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The circadian rhythm regulates branched-chain amino acids metabolism in fast muscle of Chinese perch (*Siniperca chuatsi*) during short-term fasting by Clock-KLF15-Bcat2 pathway

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Abstract

As an internal time-keeping mechanism, circadian rhythm plays crucial role in maintaining homoeostasis when in response to nutrition change; meanwhile, branched-chain amino acids (BCAA) in skeletal muscle play an important role in preserving energy homoeostasis during fasting. Previous results from our laboratory suggested that fasting can influence peripheral circadian rhythm and BCAA metabolism in fish, but the relationship between circadian rhythm and BCAA metabolism, and whether circadian rhythm regulates BCAA metabolism to maintain physiological homoeostasis during fasting remains unclear. This study shows that the expression of fifteen core clock genes as well as *KLF15* and *Bcat2* is highly responsive to short-term fasting in fast muscle of *Siniperca chuatsi*, and the correlation coefficient between *Clock* and *KLF15* expression is enhanced after fasting treatment. Furthermore, we demonstrate that the transcriptional expression of *KLF15* is regulated by Clock, and the transcriptional expression of *Bcat2* is regulated by KLF15 by using dual-luciferase reporter gene assay and *Vivo*-morpholinos-mediated gene knockdown technique. Therefore, fasting imposes a dynamic coordination of transcription between the circadian rhythm and BCAA metabolic pathways. The findings highlight the interaction between circadian rhythm and BCAA metabolism and suggest that fasting induces a switch in *KLF15* expression through affecting the rhythmic expression of *Clock*, and then KLF15 promotes the transcription of *Bcat2* to enhance the metabolism of BCAA, thus maintaining energy homoeostasis and providing energy for skeletal muscle as well as other tissues.

Key words: **Circadian rhythm: Branched-chain amino acids: Catabolism: Energy homoeostasis: Short-term fasting**

Skeletal muscle is the major organ in vertebrates, especially in fish, which represent more than 40 % of the total body weight. It maintains protein metabolic homoeostasis of the whole body by acting as a major reservoir for amino acids and stores energy in the form of proteins to cope with nutrient deficiency[1,2]. Branched-chain amino acids (BCAA) account for average thirty per cent of essential amino acids in skeletal muscle, and the breakdown of BCAA can produce alanine (Ala) which may be the most important source for gluconeogenic and protein synthesis substrate[3,4]. BCAA are also important nutrition signalling molecules that have crucial regulating effects on protein synthesis, energy homoeostasis and nutrient-sensitive signalling pathways[5–7]. BCAA are primarily catabolised and utilised as energy sources in skeletal muscle, on account of the expression of BCAA aminotransferase (BCAT2), which is a key enzyme that breaks down the first step of BCAA to produce glutamic acid (Glu) and branched-chain α-ketoacid, is high in skeletal muscle[8,9]. In the fasting state, the transamination of BCAA is significantly increased in skeletal muscle, and then the metabolite Glu converted into Ala by alanine aminotransferase (ALT); eventually, the release of Ala is taken up by the liver as substrate for gluconeogenesis to maintain energy homoeostasis[8,10,11]. The

Abbreviations: Ala, alanine; ALT, alanine aminotransferase; BCAA, branched-chain amino acids; BCAT2, BCAA aminotransferase; KLF15, Krüppel-like factor 15; Glu, glutamate.

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catabolic process of BCAA has been well known, but the adaptive regulatory mechanism and characteristic of transcriptional regulation during nutrient deficiency need further study, especially in aquatic animals.

Krüppel-like factor 15 (KLF15) plays a crucial function in regulating glycemic, lipid and amino acids metabolism[12–14]. Recently, KLF15 has been identified as a key transcriptional regulator in BCAA metabolism[14]. KLF15 can accelerate BCAA degradation and Ala production by upregulating transcriptional expression of *Bcat2* in mice and rats[15–17]. In addition, the mRNA expression of *Bcat2* is significantly decreased in *KLF15* mutant mice, and the ability to breakdown BCAA in muscle as substrate for gluconeogenesis is impaired[15]. The catabolism of BCAA is enhanced, and the mRNA transcription level of *KLF15* and *Bcat2* is significantly increased in muscle of *Oreochromis niloticus* and *Siniperca chuatsi* after short-term fasting[18,19], indicating that KLF15 is involved in dynamic regulation of BCAA catabolism in fish in response to fasting. However, the molecular mechanism by which KLF15 is involved in this process is unclear.

Circadian rhythms, also known as the circadian clock, refer to changes in behaviour, physiology and molecules that occur on a cycle length of approximately 24 h[20]. Many aspects of animal physiology and behaviour are coordinated with the light–dark cycle by circadian rhythm which is thought to be driven by molecular clock, that in mammals refer to the core clock genes[21–23]. The circadian oscillator participates in regulation of energy homoeostasis by affecting food intake, expression and activity of hormones and metabolism-related enzymes[20,24–26]. The peripheral circadian clocks play a unique and integral function in each of tissues and stimulate the rhythmic expression of specific genes participated in diverse physiological functions[27,28]. Peripheral circadian clocks also have an important effect on the whole-body metabolism[29,30]. More than 2300 genes have been identified as rhythmically expressed in skeletal muscle, and most of these genes have been identified as involved in metabolism, transcription and myogenesis[22]. When circadian clocks are disrupted, the type of muscle fibre, the structure of sarcomeric and the function of the muscle are all affected[31,32]. These data indicate a critical role for circadian clocks in skeletal muscle; however, further study is needed to reveal the regulatory mechanism of circadian clock in skeletal muscle.

The effects of fasting on expression of core circadian clocks and BCAA metabolism in skeletal muscle of fish have been studied[19,33,34]; however, the molecular mechanism of circadian rhythm regulating adaptive metabolism of fish skeletal muscle under fasting remains unclear. In this study, we investigated the expression characteristics of *KLF15*, *Bcat2* and fifteen core circadian clock genes in fast muscle of Chinese perch (*Siniperca chuatsi*) during short-term fasting, then analysed the correlation between their expression to screen out the clock gene involved in dynamic regulation of BCAA metabolism, and finally demonstrated that the circadian rhythm regulates BCAA metabolism in Chinese perch during short-term fasting by Clock-KLF15-Bcat2 pathway.

Materials and methods

Fasting and daily rhythm experimental design and sample collection

The experimental work was performed following the guidelines approved by the Animal Care Committee of Hunan Agricultural University (approval number: 20190618). A total of 216 healthy juvenile Chinese perch with body weight of about 150 g were randomly divided into four groups (fifty-four individuals per each group) that fasted for 0, 1, 5 and 7 d, respectively. Among them, the 0-d fasting group was the normal feeding group without fasting treatment and was used as the control group. The fish in each group were kept in about 10 m^3 tank which equipped with a flow-through water exchange and continuous aeration system. The fish were fed with live *Carassius auratus* twice a day at 08.00 and 17.00. Before the fasting experiment, the testing fish were acclimated to the above conditions for 1 month under 12.12 light–dark photoperiod, ZT0 was the time when light begins, and ZT12 was the time when darkness begins. After 1 month, tissue sampling was carried out at 0 (normal feeding), 1, 5 and 7 d of fasting. Before sampling, the fish were anaesthetised with 0·15 g/l tricaine methane sulphonate (MS-222). Fast muscle from dorsal myotomes were collected from five individuals in each group at ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, ZT21 and ZT24. All the muscle samples were snap-frozen in liquid N_2 and then transferred to −80°C for preservation.

cDNA synthesis and quantitative real-time PCR analysis

Total RNA were isolated from Chinese perch fast muscle using RNAiso Plus (Takara) according to the manufacturer's protocol. The RNA samples were quantified using a NanoPhotometer-NP80 (Implen), and equal amounts of RNA were reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara).

