

Molecular Identification, Tissue Distribution, and Effects of Fasting and Refeeding on the Transcription of Uncoupling Protein 2 in Yellow Catfish, *Pelteobagrus vachelli*

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ABSTRACT

Uncoupling protein-2 (UCP2), an important member of the inner mitochondrial membrane protein families, plays pivotal roles in energy expenditure, fatty acid metabolism and ROS emission in mammals. In contrast to mammals, the roles of this protein are still rarely known in fish. Here, we first identified the *ucp2* gene in yellow catfish (*Pelteobagrus vachelli*) and investigated its transcriptional changes in response to fasting and refeeding. The cDNA of *pvucp2* was 1,193 bp long and possessed a 939 bp open reading frame (ORF) encoding 312 amino acids. Multiple protein sequences alignment revealed that UCP2 protein sequences were highly conserved among vertebrates. Phylogenetic analysis suggested the evolutionary process of fish *ucp2* was consistent with species evolution, and the *pvucp2* shared a close relationship with electric eel *ucp2*. Quantitative PCRs showed that *pvucp2* was extensively expressed in all detected tissues, with the highest expression in liver. Two-week fasting significantly decreased while refeeding dramatically increased the hepatic *pvucp2* transcriptions. These findings suggested that fish UCP2 proteins are highly conserved and they might play important roles in maintaining energy homeostasis and reducing reactive oxygen species.

INTRODUCTION

Uncoupling protein 2 is an anion-carrier protein located in mitochondria inner membrane, which is widely distributed and plays important roles in regulating glucolipid metabolism, ROS emission and ATP production in mammals (Donadelli *et al.*, 2014; Zhou *et al.*, 2018). The *ucp2* gene was first identified from human in 1997 and proved to be linked with human obesity and hyperinsulinemia (Fleury *et al.*, 1997). The human UCP2 contains 388 amino acids and shares 57% and 75% identity with its paralogs UCP1 and UCP3, respectively (Dalgaard and Pedersen, 2001). Differently, human UCP2 shares 95% identity with its ortholog in mouse, suggesting

this protein may be highly conserved among mammals (Souza *et al.*, 2011). Tissue distribution analysis indicated that UCP2 was widely distributed in central and peripheral tissues, including heart, muscle, brown adipose tissues (BAT), kidney, liver, lungs, spleen, thymus, bone marrow, macrophages, brain, gastrointestinal tract, and pancreatic islets (Chan *et al.*, 2004; Fisler and Warden, 2006). Subsequently, UCP2 and UCP3 were proposed to act as critical roles in regulating cellular energy metabolism and reducing reactive oxygen species (ROS) (Echtay *et al.*, 2018). Although the UCP2 has been widely explored in mammals, its exact molecular activities and physiological roles are still needed to be further investigated.

Differently with mammals, the roles of UCP2 are rarely reported in fish. The first fish *ucp2* was identified in common carp and zebrafish in 1999, and both *ucp2* genes encoded 310 amino acids and shared an average 82% sequence identity with mammalian UCP2 proteins

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Authors' Contribution

FD performed the experiment, analyzed the data and wrote the draft manuscript. ZYW designed the study and revised the manuscript. XDW and YL provided advice and revised the manuscript.

Key words

Uncoupling protein 2, Phylogenetic analysis, Tissue distribution, Fasting and refeeding, *Pelteobagrus vachelli*

(Stuart *et al.*, 1999). Fish UCP2 was not considered to be involved in thermogenesis due to it is an ectothermic vertebrate (Stuart *et al.*, 1999). Subsequently, two *ucp2* isoforms (named as *ucp2a* and *ucp2b*) were cloned from rainbow trout, and they showed different expression patterns following the process of embryo development (Coulbaly *et al.*, 2006b). Two *ucp2* isoforms might derive from the salmonid-specific genome duplication event, and they may be involved in fatty acids metabolism in fish embryo development (Berthelot *et al.*, 2014; Glasauer and Neuhaus, 2014). In polar fish Antarctic eelpout, *ucp2* was regulated by thermal changes, suggested UCP2 might play an important role in reducing ROS produced by mitochondrion but not thermogenesis (Mark *et al.*, 2006). In Chinese perch, *ucp2* was highly distributed in liver, and its transcription in liver was significantly increased after cold accumulation but not changed after hot accumulation, suggesting fish UCP2 might play important roles in response to cold water environment (Wen *et al.*, 2015). In addition, gilthead sea bream *ucp2* was sensitive to food deprivation and hypoxia state, suggesting UCP2 might serve as metabolic and redox sensors to prevent the activation of futile cycles of energy wastage (Bermejo-Nogales *et al.*, 2014). Recently, fish UCP2 proteins were suggested to play an important role in food intake regulation and growth control (Wen *et al.*, 2015; Qin *et al.*, 2018b). However, the functional roles of fish UCP2 are still limited and more researches are needed.

