Dietary isoleucine affects muscle fatty acid and amino acid profiles through regulating lipid metabolism and autophagy in hybrid catfish (Pelteobagrus vachelli ♀ × Leiocassis longirostris ♂)

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Original Research Article

Abstract

The present study explored the impacts of Ile on muscle fatty acid and amino acid profiles, lipid metabolism, and autophagy in hybrid catfish. Seven isonitrogenous (387.8 g/kg protein) semi-purified diets were formulated to contain 5.0 (control), 7.5, 10.0, 12.5, 15.0, 17.5, and 20.0 g Ile/kg diet respectively. The fish (initial weight of 33.11 ± 0.09 g) were randomly assigned to 7 groups for a 56-day trial. Each group has 3 replicates with 30 fish per replicate, fed at 08:00 and 18:00 each day. Results showed that muscle protein and lipid, C14:0, C18:0, C22:0, C14:1, C18:1n-9, polyunsaturated fatty acid (PUFA), Arg, Ile, Ala, Cys, Gly, Tyr, essential amino acid (EAA), and total amino acid (TAA) contents and amino acid (FAA)/TAA in muscle had positive linear and/or quadratic responses to dietary Ile levels (P < 0.05). Fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), acetyl-CoA carboxylase (ACC), and lipoprotein lipase (LPL) activities had positive linear and/or quadratic responses, but carnitine palmitoyl transferase 1 (CPT1) activity had a negative response with increasing dietary Ile levels (P < 0.05). The mRNA expressions of hormone-sensitive lipase (HSL), PPAR α, PPAR γ, uncoordinated 51-like kinase 1 (ULK1), beclin1 (Becn1), autophagy-related protein 9x (Atg9x), Atg4b, Atg7, autophagy marker light chain 3 B (LC3B), and SQSTM1 in muscle had negative linear and/or quadratic responses to dietary Ile levels (P < 0.05). Finally, the mRNA expressions of PPARδ, PPARγ, uncoordinated 51-like kinase 1 (ULK1), beclin1 (Becn1), autophagy-related protein 9x (Atg9x), Atg4b, Atg7, autophagy marker light chain 3 B (LC3B), and SQSTM1 in muscle had negative linear and/or quadratic responses to dietary Ile levels (P < 0.05). The above results indicate that dietary Ile improves muscle fatty acid and amino acid profiles potentially via respectively regulating lipid metabolism and autophagy. The Ile requirement of hybrid catfish (33 to 72 g) were estimated to be 12.63, 13.77, 13.75, 11.45, 10.50, 12.53 and 12.21 g/kg diet based on the regression analysis of protein, lipid, SFA, PUFA, FAA, EAA, and TAA muscle contents, respectively.

1. Introduction

As one of the fastest-growing food production sectors globally during the past 50 years, aquaculture provides over 50% of fish available globally for human consumption since 2016 (Garlock et al., 2020). Muscle tissue is the primary edible and most commercially valued component of the fish. The tissue comprises 40% to 60% of body mass in fish including hybrid catfish.
(Pelteobagrus vachelli × Leiocassis longirostris sh) (Rescan, 2019; Valente et al., 2013; Zhao et al., 2020a). With increased consumer health awareness, people have recently begun to pay attention to muscle quality (Balico et al., 2020). Flesh quality largely depends on muscle characteristics, such as texture, color, and nutritional composition (Fuentes et al., 2010). The lipid and fatty acid contents of fish muscle are the important decisive factors to affect the nutritional and organoleptic properties of the flesh (Lefevre et al., 2015; Robb et al., 2002). Protein and amino acid (AA) contents may directly reflect the flesh nutritional quality (Pawar and Sonawane, 2013). Accumulating evidence demonstrated that muscle fatty acid and AA contents are strongly influenced by dietary AA (Wu et al., 2020; Zhao et al., 2019). Ile is 1 of 3 branched-chain AA, and the Ile requirement has been determined in some omnivorous fish species, such as hybrid catfish (Zhao et al., 2020c), Nile tilapia (Oreochromis niloticus) (Araújo et al., 2021), Jian carp (Cyprinus carpio L.) (Zhao et al., 2012), and blunt snout bream (Megalobrama amblycephala) (Ren et al., 2017). In general, Ile requirement ranges from 12.9 to 15.2 g/kg diet based on the growth of fish. Ile deficiency results in poor growth and low feed efficiency in fish (Araújo et al., 2021; Zhao et al., 2020c). Recent studies indicate Ile improves muscle AA profiles in Japanese flounder (Paralichthys olivaceus) and fatty acid profiles in finishing pigs (Wang et al., 2017; Luo et al., 2019). However, the roles of Ile in regulating muscle fatty acid and AA profiles are not elucidated in fish.