Relative transcript levels were measured by quantitative PCR using SYBR Premix Ex TaqTM (TaKaRa). Primers for the qRT-PCR assays were designed using Primer Premier 5.0 software, and the sequences were shown in Table 1. Sequences of all genes used for quantitative expression analysis were referenced from the previous transcriptome database[35] and the *Siniperca chuatsi* genome database (http://genomes.igb-berlin.de/cgi-bin/hgGateway?db=sinChu7). The method of qRT-PCR was according to our previous report[22]. *RPL13* gene was used as reference gene, and the relative expression level of target mRNA was calculated by $R = 2^{-\Delta\Delta Ct}$.

Branched-chain amino acids and BCAT2 content and enzyme activity determination

The BCAA contents in fast muscle of Chinese perch that fasted for 0, 1, 5 and 7 d were determined according to previous method[19]. The sum of leucine, isoleucine and valine represents the content of the BCAA. The Bcat2 protein content in fast muscle was detected using a Fish Bcat2 ELISA Kit from Zhuo-Chai Biotechnology Institute, and the activities of ALT were

Table 1. Primers for qRT-PCR

Genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Arntl1	GGACCTGATTCTGTGGGCTA	AGCCTCTAGCAGGGACATGA
Arntl2	AGGGACCCAAATCGCAAATG	TGTGGGGAAACAAGGGGAC
Bcat2	CTGGCGGACCCTTCATTTG	GCTATCGTAGGACCGTAGTTGC
Clock	TGCTGGAGGCTCTGGATGG	GGTTCTGGTCCACTAAGTCCGTC
Cry1	ATTTCCCGCCTGTCTTATGAGT	CAGGGTCTGGAACCGTTTGTA
Cry2	GAGAAAAGCGTGGGTGGC	CTTGCGGTAGAGGTCTGTGAG
Cry3	ATCTTGAAGGACTACCCGAACC	GCTGCCCTCTGCGTGGTTA
Cry-dash	AAGCAAGGCCTCGACTATGA	GCTCCATTCAGGCGCTATAA
KLF15	GGAAAGACTGTCAACACCAAGCG	GCCTCCAGAGCAGGGTTAGCC
Npas2	CAGATAGCGAGTTCAGCCAAGA	TGGAGAATGAAGGAGCGATGA
Nr1d1	GCCGTGGTGCTGGTGTCTG	TTGTTGAGCGTTCGCAGGTC
Nr1d2	TCTCCCCATGTGGACCCTC	GGTGCGGTCCTTCACATCG
Per1	CAACAAACTCATCCTCCTGGC	CGGTGGGTAAACAGGGTAGATT
Per2	TGGTAACGAGTCGCAAGGC	TCACCAGACTGAAGGCGTTAGA
Per3	CCGCTACAGAAGAACGAAGG	GGGAGTGAGCCATAGAGCTG
Rorα	GGTGGGTTCTACCTGGACTTCC	TGAAGGAGCAGTACGGGAAGAA
RPL13	CACAAGAAGGAGAAGGCTCGGGT	TTTGGCTCTCTTGGCACGGAT
Timeless	GAAGGCTACAGCAAAGACGGA	AGTCCTGCCACCAGTCCGT

estimated using commercial Alanine aminotransferase assay kit (Jian-Cheng Biotechnology Institute) according to instruction.

Dual-luciferase reporter gene assay

The *in silico* analysis identified two putative sites of Clock binding to *KLF15* gene promoter and two putative sites of KLF15 binding to *Bcat2* gene promoter. In order to determine whether Clock regulates transcription of *KLF15* and KLF15 regulates transcription of *Bcat2*, the psiCHECK2-KLF15-WT, psiCHECK2-KLF15-Mut1, psiCHECK2-KLF15-Mut2, psiCHECK2-Bcat2-WT, psiCHECK2-Bcat2-Mut1 and psiCHECK2-Bcat2-Mut2 dual-luciferase reporter gene expression vectors were constructed. For overexpression of Clock and KLF15 in cultured cells, full-length *Clock* and *KLF15* cDNA was subcloned into a pcDNA3.1-flag expression plasmid. As there is no muscle cell line of fish, the primary cultured Chinese perch muscle cells are unstable. So 293T cells, which are often used in luciferase reporter assays, were used for *in vitro* validation experiments in this study. Reporter plasmids were co-transfected into 293T cells with expression plasmids or control plasmids in 24-well plates using Lipofectamine 3000 (Invitrogen, L3000015). The luciferase activity was determined by Dual-Luciferase Reporter Assay System (Promega, E1910) according to the manufacturer's protocol after transfection for 48 h.

KLF15 and clock gene knockdown

The knockdown (KD) of *KLF15* and *Clock* genes in Chinese perch were achieved by injection of translation-blocking *Vivo*-morpholinos (Gene Tools) targeting the mRNA AUG translational start site or adjacent sequences. The antisense sequence used for *KLF15* and *Clock* was 5′-TACCATCCCTGGATA GTGCCAAACG-3′ and 5′-CAGTGATTTGCTCTTTAGGC GTGA-3′, respectively. Healthy juvenile Chinese perch with body weight of about 150 g were chosen for *Vivo*-morpholinos injection experiment, and three individuals were injected in each group. *Vivo*-morpholinos were injected in dorsal muscle with 12·5 mg/kg (1·25 mmol/kg) body weight, and the control group was injected with equal amount of control oligos. Fast muscle samples were taken at 2 d after injection and stored at −80°C after snap-frozen in liquid N_2.

Western blotting

For western blotting, proteins were extracted from fast muscle samples in RIPA lysis buffer, separated on 12 % SDS-PAGE gels, transferred to PVDF membranes by Trans-Blot Turbo and probed with primary antibody against KLF15 (Absin, abs113067, 1:1000), Clock (Proteintech, 18 094–1-AP, 1:1000) or *β*-actin (Proteintech, 20 536–1-AP, 1:2000). Samples were then stained with secondary antibody conjugated to HRP (Abbkine, A21020) at the dilution of 1:5000. The signal was scanned by the ChemiDoc XRS+ imaging system, and the grey values of protein signal were analysed by NIH-Image J software.

Statistical analysis. To detect variation in mRNA levels among different time points, statistical analyses were carried out with one-way ANOVA procedures by SPSS 19.0. The daily rhythmicity in relation to the expression of core circadian clock genes, *KLF15* and *Bcat2*, was assayed with Matlab 7.0. To perform a cosinor analysis, the formula $f(t) = M + Acos\ (t/pi/12\ -\phi)$ was used, and the meanings of the letters in the formula refer to previous reports[34]. Significance of cosinor analysis was defined by the noise/signal of amplitude calculated from the ratio se(A)/A (hereafter referred as *P*-value). Expression was considered to display a daily rhythm if it had both $P < 0{\cdot}05$ by ANOVA and *P*-value < 0·30 by cosinor analysis. The data were expressed as the mean ± SE (n 5).

The data in gene knockdown and luciferase assays were expressed as the mean ± SD (n 3). Two-tailed Student's t test was used for comparisons between two independent groups. For multiple comparisons, Duncan's multiple range tests were used. *P*-values less than 0·05 were considered statistically significant.