The yellow catfish (*Pelteobagrus vachelli*) belongs to the family Bagridae and the order Siluriformes, is a fish with the biggest body size in this family (Qin *et al.*, 2017). The fish is an omnivorous fish that always keeps little movement during the day but active in the evening. Because of the rapid growth and valuable taste traits, this fish has now been widely cultured as an economically important freshwater fish in China (Qin *et al.*, 2018a). In this study, we identified the *ucp2* gene in *P. vachelli* for the first time and its transcriptional changes in response to fasting and refeeding were determined. These findings will be undoubtedly helpful for better understanding the roles of UCP2 in fish and might provide novel genetic locus for molecular marker associated breeding.

MATERIALS AND METHODS

Fish sampling

Juvenile yellow catfish (weight 5.38 ± 0.31 g) were used for investigation and they were cultured at the aquarium in Neijiang Normal University. The cultural environments were set as follows: cultural tanks with volume of 100 L; an aeration water with temperature maintaining at 20-22 °C; the natural light-dark conditions (12 L/12 D).

Experimental fishes were fed with commercial feeds once a day at 19:00, and their eating residues and feces were cleaned immediately when fishes stopped eating.

After acclimation for two weeks, six fishes were randomly selected and used for cloning and tissue distribution experiments. Selected fishes were anesthetized with MS222 before decapitating and then ten tissues including adipose, brain, gill, heart, intestine, kidney, liver, muscle, spleen, and stomach were collected. For fasting and refeeding experiments, fishes were cultured at 3 experimental tanks with 20 tails per tank, and 1 tank of fishes were fed at 19:00 every day as control, while the other 2 tanks of fishes were not fed last for 2 weeks. Before sampling, fishes in control group and one fasted tank (refeeding group) were fed at 19:00, and the other fasted tank (fasted group) was still not fed. Fishes were allowed fifteen minutes for eating, and then six fishes from each tank were randomly selected and their liver tissues were collected. The samples were stored at -80°C until further utilization.

The animal experiments throughout the study were conducted according to the Chinese Ministry of Science and Technology Guiding Directives for Humane Treatment of Laboratory Animals and approved by the Animal Care and Use Committee of Neijiang Normal University.

Molecular cloning of *ucp2* in *P. vachelli*

Total RNA was extracted using the Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. After assessing the quality, 1 µg of the total RNA was reversely transcribed to cDNA using Super Script™ II RT reverse transcriptase (ThermoFisher Science, China). Meanwhile, two pairs of primers (Table 1) were designed to amplify the coding sequences, which were based on the partial mRNA sequence obtained from our previous transcriptome database (Qin *et al.*, 2017). The basic cycling conditions of the PCR were as follows: a denaturing stage at 94 °C for 30s, gene-specific annealing temperature for 45 s and elongation stage at 72 °C for 60 s, a total of 34 cycles. The target products were purified from agarose gel using the Universal DNA Purification Kit (Tiangen, China), and then cloned into a pMD-19T vector (TaKaRa, Dalian, China). The cDNA clones were sequenced at BGI-Wuhan (Wuhan, China).

Sequence analysis and data processing

The cDNA sequence of *ucp2* was obtained and the ORF was determined using online software ORF finder (<https://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Putative amino acid sequence was predicted with Primer Premier 5.0 software (Primer, Canada). Subsequently, the BioEdit software was used to analyze the electronic

point and molecular weight, and the online tool TMHMM Server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) was used to predict the transmembrane α -helix domains. In addition, multiple protein sequences alignment and functional domains prediction were as described in two previous studies (Wen *et al.*, 2015; Yang *et al.*, 2018).