As the largest tissue in fish, skeletal muscle is a major site of lipid deposition in catfish (Zheng et al., 2014). Lipid accumulation is a complex process including lipogenesis, lipid transport, and lipolysis (Guo et al., 2021; Ren et al., 2016). Fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are 2 marker genes responsible for fatty acid synthesis. As an important decisive factor, FAS is involved in de novo biosynthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA (Ganguly et al., 2018; Vestergren et al., 2013). The ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, and stearoyl-CoA desaturase (SCD) catalyzes the conversion of palmitic acid and stearic acid into the corresponding unsaturated fatty acid. They are rate-limiting enzymes in the de novo lipogenesis pathway (Lopes et al., 2021) and monounsaturated fatty acids (MUFA) biosynthesis (Zhou et al., 2021), respectively. Lipoprotein lipase (LPL) can hydrolyze TG-rich lipoprotein to produce free fatty acids (Kaneko et al., 2016). Hormone-sensitive lipase (HSL) is the rate-limiting lipase of hydrolizing triglycerides and diglycerides (Chen et al., 2014). The adenosine 5’-monophosphate-activated protein kinase (AMPK) and sterol response element-binding protein 1 (SREBP-1) are 2 key regulators in lipid metabolic processes (Lounis et al., 2017; O’Neill et al., 2013). A previous study reports that dietary Ile increases muscle fat deposition in grass carp (Ctenopharyngodon idellus) (Gan et al., 2014). Ile deficiency dramatically increases fat mobilization in white adipose tissue and reduces fat quality in mice (Du et al., 2012). Surplus Ile in pig diets may enhance the synthesis of MUFA and fat accumulation in skeletal muscle by regulating lipid metabolism (Luo et al., 2019). The above data suggests that Ile might have a potential role in regulating lipid metabolism. However, whether dietary Ile can regulate fish lipid metabolism remains largely unclear, especially in fish muscle, which is deeply related to flesh quality (Guan et al., 2021; Zhao et al., 2019).

Protein content and AA profiles affect the nutritional value and flavor of fish muscle (Bermúdez et al., 2014), which are the result of protein synthesis and degradation. The ubiquitin-proteasome and autophagy–lysosome pathways are 2 important signaling pathways involving in protein degradation (Matias et al., 2020; Wang et al., 2018; Zhao et al., 2020). Previous studies mostly focus on the ubiquitin-proteasome pathway (Todgham et al., 2016; Zhang et al., 2022). Newer evidence from Nile tilapia indicates that the autophagy-lysosome pathway exerts an important role in protein degradation (Han et al., 2019). Autophagy involves autophagosome initiation, elongation, nucleation, cargo absorption, closure/maturation, and lysosome fusion (Nakamura and Yoshimori, 2017; Parzych and Klionsky, 2014). Amin acid deficiency induces autophagy in eukaryotic cells (Lahiri et al., 2019; Shen et al., 2021). In mice, Ile deficiency induces autophagy leading to proteasomal protein degradation in the rectus femoris muscle (Wang et al., 2017). Leu negatively regulates autophagosome biogenesis via the impact of its metabolite acetyl-CoA on the target of rapamycin complex 1 (Son et al., 2020). Optimal dietary Ile may increase muscle AA contents in grass carp (Gan et al., 2014). However, whether Ile improves fish muscle AA profiles by regulating autophagy requires further study.

In recent years, the hybrid catfish has been largely cultured in China due to its advantage of flesh quality (Zhao et al., 2019; Zhao et al., 2020). Our previous studies reported that Ile improved growth, immunity, and physical barrier function of the intestine and skin in this hybrid catfish (Yin et al., 2020; Zhao et al., 2020c). However, less attention is paid to the relationship between dietary Ile and lipid metabolism, and protein degradation in fish. Hence, using the same experimental methodology (Zhao et al., 2020), the current study explores the possible influence of dietary Ile on muscle fatty acid and AA profiles, lipid metabolism, and autophagy for the first time. The results may improve our understanding of the potential regulation mechanisms of flesh growth by Ile in fish.

2. Materials and methods

2.1. Animal ethics statement

Experimental design and procedures in this study were reviewed and approved by the Animal Ethical Committee of Sichuan Agricultural University (Approval number: DKY-2018202027).