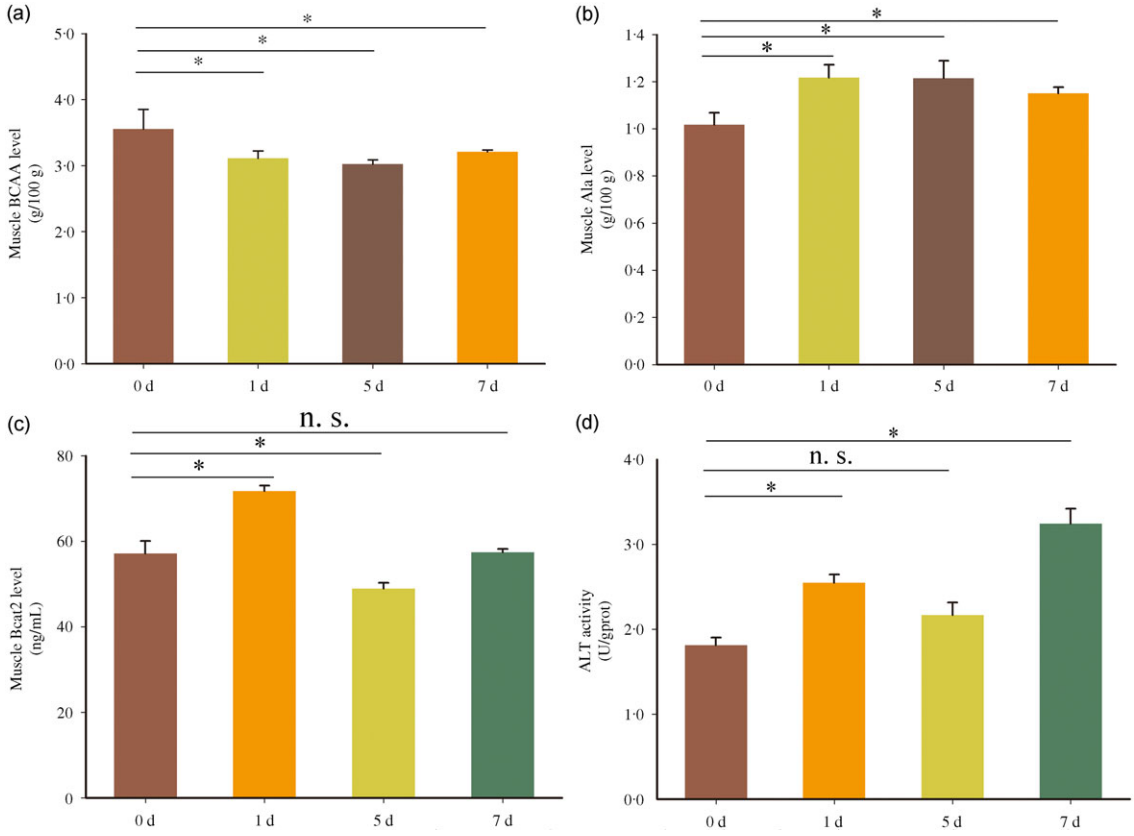

Fig. 1. Metabolic characteristics of BCAA in fast muscle of Chinese perch during short-term fasting. The A-D represents BCAA, Ala and Bcat2 contents, and ALT activity, respectively. Values in the figures are the mean ± SE, *n* 5. The asterisk indicates a significant difference between two groups ($P < 0{\cdot}05$). n.s. indicates no significant difference between two groups ($P > 0{\cdot}05$). BCAA = branched-chain amino acids; Ala = alanine; ALT = alanine aminotransferase; d = days after fasting.

The expression correlation between genes was tested by Pearson's correlation test using GraphPad Prism 7.0 software. If the coefficient r is positive, it indicates a positive correlation between genes; conversely, the genes are negatively correlated. If $0{\cdot}50 \leq |r| < 0{\cdot}70$, there is moderate correlation between genes. If $|r| \geq 0{\cdot}70$, the genes were strongly correlated.

Results

Metabolic characteristics of branched-chain amino acids in fast muscle during short-term fasting

To analyse the metabolic characteristics of BCAA in Chinese perch during short-term fasting, the contents of BCAA, Ala and Bcat2, and the activities of ALT were measured in fast muscle. The BCAA content in fast muscle was significantly decreased after 1, 5 and 7 d fasting compared with 0-d fasting (normal feeding) (Fig. 1(a)). But in contrast, the Ala content in fast muscle was significantly increased after short-term fasting treatment (Fig. 1(a)). However, the content of Bcat2 protein was dynamically adjusted during fasting, with increased after 1 d fasting, reduced after 5 d fasting and then recovered to initial value (value in 0 d fasting) after fasting for 7 d (Fig. 1(c)). The activity of ALT showed increased after 1 d and 7 d fasting but unchanged after 5 d fasting (Fig. 1(d)). The results above indicate that the catabolism of BCAA in fast muscle is enhanced during short-term fasting, and the activity or content of BCAA metabolism-related enzymes has a dynamic adjustment process during the fasting state.

The rhythmic expression of Bcat2 in fast muscle of Chinese perch after short-term fasting treatment

To investigate whether BCAA metabolism is regulated by circadian rhythm, the expression of *Bcat2* during a daily cycle was analysed in normal feeding (fasted for 0 d group) Chinese perch. The daily expression profile showed that *Bcat2* was oscillating between day and night, and *Bcat2* expression level was low during the day and high at night (Fig. 2). The result of cosinor analysis showed that the expression of *Bcat2* displayed a significant daily rhythm (P-value $< 0{\cdot}30$) with acrophase at night ($ZT = 15{\cdot}43$) (Table 2). The result indicates that the BCAA metabolism in Chinese perch fast muscle may has a circadian rhythm. To further evaluate the effects of short-term fasting on rhythmic expression of *Bcat2*, the daily expression profile of *Bcat2* was also analysed after 1, 5 and 7 d fasting (Fig. 2). After 1 and 5 d fasting, *Bcat2* in fast muscle still displayed significant daily cyclic oscillations; however, the acrophases and amplitudes were changed (Table 2). The acrophase exhibited a left shift after 1 and 5 d fasting compared with normal feeding