Table I. Primer pairs used for molecular cloning and real-time PCR.

Primer	Sequence (5'-3')
Ucp2-01-F	TCAGCTGTTTCTATGG
Ucp2-01-R	ATCCATGGTGCCCTGGT
Ucp2-02-F	AGGATCTGATCATGTTG
Ucp2-02-R	TAAGGGGAGGAAGAACT
Ucp2-01-qF	TGCCAGACGATGAAGTAT
Ucp2-01-qR	TAACCTGAGCCTGAAAACG
β -actin-qF	GGTCCAGACGCAGAAATAGC
β -actin-qR	AATCCCAAAGCCAACAGG

Phylogenetic analysis

The UCPs from fish and other vertebrates were aligned by CLUSTAL X2.1 (Wen *et al.*, 2017; Li *et al.*, 2018). The aligned amino acid data sets were used to reconstruct the phylogenetic tree with neighbor joining (NJ) approach using Mega 6.0 software (Qin *et al.*, 2018b). The best-fitting model was calculated by Mrmodeltest 2.0 and ProtTest 2.4 (Abascal *et al.*, 2005), and finally the JTT + G model was selected as the best model. The robustness of the tree topology was assessed by nonparametric bootstrap analysis with 1,000 resampling replicates, and then the tree was beautified using FigTree software. The Mouse ear cross was selected as an outgroup species.

Quantitative real-time PCRs

Total RNA isolation and first strand cDNA synthesis were performed as described above. Subsequently the real-time PCR was used to detect the hepatic *ucp2* expression with Light Cycler Real-Time system. Reverse transcription product was used for real-time PCR in a final volume of 10 μ L. The end products of PCR were verified with the melting curves that showing a single specific peak for the target gene. The relative expression levels were calculated according to the method described in two previous studies (Qin *et al.*, 2018b; Yang *et al.*, 2018). Meanwhile, four reference genes (*tuba1*, *GAPDH*, *18 S* and β -actin) were calculated by geNorm software (Wen *et al.*, 2019, 2020), and finally the β -actin was selected as the reference gene. Quantification primers used to amplify the *ucp2* and β -actin were listed in Table I.

Statistical analysis

Statistical analysis was performed with SPSS 22.0 software and all data were expressed as the mean $\pm$ SEM. One-way analysis of variance (ANOVA) was used to calculate the significant difference followed by student's t test, after confirming for data normality and homogeneity of variances. Differences were considered to be significant if $P < 0.05$.

RESULTS

Molecular cloning of the *pvucp2*

Two PCR fragments were obtained and sequenced for *ucp2*, respectively. Subsequently, these two fragments were assembled into one complete sequence. The final nucleotide sequence of *ucp2* cDNA was 1,193 bp in length, and it was deposited into GenBank database with an accession number MK625181. The *ucp2* cDNA contained a 189-bp 5'-UTR, a 65-bp 3'-UTR, and a 939-bp ORF encoding 312 amino acids (Fig. 1). Meanwhile, the predicted UCP2 protein possessed six transmembrane α -helix domains, which was considered to be highly conserved among vertebrates (see Fig. 1).