2.2. Experimental design and diets

Feed ingredients and proximate composition are shown in Table 1. The diet contains 3 protein sources (fish meal, casein, and gelatin), 2 lipid sources (soybean oil and fish oil), and one carbohydrate source (wheat flour). The crude protein and crude fat contents were 387.8 and 71.0 g/kg respectively. Dietary AA and fatty acid contents are shown in Tables 2 and 3. Seven experimental diets were supplemented with crystalline AA to simulate whole body AA compositions of hybrid catfish, except for Ile, as previously described (Zhao et al., 2020c). The diets were supplemented with crystalline Ile to provide Ile at the levels of 5.0 (control), 7.5, 10.0, 12.5, 15.0, 17.5, and 20.0 g/kg diet (Table 2), and balance nitrogen via L-Gly inclusion. Feed ingredients were crushed and sieved through a 60-mesh sieve and weighed according to the formulation. Then fish oil, soybean oil, and water were added and mixed well. Each diet was extruded and pelleted through a 3.0-mm die using a MY-165 twin-screw extruder (EXT50A, Yang Gong Machine, China) according to Zhao et al. (2020c). The extruded particles were dried at 40 °C and sealed in plastic bags. The diets were kept at −20 °C but stored at 4 °C 1 day before use.
During the feeding trial period, each diet was randomly assigned and fed to 3 replicates for 8 weeks. The volume of each tank was replaced with aerated tap water every day. Continuously aerated using air stones. One-third of the total water was suitable for the growth of this hybrid catfish. The feeding trial was conducted in summer (3rd June to 28th July) and water temperature was monitored daily using YSI Professional Plus Multiparameter Instrument (YSI Incorporated, Yellow Springs, OH, USA). The water temperature (ranged from 23 to 27 °C), dissolved oxygen (above 6.0 mg/L), pH (ranged from 6.9 to 7.5), nitrite (low; 0.05 mg/L), and NH₄-N (less than 0.5 mg/L) contents were recorded throughout the experiment.

### Table 1
Composition and nutrient content of basal diet (air-dry basis, g/kg).

| Item         | Content |
|--------------|---------|
| Ingredient   | 170.0   |
| Pellet meal   | 10.0    |
| Gelatin      | 10.0    |
| Corn starch  | 168.3   |
| Alpha-starch | 30.0    |
| Menhaden fish | 26.0   |
| Soybean meal | 210.0   |
| Monocalcium phosphate | 40.0 |
| Vitamin premix | 10.0 |
| Ile premix   | 120.0   |
| Mineral premix | 20.0 |
| Crystal amino acid premix | 170.0 |
| Choline chloride (50%) | 10.0 |
| Ethoxy quinoline (3%) | 0.2   |
| Total        | 1000.0  |
| Nutrient contents |
| Moisture     | 92.38   |
| Crude protein| 387.8   |
| Crude lipid  | 71.0    |
| Crude ash    | 145.2   |

1. Per pet fish meal and menhaden oil were provided by Guangzhou FishTech Fisheries Science & Technology Co. Ltd (Guangzhou).
2. One kilogram of premix provides the following vitamins: DL-α-tocopherol acetate (500 g/kg), 53.600 g; menadione (230 g/kg), 0.217 g; retinyl acetate (50,000 IU/g), 8,063 g; cholecalciferol (50,000 IU/g), 0.100 g; thiamin nitrate (900 g/kg), 0.370 g; cyanocobalamin (10 g/kg), 0.100 g; niacin (990 g/kg), 3.143 g; folic acid (960 g/kg), 0.521 g; meso-inositol (990 g/kg), 52.323 g; D-biotin (20 g/kg), 5.0 g; riboflavin (500 g/kg), 2.667 g; pyridoxine hydrochloride (810 g/kg), 1.125 g; ascorbyl acetate (930 g/kg), 86.022 g.
3. One kilogram of mineral element premix provides the following minerals: FeSO₄·7H₂O (300 g/kg Fe), 13.333 g; CuSO₄·5H₂O (250 g/kg Cu), 1.300 g; ZnSO₄·7H₂O (345 g/kg Zn), 13.043 g; MnSO₄·H₂O (318 g/kg Mn), 4.717 g; KI (38 g/kg I), 1.447 g; Na₂SeO₃ (10 g/kg Se), 1.000 g. All ingredients were diluted with CaCO₃ to 1 kg.
4. One kilogram of crystal amino acid premix provides the following amino acids: Lys (780 g/kg), 125.204 g; Met (990 g/kg), 60.071 g; Trp (980 g/kg), 11.723 g; Arg (997 g/kg), 116.491 g; His (997 g/kg), 27.508 g; Thr (980 g/kg), 163.764 g; Leu (985 g/kg), 20.824 g; Phe (996 g/kg), 17.364 g; Val (992 g/kg), 11.302 g; Cys (999 g/kg), 11.432 g. All ingredients were diluted with corn stach to 1 kg.
5. Crude protein, crude fat, and crude ash, and ile contents were measured values.