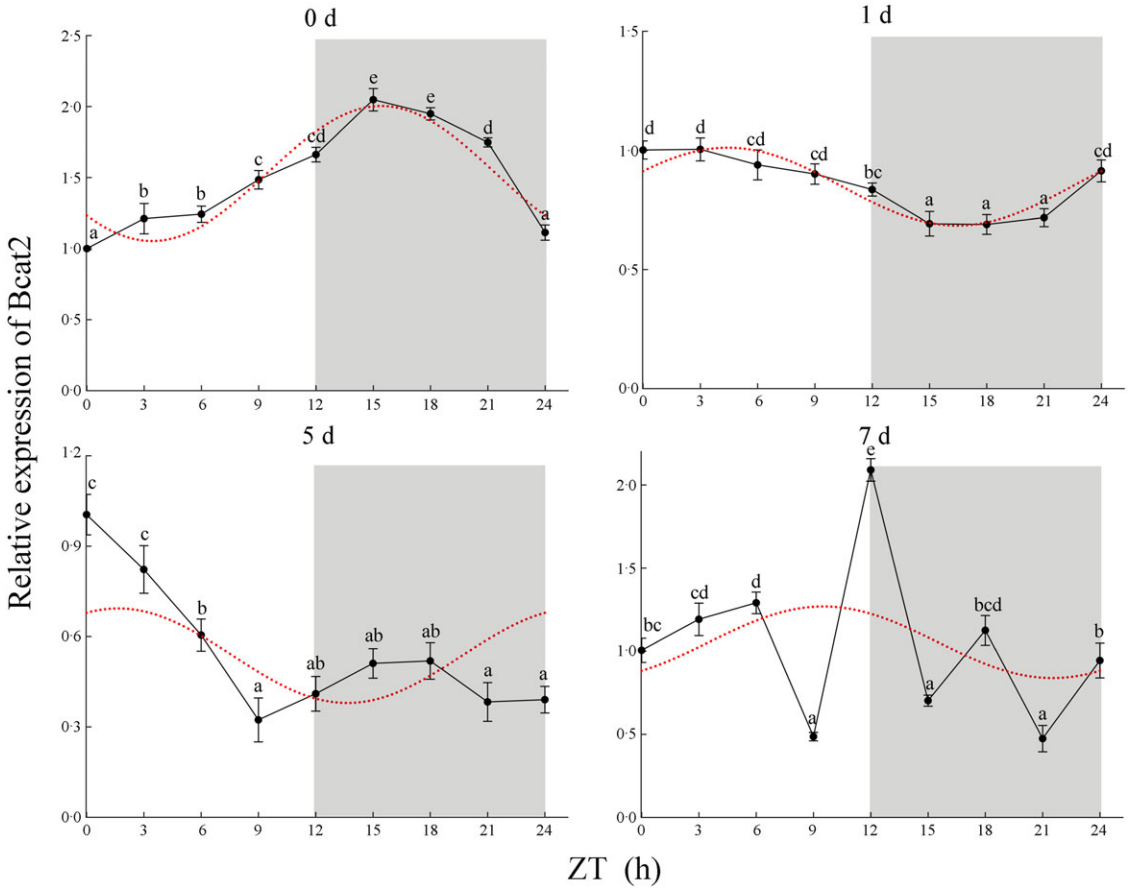

Fig. 2. Cosinor analyses of *Bcat2* expression in fast muscle of Chinese perch during a daily cycle after short-term fasting. The values are mean ± SE (n 5). Letters on the error line indicate significance markers, and different letters represents statistical difference between different time point ($P < 0{\cdot}05$). The red dotted lines show the periodic sinusoids fitted based on the periodic parameters of each gene expression. ZT = zeitgeber time; 0, 1, 5 and 7 d = 0, 1, 5 and 7 d after fasting.

Table 2. Circadian parameters of *Bcat2* and *KLF15* expression during short-term fasting

Gene	Amplitude	Mesor	Acrophase (h)	*P*	Fasting time
Bcat2	0·48	1·53	15·43	< 0·01	0 d
Bcat2	0·16	0·85	4·44	< 0·01	1 d
Bcat2	0·16	0·54	1·66	0·26	5 d
Bcat2	0·22	1·05	9·52	0·63	7 d
KLF15	0·36	1·34	10·47	0·29	0 d
KLF15	0·26	0·99	6·34	0·08	1 d
KLF15	0·25	0·61	15·28	0·26	5 d
KLF15	0·38	0·98	8·25	0·08	7 d

The amplitude is half of the distance between two waveform peaks fitted. Median is the average of the curve. Acrophase is the radian corresponding to the time point of the highest amplitude. *P*-value is the noise/signal amplitude ratio in cosine analysis.

Chinese perch (Fig. 2 and Table 2). Whereas, fasting for 7 d disrupted circadian periodicity for *Bcat2* which the rhythmic expression was disappeared (*P*-value = 0·63) (Fig. 2).

The rhythmic expression of KLF15 *and correlation analysis of* KLF15 *and* Bcat2 *expression during short-term fasting*

Considering that KLF15 can affect the expression of *Bcat2*, we speculated whether KLF15 is involved in regulating the rhythmic expression of *Bcat2* in fast muscle of Chinese perch that under normal feeding or fasting state. To test this hypothesis, the daily expression profile of *KLF15* and the cosinor analysis of its expression was analysed after 0, 1, 5 and 7 d fasting. The daily expression profile showed that *KLF15* was oscillating between day and night, and its expression displayed a significant daily rhythm (*P*-value < 0·30) in fast muscle of normal feeding (fasted for 0 d group) and 1, 5 and 7 d fasting Chinese perch (Fig. 3). But the acrophases were changed during 1, 5 and 7 d fasting (Table 2). The results suggest that the transcription of *KLF15* is also regulated by circadian rhythm, and its rhythmic expression can be dynamically adjusted with different periods of fasting. Next, the correlation between the circadian rhythmic expression of *KLF15* and *Bcat2* was analysed. In the normal feeding and fasting for 5 d fish, the transcript levels of *KLF15* and *Bcat2* displayed low positive correlation ($r = 0{\cdot}24 < 0{\cdot}50$). Interesting, *KLF15* displayed moderate positive correlation with *Bcat2* after 1 d fasting ($0{\cdot}50 < r = 0{\cdot}57 < 0{\cdot}70$), and strong positive correlation with *Bcat2* after 7 d fasting ($r = 0{\cdot}74 > 0{\cdot}70$).

The expression of Bcat2 *is regulated by KLF15*

Although KLF15 has been reported to induce *Bcat2* expression in mammals, it is not clear how KLF15 regulates the expression of *Bcat2* in fish. To verify whether KLF15 regulates *Bcat2*