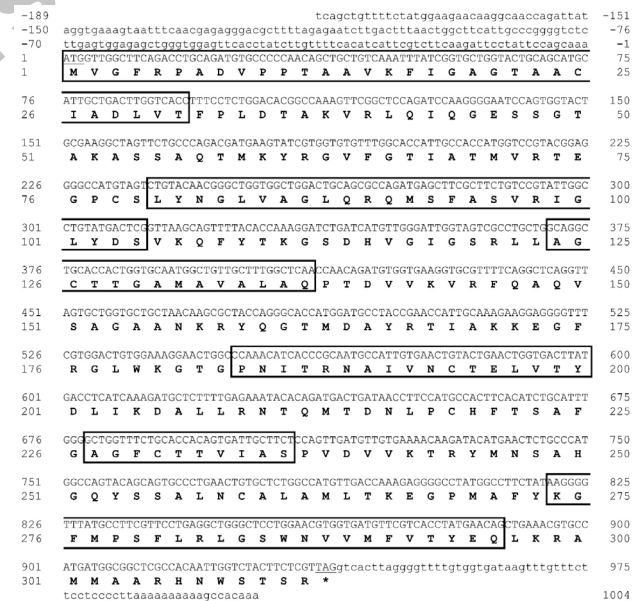


Fig. 1. Complete CDS sequences and predicted amino acids of uncoupling protein 2 (UCP2) identified from *P. vachelli*. Positions of nucleotide and amino acid sequences were indicated on both sides. Putative transmembrane conserved domains were shown by boxes. The amino acids sequences were presented by uppercase in bold. Letters in lower case represented the 5' and 3' untranslated regions. Underlines showed initiation codon and termination codon. Stop codon was indicated by (*).

Table II. Sequence identity matrix between yellow catfish UCP2 and its counterparts from other vertebrate species.

	<i>A. mexicanus</i>	<i>P. vachelli</i>	<i>D. rerio</i>	<i>S. chuatsi</i>	<i>R. norvegicus</i>	<i>H. sapiens</i>	<i>X. tropicalis</i>
<i>A. mexicanus</i>	ID	0.913	0.871	0.82	0.807	0.81	0.807
<i>P. vachelli</i>	0.913	ID	0.862	0.807	0.798	0.801	0.81
<i>D. rerio</i>	0.871	0.862	ID	0.81	0.809	0.812	0.809
<i>S. chuatsi</i>	0.82	0.807	0.81	ID	0.788	0.772	0.775
<i>R. norvegicus</i>	0.807	0.798	0.809	0.788	ID	0.951	0.828
<i>H. sapiens</i>	0.81	0.801	0.812	0.772	0.951	ID	0.822
<i>X. tropicalis</i>	0.807	0.81	0.809	0.775	0.828	0.822	ID

