growth and development, which provide essential fatty acids, promote lipid-soluble vitamin absorption, and facilitate many other physiological functions (Wang *et al.*, 2005).

Variability in fat content of foods play a regulatory role in CPT-I and CPT-II, ACC, LPS, and LPL enzyme activity or content. Studies have shown that ACC, CPT-II, and lipase contents were significantly different in *Carassius auratus gibelio* when fed a different lipid source (Zhang *et al*., 2012). In our study, the fat content in the three diets were 14.92%, 24.78%, and 13.22% (dry weight), respectively. CPT-I and CPT-II content in the liver as well as ACC, LPS, and LPL activity values in the high-fat diet group (diet 2) of *C. nasus* were significantly different from the other two ($P<0.05$). This suggests that appropriate dietary lipid levels can be raised based on lipid metabolism enzyme content and activity in the liver, but excessive levels may have negative effects (Ji *et al*., 2007; Om *et al*., 2001; Hong *et al*., 2013). Further study is need to determine the appropriate amount of fat content in the diet of *C. nasus* juveniles.

Fat content in diets affects Fads6 *gene expression*

HUFAs are important indicators to measure quality of fish. The Δ^6-desaturase is the rate-limiting enzyme involved in HUFAs biosynthesis, which is responsible for the first step in the desaturation/elongation process in HUFAs synthesis. Therefore, fat content in feed has an effect on *Fads*6 gene expression (Christiansen *et al*., 1991; Pauloin, 2010; Rodriguez, 2011). Our research showed that *Fads*6 gene expression in the liver of *C. nasus*, when fed the high fat content (diet 2 group) was substantially higher than the other two groups. It suggests that high fat content (diet 2 group) in feed induces *Fad* gene expression and may improve HUFAs synthesis capabilities. The appropriate dietary lipid levels in feed raise the content and activity of lipid metabolic enzymes in the liver, and increase the level of *Fads*6 gene expression, perhaps enhancing the synthesis of HUFAs.

To better understand what food components are responsible for growth and digestive enzymes, according to the diet of juveniles of *C. nasus*, it is necessary to know the composition of artificial diets. This investigation provided information on the outcome of the effect of different artificial diets. Moreover, composition of different diets would give data on utilization of nutrients and development of the digestive system. Lastly, because it is difficult to rear juveniles of *C. nasus*, development of an artificial diet is critical for propagation.

CONCLUSIONS

In conclusion, this study is the first to successfully rear *C. nasus* under aquaculture conditions using artificial diets and successfully clone Δ^6 fatty acid desaturase genes. The *Fads*6 consists of the typical structure of Δ^6 fatty acid desaturases that are identical to the Δ^6-desaturase of *Muraenesox cinereus* and the *Human* Δ^6-desaturase. Genes for *Fads*6 are expressed in various tissues of *C. nasus*, and highly expressed in brain, intestine, muscle and liver. Our results show that *Fads*6 has distinctive expression levels under various nutritional regimens. Results have provided new information on how changes in *Fads*6 gene expression in different tissues occur, and how lipid enzyme-specific activities in liver respond to artificial diets and *Daphnia*. *Fads*6 gene expressions in liver, intestine and gill were significantly increased in *C. nasus* when fed with artificial diets, and lipid metabolism, enzyme-specific activities in liver increase when fish feed on artificial diets. This is consistent with evidence that the liver is the major tissue for regulating overall fatty acid metabolism in the body. Our study supports the hypothesis that regulation of fatty acid desaturase expression may play an important role in managing hepatic lipid composition in response to changes in diet. Further work is in progress to determine the mechanism of differential expressions of *Fads*6 genes in various tissues and the roles of transcription factors in regulating HUFAs synthesis.

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Conflict of interest statement

The authors have declared that no competing interests exist.

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