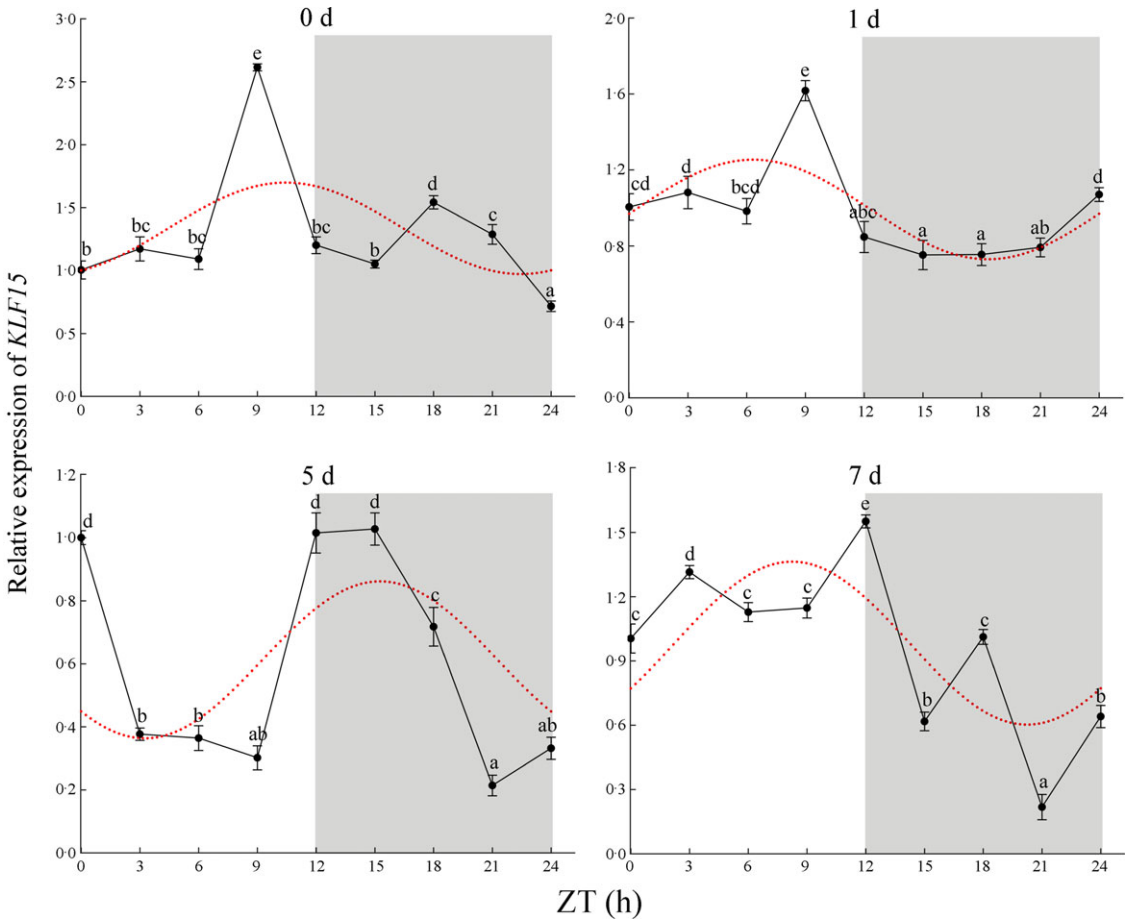

Fig. 3. Cosinor analyses of *KLF15* expression in fast muscle of Chinese perch during a daily cycle after short-term fasting. The values are mean ± SE (n 5). Letters on the error line indicate significance markers, and different letter represents statistical difference between different time point ($P < 0{\cdot}05$). The red dotted lines show the periodic sinusoids fitted based on the periodic parameters of each gene expression. ZT = zeitgeber time; 0, 1, 5 and 7 d = 0, 1, 5 and 7 d after fasting.

transcription, we analysed the 2-kb DNA sequence upstream of the *Bcat2* transcriptional start site for evidence of G-rich element which has been reported as KLF15 binding site[36]. The analysis identified two putative G-rich elements (designated as G-rich 1 and G-rich 2, respectively) in the *Bcat2* gene promotor. The sequence of one site is 5′-GGGGAGGGGA-3′ (G-rich 1), and the other is 5′-AAACCCCCCCCC-3′ (G-rich 2, the complementary strand is 5′-GGGGGGGGGTTT-3′). Therefore, we used luciferase assays to determine whether these sites are involved in KLF15-regulating transcriptional expression of *Bcat2*. First, the dual-luciferase reporter vector containing G-rich 1 and G-rich 2 was generated, and the luciferase assays were performed in cells which transfected with the luciferase reporter vector and KLF15 overexpression vector or negative control vector. The result showed that overexpression KLF15 enhanced the luciferase activity of the reporter vector containing G-rich 1 and G-rich 2 element (Fig. 4(a)). Next, to confirm which element is regulated by KLF15, the G-rich 1 or G-rich 2 sequence in the reporter vector was disrupted, respectively, by site-directed mutagenesis. The result showed that G-rich 1 mutant abolished KLF15 regulation of the reporter activity, instead of the reporter construct containing G-rich 2 mutant (Fig. 4(a)), indicating that this G-rich 1 is a crucial site for KLF15-regulating *Bcat2* transcriptional expression.

Considering that the 293T cells, which were used for *in vitro* validation in this study, differ significantly from fast muscle cells, a gene-specific antisense oligonucleotide, *Vivo*-morpholino, was designed to knock down endogenous KLF15 expression and to test the effect on *Bcat2* expression. Compared with the control group, KLF15 protein expression level was significantly reduced in morpholino injection group (Fig. 4(b) and (c)), indicating the morpholino had successfully knock down KLF15. In addition, KLF15 knockdown significantly inhibited the transcriptional level of *Bcat2* (Fig. 4(d)). Together, the *in vitro* and *in vivo* experiments demonstrate that *Bcat2* expression may be directly regulated by KLF15. Interesting, the mRNA level of *KLF15* was also decreased when knocked down KLF15 protein expression (Fig. 4(d)). Study in mouse has found that BCAA negatively regulated *KLF15* expression[37]; therefore, the decreased *KLF15* might be attributed to the accumulation of BCAA when BCAA metabolism was inhibited by KLF15 knockdown.

The expression of core clock genes during short-term fasting and its correlation with KLF15

Previous study has identified that the *KLF15* expression is regulated by core clock machinery in mammal[38], and KLF15 is an

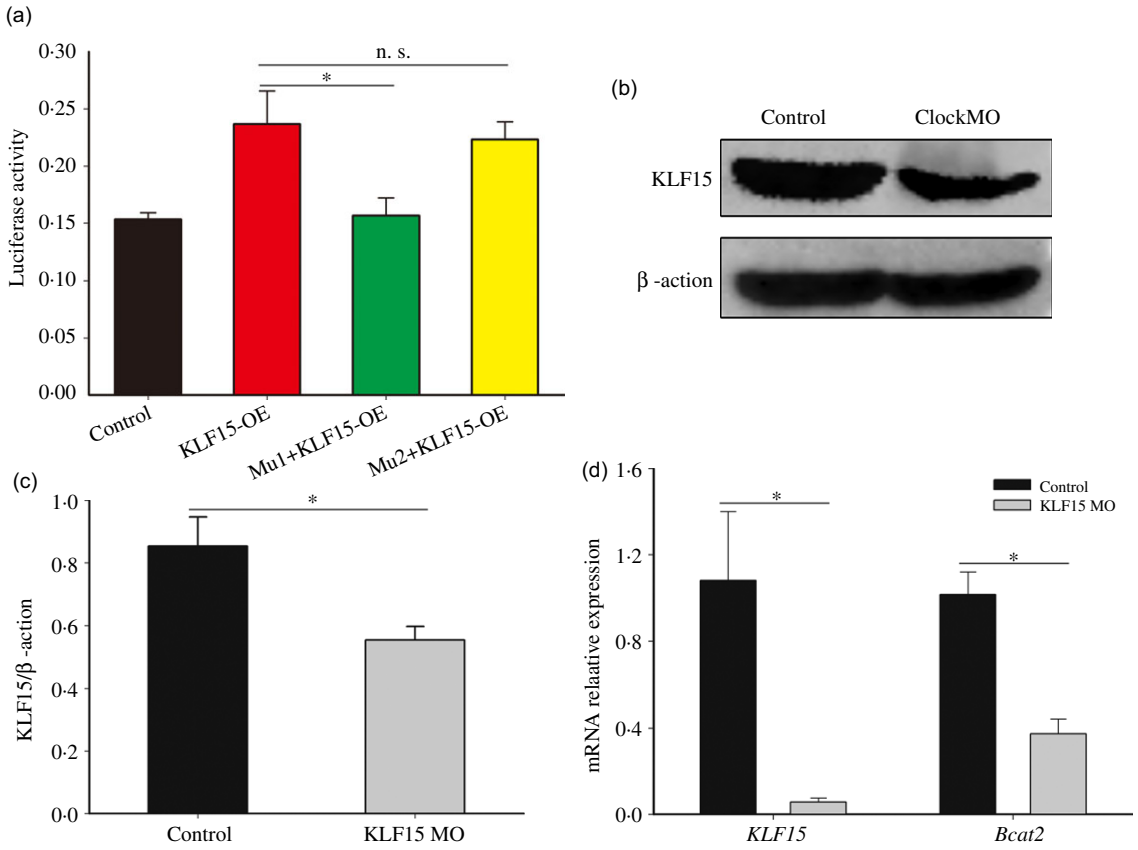

Fig. 4. The transcriptional expression of *Bcat2* is regulated by KLF15. (A) Luciferase activity in cells transfected with *KLF15* overexpression vector and reporter vector containing G-rich 1 and G-rich 2 element, or in cells transfected with *KLF15* overexpression vector and reporter vector containing G-rich 1 or G-rich 2 element mutant (Mu1 or Mu2). (B) The KLF15 protein in control and KLF15 morpholino group by western blotting. (C) The relative protein level of KLF15 in control and morpholino group by grey scale analysis. (D) The mRNA expression of *KLF15* and *Bcat2* in control and KLF15 morpholino group. The asterisk indicates significant difference between two groups ($P < 0{\cdot}05$). n.s. indicates no significant difference between two groups ($P > 0{\cdot}05$). OE = over expression; Mu = mutant; MO = morpholino.