### Table 2
Amino acid composition of experimental diets (DM basis, g/kg).

| Item   | Dietary Ile levels, g/kg |
|--------|--------------------------|
|        | 5.0 (Control) | 7.5 | 10.0 | 12.5 | 15.0 | 17.5 | 20.0 |
| Thr    | 3.19          | 3.28 | 3.17 | 3.24 | 3.32 | 3.22 | 3.25 |
| Val    | 8.3           | 7.5  | 8.0  | 8.5  | 8.4  | 7.7  | 7.9  |
| Met    | 13.7          | 12.8 | 13.6 | 13.1 | 14.1 | 13.3 | 13.0 |
| Ile    | 4.7           | 7.7  | 9.7  | 12.9 | 14.3 | 17.8 | 20.6 |
| Leu    | 13.8          | 12.7 | 13.0 | 13.3 | 13.1 | 12.9 | 13.4 |
| Phe    | 8.2           | 7.6  | 8.5  | 7.9  | 8.3  | 7.7  | 8.1  |
| His    | 8.2           | 7.8  | 8.5  | 8.4  | 8.7  | 7.9  | 8.3  |
| Lys    | 26.1          | 25.5 | 26.4 | 26.6 | 25.8 | 26.3 | 26.7 |
| Arg    | 26.8          | 27.3 | 27.7 | 28.1 | 27.1 | 28.5 | 28.3 |
| Trp    | 3.7           | 3.4  | 2.8  | 3.6  | 4.2  | 2.9  | 3.7  |
| Cys    | 2.8           | 2.7  | 3.3  | 3.4  | 3.0  | 3.5  | 3.2  |
| Tyr    | 1.8           | 2.4  | 2.1  | 1.9  | 2.0  | 2.5  | 2.3  |

### Table 3
Fatty acid composition of experimental diets (% of total FA methyl esters).

| Item   | Dietary Ile levels, g/kg |
|--------|--------------------------|
|        | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 17.5 | 20.0 |
| SFA    | 31.16 | 31.07 | 31.91 | 31.04 | 32.63 | 29.79 | 32.44 |
| MUFA   | 45.79 | 45.21 | 44.20 | 45.85 | 45.61 | 45.85 | 46.35 |
| PUFA   | 16.1 | 3.55 | 3.27 | 3.63 | 3.33 | 3.58 | 3.24 |
| C18:1n-7 | 2.94 | 3.13 | 3.12 | 3.05 | 3.47 | 3.19 | 3.03 |
| C18:1n-9 | 3.18 | 2.48 | 2.39 | 2.19 | 2.27 | 2.21 | 2.09 |
| C18:2n-6 | 7.02 | 5.48 | 5.28 | 4.95 | 5.37 | 5.02 | 4.77 |
| C20:4n-6 | 0.31 | 0.28 | 0.23 | 0.27 | 0.19 | 0.32 | 0.26 |
| C22:5n-6 | 0.83 | 0.77 | 0.72 | 0.75 | 0.68 | 0.81 | 0.74 |
| C22:6n-3 | 5.23 | 4.98 | 5.16 | 5.57 | 5.33 | 5.65 | 5.38 |