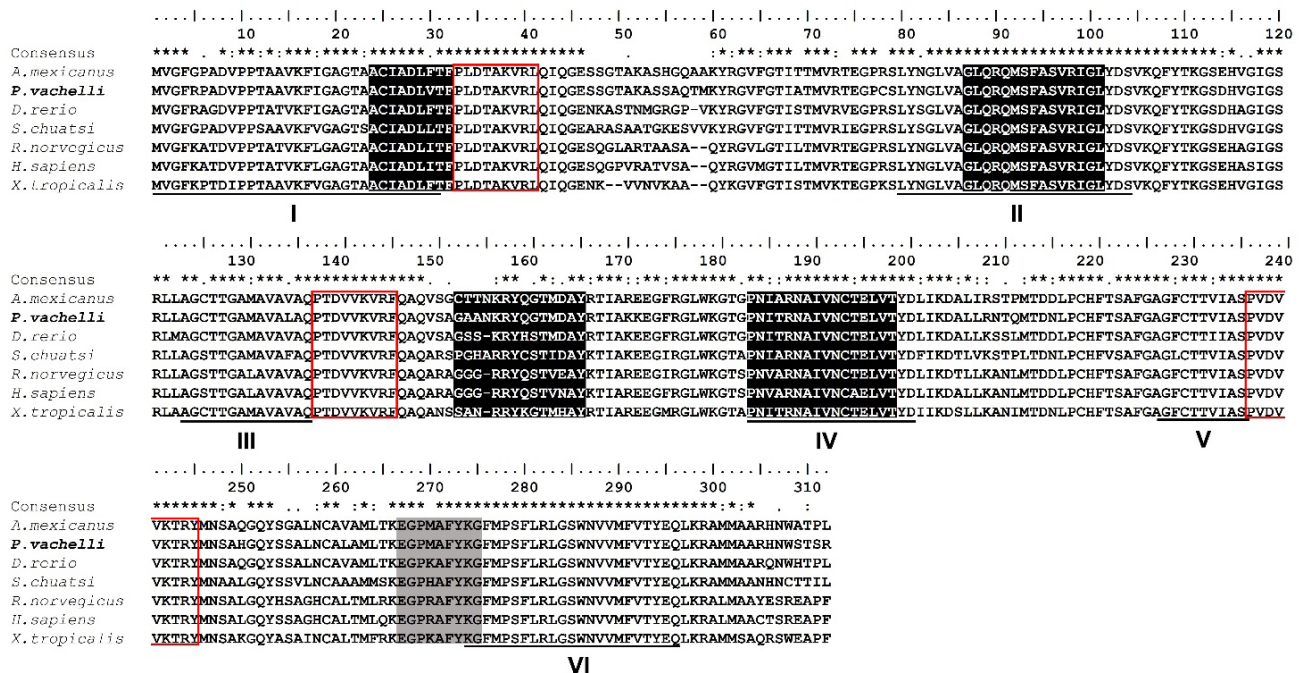


Fig. 2. Multiple alignments of the UCP2 amino acid sequences of *P. vachelli* with those of other vertebrates. The dashes in amino acid sequence indicate the amino acid gaps that are necessary to align these sequences. Asterisks (*) indicated the highly conserved amino acids among all members of the proteins. Six transmembrane α -helix domains were underlined and labeled I-VI. Three mitochondrial carrier protein motifs were labelled by red box. Purine-nucleotide binding domain was indicated by grey shade. UCP signature motifs were shown in white letters on black. The protein sequences IDs were shown in supplemental Table II.

Multiple protein sequences alignment of the vertebrate UCP2

Multiple protein sequences alignment could be helpful for better understanding the structural and functional properties of proteins. Here we demonstrated that UCP2 was highly conserved across vertebrates, sharing similar structural characteristics (six conserved transmembrane α -helix domains, four UCP signature motifs, three mitochondrial carrier protein motifs, and a purine-nucleotide binding site) with its homologues (Fig. 2). Meanwhile, sequence identity matrix showed that the pvUCP2 shared 80.1%, 79.8%, 81% identity

with its counterparts from human, mouse and frog, and shared higher identity (91.3%, 86.7% and 80.7%) with its homologs from Mexican cavefish, zebrafish and Chinese perch (Table II). Furthermore, the electronic point and molecular weight of the pvUCP2 was calculated to be 10.17 and 33.66 KD, respectively.

Phylogenetic analysis

Phylogenetic analysis was performed to better understand the evolutionary relationship among various vertebrate UCPs. The NJ tree was reconstructed based on 51 UCPs protein sequences of vertebrates. It appeared that