important regulator of daily rhythmicity in skeletal muscle[39]. This suggests that the rhythmic expression of *KLF15* is also controlled by core clock in Chinese perch. To identify which core clock gene regulates *KLF15* expression, the rhythmic expression patterns of fifteen core clock genes were determined in fast muscle of normal feeding and short-term fasting Chinese perch. The circadian parameters of core clock genes in fast muscle of Chinese perch, which was fasted for 0, 1, 5 and 7 d, were shown in Supplementary Table S1–S4. The expression of tested genes showed a significant daily rhythm in normal feeding Chinese perch, except for *Arntl2* and *Cry-dash* (Fig. 5 and online Supplementary Fig. S1). After 1 d fasting, the rhythmic expression of *Cry1* was disappeared, and the expression of *Arntl2* and *Cry-dash* began to show a rhythm (Fig. 5 and online Supplementary Fig. S2). After 5 d fasting, the rhythmic expression of *Cry1*, *Rorα* and Timeless (Tim) was disappeared (Fig. 5 and online Supplementary Fig. S3). The rhythmic expression of core clock gene was seriously disrupted during 7 d fasting, with only *Clock*, *Arntl1*, *Arntl2*, *Rorα* and *Per2* still displayed significant daily cyclic oscillations (Fig. 5 and online Supplementary Fig. S4). These results indicate that the core clock genes are dynamic adjustment during short-term fasting. Among the fifteen core clock genes, only *Clock*, *Arntl1* and *Per2* had always displayed daily cyclic oscillations during the short-term fasting (Fig. 5).

The correlation of circadian rhythm expression between *KLF15* and 15 core clock genes was analysed. In the fast muscle of normal feeding fish, the transcript level of *KLF15* displayed moderate positive correlation with *Clock* and *Rorα* ($0{\cdot}50 \leq r < 0{\cdot}70$), and strong negative correlation with Timeless ($|r| > 0{\cdot}70$) (Table 3). After 1 d fasting, *Clock* and *Tim* displayed strong positive and negative correlation with *KLF15*, respectively (Table 3). Meanwhile, *Rorα*, *Nr1d2*, *Per1* and *Per3* displayed moderate positive correlation with *KLF15* (Table 3). After 5-d fasting, *Clock* displayed strong positive correlation with *KLF15*, *Arntl1* and *Npas2* displayed moderate positive correlation with *KLF15*, and *Cry-dash* displayed moderate negative correlation with *KLF15* (Table 3). After 7-d fasting, the correlation between core clock genes and *KLF15* was enhanced. *Clock*, *Arntl1*, *Per2*, *Per3*, *Cry1* and *Cry3* displayed strong positive correlation with *KLF15*, and the negative correlation between *Time* and *KLF15* was disappeared (Table 3). The correlation analysis results showed that *Clock* always displayed positive correlation with *KLF15*, suggesting that the rhythmic expression of *KLF15* is likely to be regulated by core clock gene Clock.

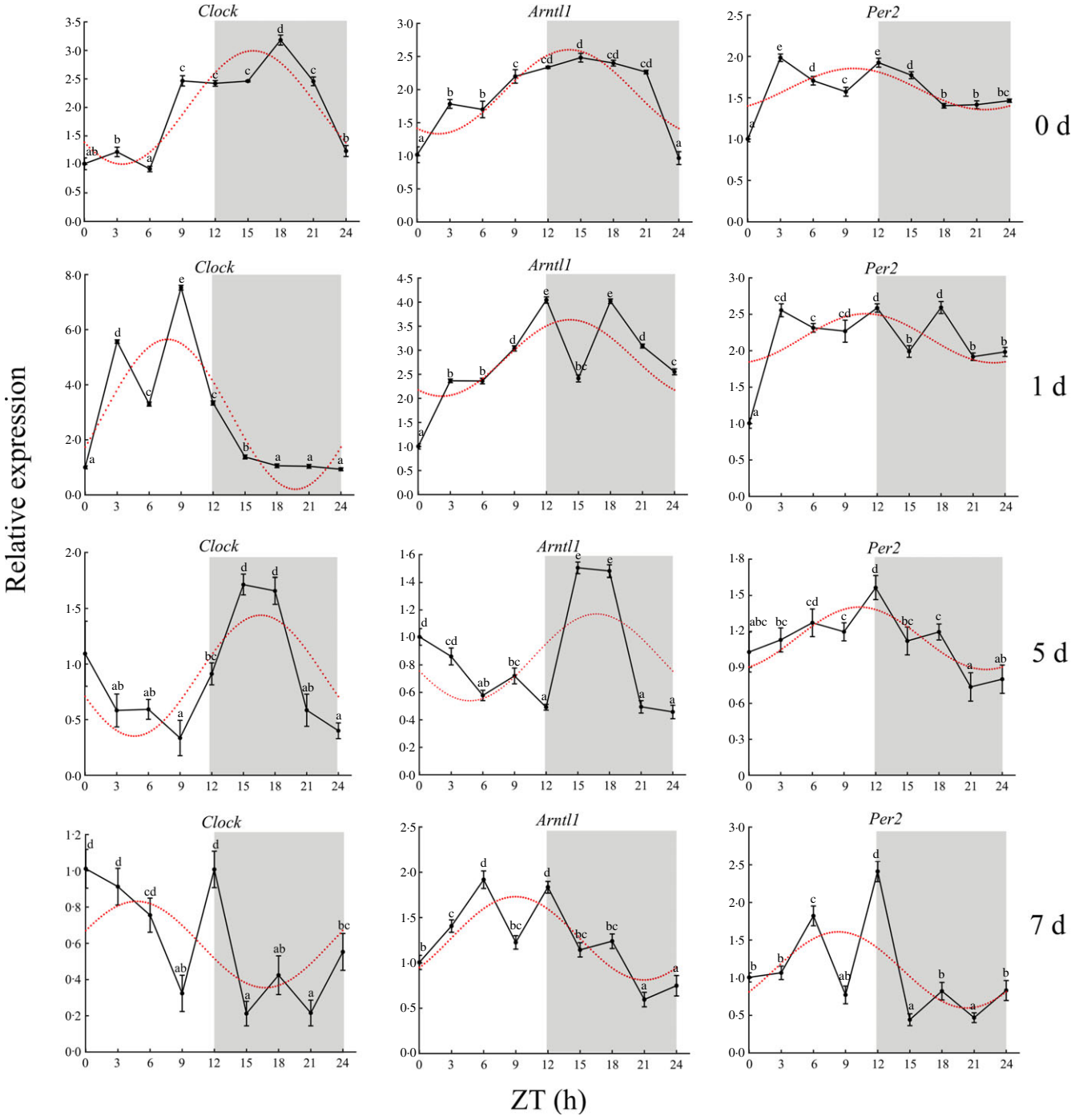

Fig. 5. Cosinor analyses of *Clock*, *Arntl1* and *Per2* expression in fast muscle of Chinese perch during a daily cycle after short-term fasting. The values are mean ± SE (n 5). Letters on the error line indicate significance markers, and different letter represents statistical difference between different time point ($P < 0{\cdot}05$). The red dotted lines show the periodic sinusoids fitted based on the periodic parameters of each gene expression. ZT = zeitgeber time; 0, 1, 5 and 7 d = 0, 1, 5 and 7 d after fasting.