### 2.3. Fish feeding and management

The feeding trial was executed at the Ya'an Aquaculture Station of Sichuan Agricultural University (Ya'an, Sichuan). Hybrid catfish from a local commercial farm were fed twice daily (08:00 and 18:00) with a basal diet of about 3% of body weight and acclimated for 1-week at laboratory conditions. A total of 630 hybrid catfish (33.11 ± 0.09 g) were randomly and equally assigned into 21 outdoor concrete tanks giving 30 fish per tank. Each tank had a water volume of 2100 L (200 cm × 100 cm × 105 cm). Water was continuously aerated using air stones. One-third of the total water volume of each tank was replaced with aerated tap water every day. Each diet was randomly assigned and fed to 3 replicates for 8 weeks. During the feeding trial period, fish were hand-fed daily to apparent satiation at 08:00 and 18:00 under the natural photoperiod. Forty minutes after feeding, uneaten feed was removed, dried, and weighed to calculate feed intake. The feeding trial was conducted in summer (3rd June to 28th July) and water temperature was suitable for the growth of this hybrid catfish. Water parameters were monitored daily using YSI Professional Plus Multiparameter Instrument (YSI Incorporated, Yellow Springs, OH, USA). The water temperature (ranged from 23 to 27 °C), pH (ranged from 6.9 to 7.5), nitrite (low; 0.05 mg/L), and NH₄-N (less than 0.5 mg/L) contents were recorded throughout the experiment.

### 2.4. Sample collection

At the end of the feeding trial, fish were deprived of feed for 24 h and then captured from each replicate, counted, and bulk weighed. Then, 12 fish from each replicate were anesthetized with benzocaine solution (50 mg/L). The left side muscle of 6 fish were quickly

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sampled and frozen in liquid nitrogen from each replicate. The samples were then stored at −80 °C for detecting gene expression and protein levels. The right-side muscle of the same 6 fish were sampled for muscle composition and AA analysis. Another 6 fish from each replicate were randomly selected to sample the left and right muscles. The samples were stored at −80 °C for fatty acid and biochemical analysis.

2.5. Muscle amino acid and fatty acid analysis

The AA concentrations were measured using gas chromatography (GC-2010, Shimadzu, Japan) according to Zhao et al. (2019). Briefly, lipids were extracted from the diet and muscle samples by homogenization with a mixture of chloroform and methanol (2:1, vol:vol), respectively. The lipid extracts were hydrolyzed by 2 ml KOH-ethanol at 80 °C saponification for 60 min. Then, they were esterified with 7% BF3-methanol solution at 80 °C for 20 min. Fatty acid methyl esters (FAME) were separated by 20 min. Fatty acid methyl esters (FAME) were separated by 20 min. Fatty acid methyl esters (FAME) were separated by 20 min. Fatty acid methyl esters (FAME) were separated by 20 min. Fatty acid methyl esters (FAME) were separated by 20 min.

2.6. Biochemical and gene expression analysis

The sample (100 mg) was weighed and homogenized in ice-cold 0.9% sterile saline (wt:vol, 1:10). The homogenate was centrifuged at 6,000 g for 20 min and the supernatant was used to assay FAS, ACC, LPL, HSL, SCD, and carnitine palmitoyltransferase 1 (CPT1) activities by the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu). Total RNA was extracted from the sample by TRIzol reagent (TaKaRa, Dalian, Liaoning). The integrity and purity of RNA samples were evaluated respectively by electrophoresing on a 1% agarose gel and the ratio of absorbance at 260/280 nm. The reverse transcription of RNA was conducted using RT Reagents (TaKaRa, Dalian, Liaoning). The mRNA expressions of target genes including FAS, ACC, SCD, HSL, CPT1, LPL, AMKP, SREBP-1c, peroxisome proliferator-activated receptor α (PPARα), PPARγ, uncoordinated 51-like kinase 1 (ULK1), autophagy-related protein 9a (Atg9a), autophagy marker light chain 3 B (LC3B), beclin 1 (Becn1), Atg5, Atg7, sequestosome 1 (SQSTM1), and target of rapamycin (TOR) were analyzed by real-time quantitative PCR (RT-qPCR) using CFX96 RT PCR Detection System (Bio-Rad, Hercules, CA, USA). The specific primer sequences of RT-qPCR are provided in Table 4. Each sample was subjected to RT-qPCR according to the following steps: 2 min at 95 °C, followed by 39 cycles of 5 s at 95 °C, and 30 s at an optimal annealing temperature (Table 4). The gene expression was computed using the 2−ΔΔCT method and the housekeeping genes (β-actin and 18 S rRNA) were used as the reference genes to normalize the target gene expression.