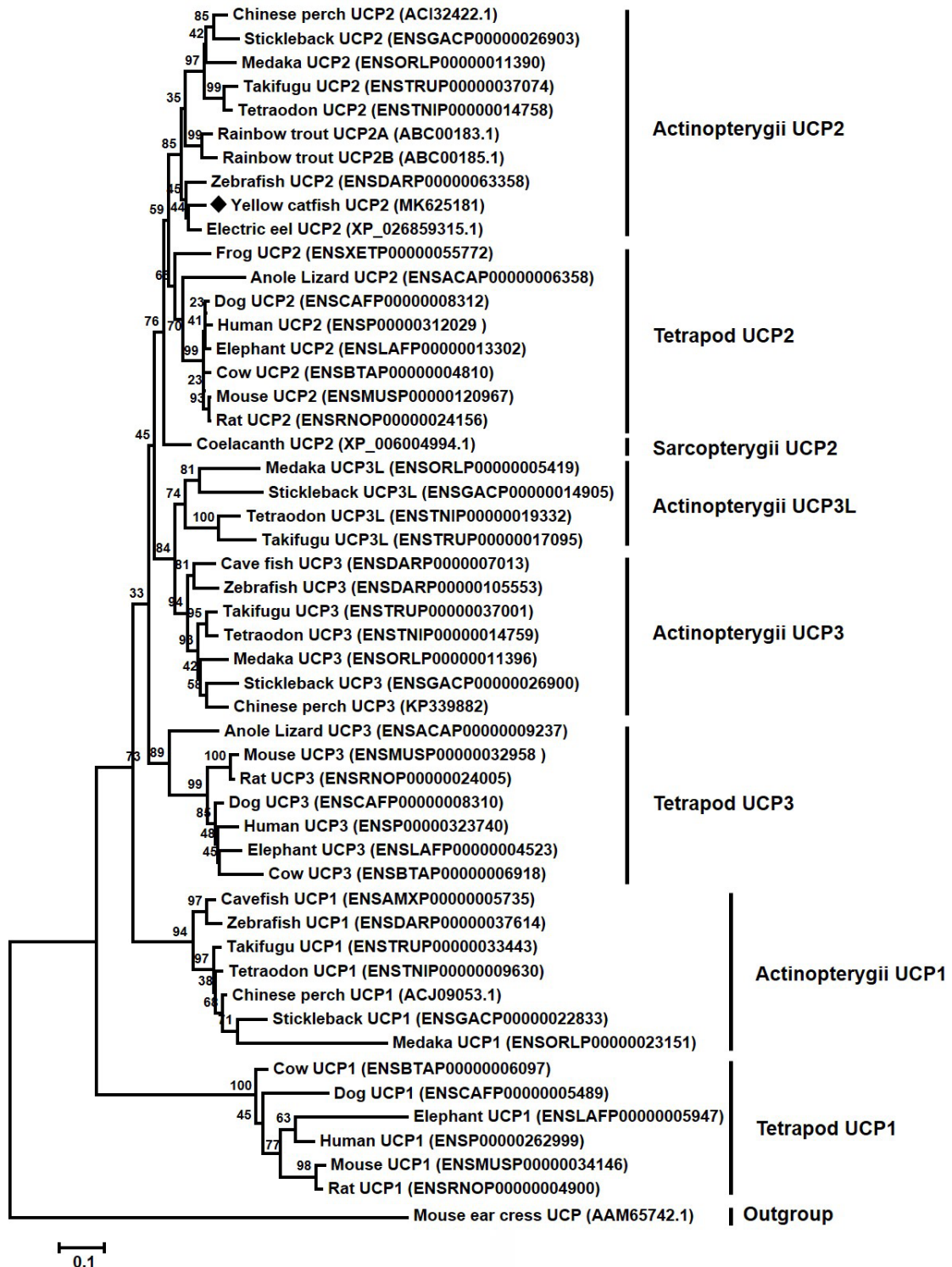


Fig. 3. Neighbor-joining tree reveals the phylogenetic relationship of vertebrate UCPs. The phylogenetic tree was constructed based on 51 protein sequences using MEGA 6.0 program. The values at the nodes represented bootstrap from 1000 replicates. The yellow catfish UCP2 was marked with diamond. The Mouse ear cress was selected as an outgroup species. Protein IDs were shown behind the taxon names.

the tree was clustered into six monophyletic groups, and the tetrapod and fish UCP1 monophyletic groups were located at the bottom of the tree while the tetrapod and fish UCP3 monophyletic groups were clustered at the top of the tree (Fig. 3). Meanwhile, a fish-specific UCP3L clade was identified and two UCP2 homologs were found in rainbow trout (Fig. 3). Moreover, the tree showed that yellow catfish UCP2 shared a close relationship with electric eel UCP2 (Fig. 3).

Tissue distribution of the *pvucp2*

Quantitative real-time PCRs were performed to determine the tissue distribution pattern of the *pvucp2*. A total of ten tissues were collected and examined (see more details in Materials and Methods). Our data showed that the *pvucp2* was widely expressed in detected tissues, including the adipose, brain, gill, heart, intestine, kidney, liver, muscle, spleen and stomach (Fig. 4). The highest transcription of the *ucp2* was detected in the liver, and a less extent high expression was found in muscle, spleen and stomach, and low expression was detected in adipose, brain, gill, heart, intestine and kidney tissues (Fig. 4).

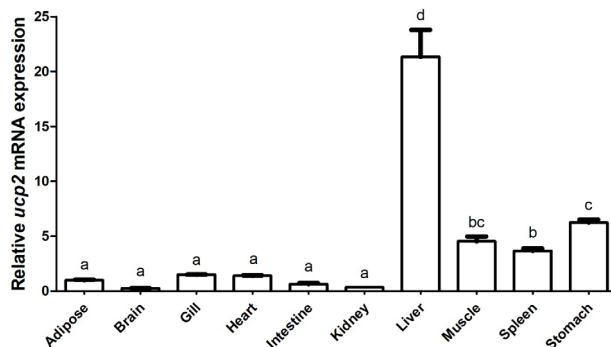


Fig. 4. Relative mRNA expression of *ucp2* in *P. vachelli*. Ten different tissues were calculated in the present study. The results were expressed as relative expression levels and standardized by β -actin gene. Error bars represent standard error of the mean (n = 6).