The expression of KLF15 *is regulated by Clock*

To verify whether the transcriptional expression of *KLF15* is regulated by Clock, we analysed the 2-kb DNA sequence upstream of the *KLF15* transcriptional start site for presence of E-box which is a canonical regulatory element for circadian clock. Two putative E-box elements (named E-box 1 and E-box 2) were identified in Chinese perch *KLF15* gene. The sequence of one site is 5′-GCCACGTGCG-3′ (E-box 1), and the other is 5′-AACACGTGCA-3′ (E-box 2). Next, the luciferase reporter vector containing E-box 1 and E-box 2 was generated, and the luciferase assays were performed in cells which transfected with above reporter vector, and a Clock overexpression

Table 3. The correlation analysis between core clock genes and *KLF15* expression

Gene pairs	Normal feeding (*r*)	Fasting for 1 d (*r*)	Fasting for 5 d (*r*)	Fasting for 7 d (*r*)
Clock:KLF15	**0·50**	**0·79**	**0·77**	**0·72**
Arntl1:KLF15	0·47	–0·18	**0·54**	**0·82**
Npas2:KLF15	0·06	–0·30	**0·61**	**0·67**
Cry2:KLF15	0·26	0·02	0·13	**0·58**
Rorα:KLF15	**0·60**	**0·60**	0·40	0·46
Per2:KLF15	0·02	0·00	0·48	**0·76**
cry-dash:KLF15	–0·02	0·06	**–0·60**	**0·56**
Cry1:KLF15	–0·23	0·31	0·22	**0·78**
Cry3:KLF15	–0·10	–0·05	0·30	**0·71**
Nr1d1:KLF15	–0·09	0·35	–0·05	0·44
Nr1d2:KLF15	–0·43	**0·66**	–0·22	**0·57**
Arntl2:KLF15	**–0·51**	–0·10	0·09	0·25
Timeless:KLF15	**–0·72**	**–0·73**	**–0·64**	–0·07
Per1:KLF15	–0·16	**0·62**	–0·21	**0·61**
Per3:KLF15	–0·36	**0·68**	–0·09	**0·70**

The *r* values were set to define the degree of correlation, data are moderately correlated if $0{\cdot}5 \leq |r| < 0{\cdot}7$ and there is a strong correlation when $|r| \geq 0{\cdot}7$. If *r* is a positive number, it means a positive correlation, and the opposite means a negative correlation.

vector or negative control vector was performed. The result showed that overexpression of Clock increased the activity of luciferase in cells which transfected with the luciferase reporter construct (Fig. 6(a)). Then to confirm which site was the regulatory element recognised by Clock, the E-box 1 or E-box 2 sequence in the reporter vector was disrupted, respectively, by site-directed mutation technique. The luciferase assays showed that the E-box 1 mutant inhibited Clock regulated the luciferase activity, rather than the reporter vector with E-box 2 mutant (Fig. 6(a)). The results indicate that the E-box 1 is important for Clock regulation of *KLF15* transcriptional expression. Furthermore, in order to evaluate whether Clock regulates *KLF15* expression *in vivo*, a Clock-specific *Vivo*-morpholino was designed to knock down endogenous Clock expression. Compared with the control group, Clock morpholino injection significantly reduced the protein level of Clock and inhibited the transcriptional level of *KLF15* and *Bcat2* (Fig. 6(b)–(d)).

Discussion

The behaviour and physiology of organisms are affected by rotation of the earth[40,41]. In animals, diurnal cycle has driven the evolution of molecular clocks, syncing physiological and cellular processes to a cycle about 24 h. The past few decades have identified components and function of the central clock in mammals, but the function of clocks in the peripheral tissues is not fully understood, especially in non-mammal. As a highly adaptive and plasticity tissue, skeletal muscle has circadian rhythms[42]. The daily rhythmicity of many clock genes was observed in skeletal muscle of fish[42–44], indicating that circadian rhythm has a potential role in regulating the physiology or metabolism in fish skeletal muscle. This study shows that circadian clock involves in regulating BCAA catabolism in fast muscle of Chinese perch during short-term fasting through a Clock crosstalk pathway to *KLF15*.

Precise regulation of metabolic processes is an important cornerstones of energy balance, and tight control of this homoeostatic process is essential for health and continuance of organisms. The circadian clock is the primary regulator of metabolism because there is growing evidence that the core clock machinery plays a central role in regulating metabolic homoeostasis[45–48]. Previously, research in mouse and human has showed that nitrogen homoeostasis exhibits a 24-h periodicity and demonstrates that nitrogen homoeostasis is a conserved intrinsic circadian process in mammals[41]. BCAA are critical for the whole-body anabolism and energy homoeostasis, whether clock-driven oscillations in BCAA impact protein turnover is an attractive hypothesis. A recent study in muscular atrophy links disruption of circadian rhythm in regulation of skeletal muscle BCAA catabolism to severity of phenotypes[49]. Microarray analyses suggest that the BCAA α-ketoacid dehydrogenase is regulated by circadian clock at the transcriptional level[50]. Interesting, the daily expression profile of *Bcat2* in Chinese perch fast muscle displayed significant daily cyclic oscillation, indicating that the BCAA metabolism in Chinese perch is under the control of circadian rhythm.

Previous studies have identified KLF15 as an important regulator of diurnal rhythmicity in skeletal muscle, heart and liver[15,39,41]. Research in mouse has identified KLF15 as a clock-driven peripheral clock factor critical for coordinating the transport of carbon skeletons and linked the clock to nitrogen homoeostasis by a KLF15-dependent way[41]. Furthermore, the rhythm of *KLF15* is disrupted in some mouse lines with circadian clock gene mutant, which supports transcriptional expression of KLF15 is directly regulated by circadian clock[22]. This study showed that KLF15 regulated the transcriptional expression of *Bcat2*, and its expression exhibited 24 h periodicity and regulated by core clock gene Clock. Clock has been reported as a positive regulator of *KLF15*, and *KLF15* rhythmicity is broken in core clock machinery mutant mouse[41]. The E-box is a critical cis-regulatory element that can be recognised by circadian clock to regulate transcription of flanking genes. The promoter region of mouse *KLF15* gene revealed four canonical E-box regions for the core clock gene Clock[41]. Interestingly, two E-box binding sites were also found in 2 kb of the promoter region of Chinese perch *KLF15* gene. Although only E-box 1 was verified to be the Clock regulatory site in Chinese perch, it suggests that the E-box is a conserved binding site for Clock regulation in at least vertebrates.