2.7. Protein extraction and Western blot analysis

The extraction of each sample protein was conducted in RIPA buffer and protease phosphatase inhibitor mixture after homogenization (Beyotime, Shanghai). Protein concentration was quantified by a protein quantification kit (Beyotime, Shanghai). Then, protein extractions were mixed with 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Inc., Bedford, USA). After treatment with blocking buffer, the membranes were exposed to primary antibodies of AMPK (Proteintech, catalogue no. 66536–1-lg), p-AMPK (phospho Thr172-AMPK, AbClonal, catalogue no. AP0432), ULK1 (AbClonal, catalogue no. A8529), SQSTM1 (AbClonal, catalogue no. A11250) and β-actin (Cell Signaling, catalogue no. D6A8), respectively, at 4 °C for 12 h. Next, the membranes were washed with TBS/T 5 times for 5 min each and incubated with secondary antibodies at room temperature for 1 h. Finally, membranes were visualized by enhanced chemiluminescence (ECL). Band density was quantified by Gel-Pro Analyzer (Media Cybernetics Bethesda, MD, USA) after scanning. The results were normalized to β-actin.

### Table 4

| Name     | Sequences (5' to 3')     | AT  | GenBank ID |
|----------|--------------------------|-----|------------|
| FAS-QF   | AAGCACTGAAAGAAGACCAAC   | 57.0| MH253823.1 |
| FAS-CR   | GCTATGACCTCACCTCA       | 57.0| MH253822.1 |
| ACC-QF   | ATACGACCTGCTTGGAT       | 59.4| MH253821.1 |
| ACC-CR   | CATAAACAGCAGCCAGTCAA    | 63.3| KJ183103.1 |
| SCD-QF   | AAATGCCTGACAGAGCCGCA   | 59.4| XM_02712606 |
| SCD-CR   | GGATCGACAGACATCACA       | 64.2| JNS7121.2 |
| HSL-QF   | CTGTCATGATGAGACTG       | 59.4| XM_02716220.1 |
| HSL-CR   | TCTGCGCATCCAGCTTG       | 63.0| XM_02715933.1 |
| LPL-QF   | GTTGATCCCCCTGATTTTT      | 59.3| MH570217.1 |
| LPL-CR   | CTGGCATCCTGCACTTG       | 60.3| XM_027140763.1 |
| PPARα-QF | GAATAACCTGAAAACACAGC    | 64.5| KR020013.1 |
| PPARα-CR | GTTCATGACCTCACCTG       | 65.0| XX52428.1 |
| AMPK-QF  | GCCTGATCTGCTCCCTCC      | 59.0| XM_02771683.1 |
| ULK1-QF  | GACGATTTGCCCCTGCCC       | 59.0| XM_02771676.1 |
| ULK1-CR  | CTTCATGATGAGACTG       | 59.0| XM_02771600.1 |
| Becn1-QF | CTCACACAGCAGCCGCTGTAAT  | 59.0| XM_0277140736.1 |
| Becn1-CR | CTCACACAGCAGCCGCTGTAAT  | 59.0| XM_0277140736.1 |
| Atg9a-QF | GTCCAGCCTTGGCTGCTTTACCA | 65.0| XM_027142736.1 |
| Atg9a-CR | GTCCAGCCTTGGCTGCTTTACCA | 65.0| XM_027142736.1 |
| Atg4b-QF | TCTGCTCTGAGAGCTGAGGAGTGT | 65.0| XM_027142736.1 |
| Atg5-QF  | CAGACGTCCGTATTCCTCCAC    | 65.0| XM_027142736.1 |
| Atg5-CR  | CTCATGATGAGACTG       | 59.0| XM_02771683.1 |
| Atg7-QF  | CTCTATTTCAAAAGGATGGCTCA | 65.0| XM_0277140727.1 |
| Atg7-CR  | ATACGTGCCTCCTAGTTTCCTCC | 65.0| XM_0277140727.1 |
| LC3B-QF  | CTCACACAGCAGCCGCTGTAAT  | 65.0| XM_0277140727.1 |
| LC3B-CR  | CTCACACAGCAGCCGCTGTAAT  | 65.0| XM_0277140727.1 |
| SQSTM1-QF | GTTCTAACTCATGAAAGAAGAC | 63.3| KY062772 |
| SQSTM1-CR | GTTCTAACTCATGAAAGAAGAC | 63.3| KY062772 |
| 18 S-QF  | CCCATGATGAGACTG       | 57.1| KP938527 |
| 18 S-CR  | CCCATGATGAGACTG       | 57.1| KP938527 |
| β-actin-QF | CCTAAACCCCAACGGGAAA    | 59.0| EU161066 |
| β-actin-CR | CCTAATGCGGAGCAATAC    | 59.0| EU161066 |