Effects of fasting and refeeding on the transcriptions of the hepatic *pvucp2*

To investigate transcription changes of the *ucp2* in response to starvation and feeding schemes, hepatic *ucp2* relative expressions were measured after two-week food deprivation and refeeding experiments. As shown in Figure 5, the expression of hepatic *pvucp2* was significantly decreased after two-week fasting in comparison with the control group (fed group), and then dramatically increased after refeeding. Groups that differ significantly were indicated by different letters above bars. Data were shown as mean $\pm$ SEM (n = 6).

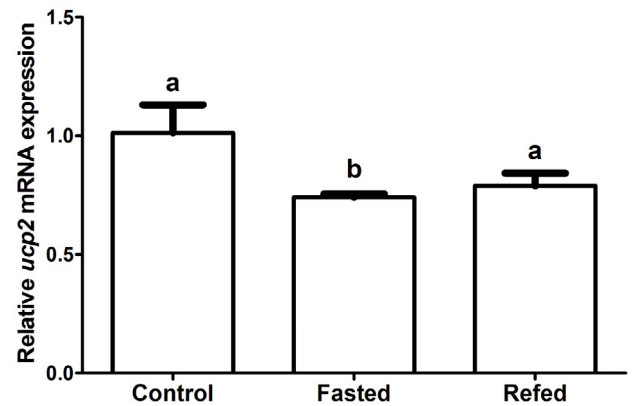


Fig. 5. Effects of two weeks fasting and refeeding on the mRNA expression of hepatic *ucp2* in *P. vachelli*. β -actin was chosen as reference gene. Significant differences ($P < 0.05$) between groups were detected using a one-way ANOVA. Groups that differ significantly were indicated by different letters above bars. Data were shown as mean $\pm$ SEM (n = 6).

DISCUSSION

In mammals, the uncoupling protein 1 (UCP1) mediates an inducible proton-leak that is directly linked to non-shivering thermogenesis in BAT (Rial and Zardoya, 2009), and now its role in thermogenesis has been well established. Following the *ucp1* identified from mammals, the paralogs named *ucp2* and *ucp3* were also found in mammals. The *ucp2* is widely distributed and has been considered to dissipate the proton gradient to prevent the proton-motive force from becoming excessive, thus decreasing reactive oxygen species (ROS) produced by electron transport (Donadelli et al., 2014). As to *ucp3*, it is mainly expressed in the muscle tissue and has been proposed to prevent the mitochondrion damage through transporting the fatty acid out from the mitochondrial matrix (Krauss et al., 2005). Interestingly, the orthologs of these genes were also found in the ectotherm fishes, which suggested the UCPs are widespread in vertebrate (Hughes and Criscuolo, 2008). However, the roles of UCPs seem not to be totally similar to their counterparts from mammals because this lineage is an allotherm and usually inhabits in variable environments (Stuart et al., 1999). To date, researches are still rarely focused on these proteins in fish, and more relative studies are helpful for better understanding the functional properties of these proteins.

In present study, the *ucp2* was identified from an economically cultured fish *P. vachelli*. The ORF length of *pvucp2* was 939 bp long encoding 312 amino acids, which showed a similar length with its orthologs reported in common carp (Stuart et al., 1999), sea bass (Tine et

al., 2012), gilthead sea bream (Bermejo-Nogales *et al.*, 2014), and mandarin fish (Wen *et al.*, 2015), suggesting the UCP2 may be highly conserved in fish. Further analysis provides more powerful evidence to support this view, multiple protein sequences alignment indicated that UCP2 was conservative among vertebrates because this protein in all species contained six highly conserved transmembrane α -helix domains, four UCP signature motifs, three mitochondrial carrier protein motifs, and a purine-nucleotide binding site (see more detail in Fig. 2), suggested UCP2 may play similar physiological roles in vertebrates (Chan *et al.*, 2004; Echtay *et al.*, 2018). In addition, the pvUCP2 shared obviously different identity with fish species compared to tetrapods, which also can be observed in some other studies (Bermejo-Nogales *et al.*, 2014; Wen *et al.*, 2015), suggesting that fish UCP2 also might play differential roles from its homolog in tetrapod.