Fasting is a dynamic adaptive metabolic state when the intake of exogenous nutrient is lacking. In state of fasting, BCAA were preferential catabolism in the dorsal muscle of *Carassius auratus gibelio* as energy substrates, and BCAA catabolism in mice skeletal muscle is required to provide carbon substrates for gluconeogenesis to maintain glucose homoeostasis[15,51]. Meanwhile, the circadian clock acts as an internal time-keeping mechanism to maintain homoeostasis in response to environment changings. Changes in expression of core clock genes in skeletal muscle after fasting have been reported in fish[33,34]. This study also showed that fasting significantly changed the rhythmic expression of circadian genes, for example, the acrophases of *Clock* were dramatically changed during 1, 5 and 7 d fasting. This indicates that the circadian rhythm responds strongly to fasting and is highly dynamic and adaptive in response to different nutritional states. Although several reports

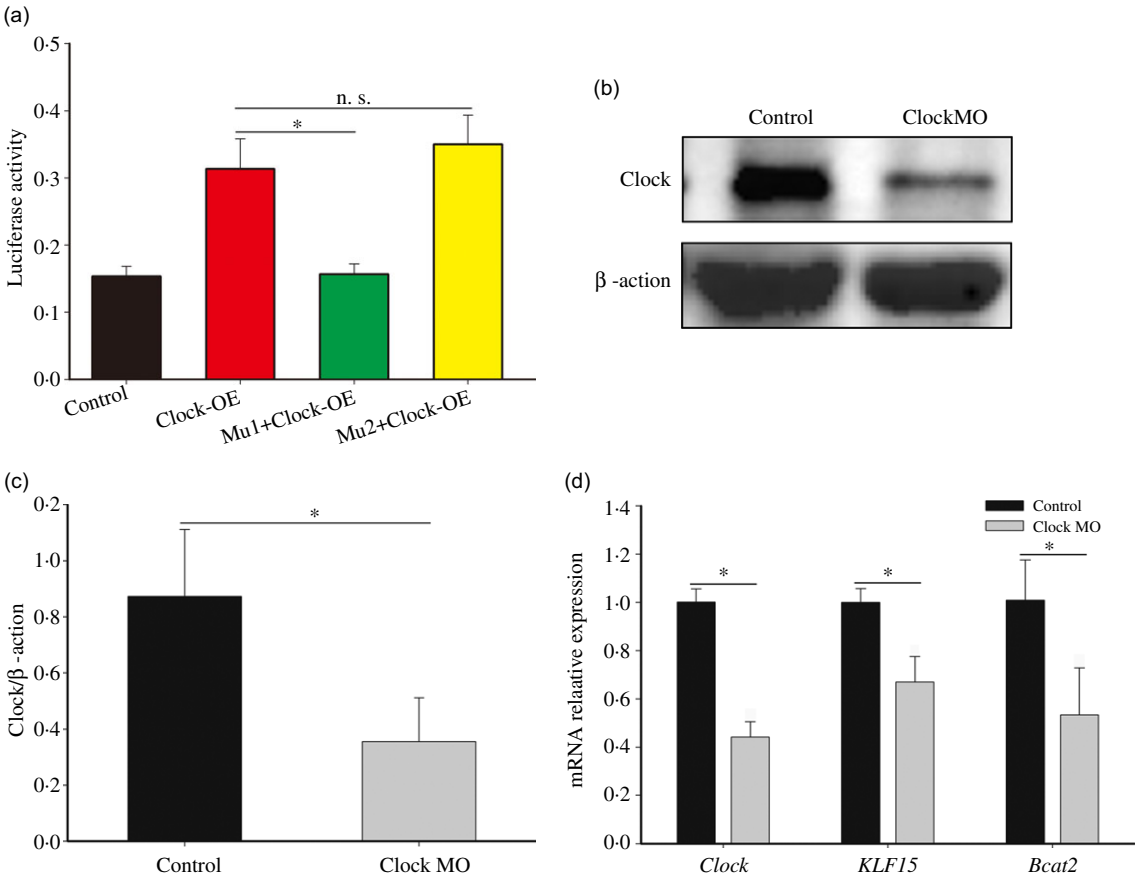

Fig. 6. The expression of *KLF15* is regulated by Clock. (A) Luciferase activity in cells transfected with *Clock* overexpression vector and reporter vector containing E-box1 and E-box2 element, or in cells transfected with *Clock* overexpression vector and reporter vector with the E-box 1 or E-box 2 mutant (Mu1 or Mu2). (B) The protein expression of Clock in control and *Clock* morpholino group by western blotting. (C) The relative protein level of Clock in control and *Clock* morpholino group by grey scale analysis. (D) The mRNA expression of *Clock*, *KLF15* and *Bcat2* in control and *Clock* morpholino group. The asterisk indicates significant difference between two groups ($P < 0{\cdot}05$). n.s. indicates no significant difference between two groups ($P > 0{\cdot}05$). OE = over expression; Mu = mutant; MO = morpholino.

have linked fasting to circadian rhythms[52–54], it is unclear how fasting affects circadian rhythm to regulate energy metabolism homoeostasis. Recent report shows that fasting imposes specialised dynamics of transcriptional coordination between the circadian clock and nutrient-sensitive pathways, resulting in a switch to fasting-specific temporal gene regulation[55].

As an important regulator of cellular metabolism, KLF15 plays a crucial role in transmitting circadian rhythm to the release and utilisation of BCAA. Our study showed that the expression of *KLF15* and *Clock* was highly responsive to fasting in fast muscle of Chinese perch. In addition, the expression correlation between *KLF15* and *Clock* was enhanced after fasting treatment. The result indicates that fasting induces a switch in *KLF15* expression through affecting the expression of *Clock*, thereby regulating BCAA metabolism. However, the fine regulatory network and mechanism of multiple circadian clock genes that coordinately regulate BCAA metabolism have not been revealed. For example, the Tim, a negative-feedback arm of the mammalian molecular clockwork, showed strong negative correlation with *KLF15* during normal feeding, whereas the negative correlation was disappeared after 5 d fasting. This suggests that Tim may negatively regulate *KLF15* under normal feeding condition and induce its negative regulation to be relieved under fasting condition, but the function and mechanism of Tim in regulating metabolic homoeostasis of BCAA need further study in the future.

Conclusions

In summary, the study showed that the transcriptional expression of core clock genes as well as *KLF15* and *Bcat2* was highly responsive to fasting, and the circadian clock involved in regulation of BCAA metabolism under fasting condition. Furthermore, we demonstrate that the transcriptional expression of *Bcat2* is regulated by KLF15, and the transcriptional expression of *KLF15* is regulated by Clock. Therefore, these findings suggest that fasting induces a switch in *KLF15* expression through affecting the rhythmic expression of *Clock*, and KLF15 promotes the expression of *Bcat2* to enhance the transamination of BCAA in fast muscle, then the Glu converts into Ala through ALT, finally the Ala releases into the circulation and absorbed by the liver as substrate for gluconeogenesis to provide energy for other tissues (Fig. 7). This study provides a mechanistic link between circadian rhythms and BCAA metabolism in teleost and opens a new field for the study in regulation of nutrient metabolism in fish.

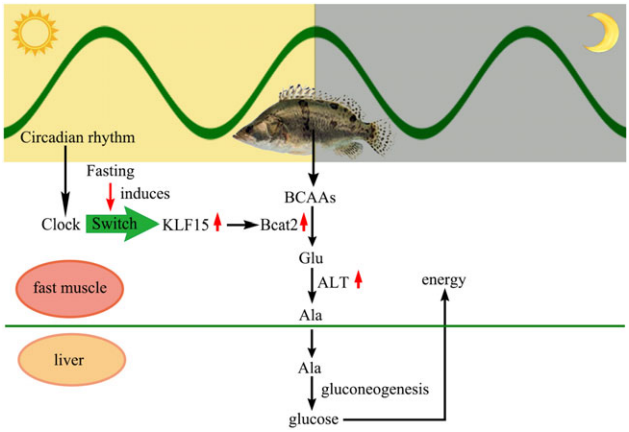

Fig. 7. The mechanism of circadian rhythms regulates BCAA metabolism during short-term fasting. Glu = glutamate; ALT = alanine transaminase; Ala = alanine.

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W. C., X. Z. and J. Z. conceived and designed the experiments. J. L., M. C. and Y. P. performed the experiments. J. L., X. Z. and L. B. analysed the data. X. Z. wrote the original manuscript. W. C., J. Z. and P. W. reviewed and edited the manuscript. All authors read and approved the final manuscript.

We declare that this study has no conflict of interest with other people or organisations.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114522003646

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