**AT** = annealing temperature; **FAS** = fatty acid synthase; **ACC** = acetyl-CoA carboxylase; **SCD** = stearoyl-CoA desaturase; **HSL** = hormone sensitive lipase; **CPT1** = carnitine palmitoyltransferase 1; **FABP** = fatty acid binding protein 4; **PPAR** = peroxisome proliferator-activated receptor; **ULK1** = uncoordinated 51-like kinase 1; **Becn1** = beclin 1; **Atg9a** = autophagy-related protein 9a; **Atg4b** = autophagy-related protein 4 b; **Atg5** = autophagy-related protein 5; **Atg7** = autophagy-related protein 7; **LC3B** = autophagy marker light chain 3 B; **SQSTM1** = sequestosome 1.
2.8. Statistical analysis

Data expressed as percentages or ratios were subjected to data transformation before statistical analysis. The Shapiro–Wilk and Levine’s tests were used to evaluate the distribution normality and variance homogeneity of data. The one-way ANOVA was used to determine any significant differences according to dietary Ile levels. Duncan’s multiple range test was used to analyze differences between the means. In addition, orthogonal polynomial contrasts were used to test linear and quadratic effects of dietary Ile level as described by Wei et al. (2019). All analyses were performed using statistical software SPSS 20.0 and the P < 0.05 was used to show statistical significance. The results were expressed as means ± SEM.

3. Results

3.1. Muscle fatty acid and amino acid profiles

The present study continues to explore the effects of Ile on muscle fatty acid and AA profiles. According to Table 5, the contents of C14:0, C17:0, C18:0, C22:0, C14:1, C18:1n-9, C18:2n-6, C20:5n-3, saturated fatty acid (SFA), MUFA, polyunsaturated fatty acid (PUFA), and essential fatty acid (EFA) were significantly affected by dietary Ile level (P < 0.05). With increasing dietary Ile level, the contents of C14:0, C18:0, C14:1, C18:1n-9, and PUFA increased quadratically (P < 0.05). The C22:0 content increased linearly and quadratically (P < 0.05). No significant linear or quadratic trends were found between the increasing levels of Ile and the responses of C17:0, C18:2n-6, C20:5n-3, SFA, MUFA, and EFA. A positive quadratic trend was found between dietary Ile levels and the contents of C15:0 and n-6 PUFA.

The AA contents are shown in Table 6. The contents of Met, Arg, Ile, Phe, Ala, Cys, Gly, Tyr, essential amino acids (EAA), flavor amino acid (FAA), total amino acids (TAA), EAA/non-essential amino acid (NEAA), EAA/TAA, and FAA/TAA were affected by dietary Ile level (P < 0.05). The Ala, Cys, and Gly contents increased and decreased linearly with increasing dietary Ile level (P < 0.05). The contents of Ile, Tyr, FAA, and TAA increased quadratically (P < 0.05). The Arg content increased linearly and quadratically (P < 0.05). The FAA/TAA increased linearly (P < 0.05). No significant linear or quadratic trends were found between the increasing levels of Ile and the responses of Met, Phe, FAA/NEAA, and FAA/TAA. The positive quadratic trend was found between dietary Ile levels and the contents of Thr, Leu, Val, Ser, and NEAA.

3.2. Enzymes activities and gene expressions related to lipid metabolism in muscle

The SCD, ACC, LPL, and CPT1 activities increased quadratically with increasing dietary Ile level (Table 7, P < 0.05). The FAS activity significantly increased linearly and quadratically (Table 7, P < 0.05). No significant difference in HSL activity was detected among treatments (Table 7). With increasing dietary Ile level, the PPARγ mRNA expression decreased linearly (Table 8, P < 0.05). The SCD, ACC, and LPL mRNA expression increased quadratically (Table 8, P < 0.05). The HSL and PPARα mRNA expression decreased quadratically (Table 8, P < 0.05). The FAS, fatty acid binding protein 4 (FABP4), FATP1, and SREBP-1c mRNA expression increased linearly and quadratically (Table 8, P < 0.05). The CPT1 (Table 8) and AMPK (Fig. 2) mRNA expressions decreased linearly and quadratically (P < 0.05). The p-AMPK level decreased quadratically (Fig. 2, P < 0.05).