For better understanding the evolutionary process of *ucp2* in vertebrates, a neighbor joining phylogenetic tree was constructed based on a dataset of UCP protein sequences. The phylogeny revealed that UCP1 is the ancestral UCP of the uncoupling protein family as demonstrated in previous studies (Hughes and Criscuolo, 2008; Wen *et al.*, 2015). However, fish UCP1 seems not to play thermogenesis role due to this function only found in brown adipose tissue (BAT) that is not existed in fish (Wen *et al.*, 2015; Qin *et al.*, 2018b). Meanwhile, we observed that UCP2 is the newest member of UCPs derived from UCP3, suggesting these two UCPs share close relationship as observed in previous study (Krauss *et al.*, 2005). Moreover, the yellow catfish UCP2 was close to electric eel UCP2, suggesting the evolutionary process of UCPs was consistent with the species evolution. In addition, two isoforms of *ucp2* were identified in rainbow trout, which may be caused by the salmonid-specific genome duplication event (Berthelot *et al.*, 2014; Glasauer and Neuhauss, 2014). Interestingly, a UCP3-like cluster was only identified in fish, indicating that the evolutionary process of fish UCPs might be quite complex than those in tetrapods and their roles may be various in fishes (Tine *et al.*, 2012). Consistent with previous studies, the fish-specific UCP3L genes might be derived from the whole genome duplication occurred in fish lineage and their exact roles needs more studies to declare (Wen *et al.*, 2015, 2020).

Quantitative real-time PCR was used to determine the tissue expression of *pvucp2*. We observed that the *pvucp2* was widely expressed in all examined tissues, with the highest transcription level in liver, to a less extent in muscle, spleen and stomach. Which was similar to the pattern in Chinese perch (Wen *et al.*, 2015), but different from the patterns in gilthead sea bream (with the highest *ucp2* expression in heart) (Bermejo-Nogales *et al.*, 2014)

and rainbow trout (with the highest *ucp2* expression in ovary) (Coulibaly *et al.*, 2006a), suggesting that the distribution pattern of fish *ucp2* genes are species-specific and their roles may also be species-specific. The *pvucp2* was also detected in other tissues, indicating it may play various roles in these tissues.

To check whether UCP2 plays an important role in appetite regulation and energy balance or not in yellow catfish, we detected the transcriptional changes of hepatic *pvucp2* in response to fasting and refeeding stimuli. Results showed that the *pvucp2* was significantly decreased after two-week food deprivation, and it was dramatically increased after refeeding (see Fig. 5). These changes were different from the findings in gilthead sea bream, which showed that the *ucp2* were significantly increased after fasting both in muscle and heart tissues (Bermejo-Nogales *et al.*, 2014), suggesting this gene might be species-specific as well as play different roles in various tissues or species. In the present study, the hepatic *ucp2* was significantly decreased which probably caused by the low ROS in mitochondrion since there was no enough fuel to produce ROS by oxidative phosphorylation after long-term fasting (Qin *et al.*, 2018b), and also probably caused by the inner needs to reduce the energy waste by uncoupling function and thus maintain the energy balance. In addition, the oxidative phosphorylation in liver mitochondrion could be much stronger than the status in fasting period, and thus more ROS was produced (Bermejo-Nogales *et al.*, 2014; Donadelli *et al.*, 2014; Wen *et al.*, 2015), which could explain why the hepatic *ucp2* was dramatically increased after refeeding.

CONCLUSIONS

In summary, we identified the *ucp2* gene from the *P. vachelli* for the first time and its potential roles in response to different nutritional states were investigated. Our data suggested that fish UCP2 was highly conserved and it may play various roles in fishes due to its species-specific distribution patterns. Functional experiments suggested that fish UCP2 might play important roles in maintaining energy homeostasis and reducing ROS. These findings are helpful for better understanding the evolutionary process and functional roles of UCP2 in fishes, as well as provide useful genetic information for further functional experiments.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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