3.3. Autophagy-related gene mRNA expression and protein level in muscle

There was a linear effect of dietary Ile level on ULK1 mRNA expression (Table 9, P < 0.05). Dietary Ile level had quadratic effects on Bec1 and Arg9a mRNA expressions (Table 9, P < 0.05). There was also a linear and quadratic effect of dietary Ile levels on Atg4b, Atg7, LC3B, and SQSTM1 mRNA expression (Table 9, P < 0.05). With increasing dietary Ile level, ULK1 protein level decreased linearly (Fig. 3, P < 0.05), SQSTM1 protein level increased quadratically (Fig. 3, P < 0.05).

3.4. Optimal dietary Ile levels for hybrid catfish

As shown in Table 10 and Table S1, based on the quadratic regression analysis for muscle protein, lipid, SFA, PUFA, FAA, EAA, and TAA contents, Ile requirement of hybrid catfish (33 to 72 g) were estimated to be 12.63, 13.77, 13.75, 11.45, 10.50, 12.53, and 12.21 g/kg diet, respectively. Table 11.

4. Discussion

The present study builds on the growth experiment reported in our previous study, which revealed that dietary optimal Ile improved the growth of hybrid catfish (Zhao et al., 2020c). Our previous report demonstrated that the final body weight and percent weight gain are improved with increasing dietary Ile levels up to 12.5 g/kg diet in hybrid catfish (Zhao et al., 2020c). The results showed that muscle lipid (linear and quadratic) and protein (quadratic) content significantly increased with increasing dietary Ile levels (Jiang et al., 2021). Fish growth primarily depends on the depositions of lipid and protein in muscle (Tie et al., 2019). The present study showed dietary 12.5 g/kg Ile resulted in higher lipid and protein depositions in the muscle compared to the other diets, which was consistent with the growth of hybrid catfish. These findings suggested that appropriate Ile level in diets may improve utilization of dietary lipid and protein for hybrid catfish growth. This outcome also agreed with previous results, which showed that dietary Ile observably increased muscle protein and lipid contents in grass carp (Gan et al., 2014) and Nile tilapia (Neu et al., 2017). As far as we know, fish muscle fatty acid and AA contents are 2 important factors affecting flesh quality (Cai et al., 2021). Thus, the effects of dietary Ile on muscle fatty acid and AA profiles and its potential mechanisms were explored in the present study.

4.1. Dietary Ile changes muscle fatty acid profiles by regulating lipid metabolism

As one of the major tissues of lipid deposition, fish muscle is closely related to fish growth (Nanton et al., 2007). The content of muscle lipid is an important factor in determining flesh quality because it not only contains EPA, but also influences taste and sensory quality (Lefevre et al., 2015; Robb et al., 2002; Zhao et al., 2019). The present study found dietary Ile could improve muscle lipid, SFA, and MUFA contents, and modify muscle fatty acid profiles. The result was consistent with the improved growth performance of the hybrid catfish (Zhao et al., 2020c). Thus, it might be inferred that the dietary optimal Ile level could allow a better lipid deposition for muscle growth and change muscle fatty acid composition. In addition, the finding was in line with reports in pigs demonstrating that excess dietary Ile intake improved fat accumulation and modified fatty acid profiles in skeletal muscle (Luo et al., 2019) and high Ile and Val intake improves intramuscular fat content (Duan et al., 2016). The SFA and MUFA contents have a positive relationship with muscle flavor (Cameron et al., 2000). As one of several n-3
PUFA, eicosapentaenoic acid (EPA, C20:5n-3) exerts important physical functions in preventing or attenuating cardiovascular problems and inflammatory responses (Yang et al., 2019). Excitingly, the present study reported for the first time that dietary Ile increased SFA, MUFAs, and EPA proportions in fish muscle. The change of lipid content is a result of lipid accumulation, which primarily depends on the metabolism of lipogenesis and lipolysis. The FAS and ACC are key regulatory enzymes for fatty acid biosynthesis. The SCD (Paton and Ntambi, 2009) introduces the first double bond and converts SFA (C16:0 and C18:0) to MUFAs (C18:1n-9). The present study indicated that dietary 12.5 g/kg Ile level remarkably improved FAS, ACC, as well as SCD activities, indicating that dietary Ile enhanced the lipogenesis in fish muscle. These results coincide with the report on pig skeletal muscle (Luo et al., 2019). The activities of these enzymes are tightly related to their transcriptional levels. The present study therefore further detected the effect of Ile on gene transcription of enzymes related to lipogenesis in hybrid catfish muscle. The present results demonstrated that the mRNA expression of FAS, ACC, and SCD responded to the dietary Ile level in a positive linear and/or quadratic manner. These results might be linked to the increased lipid, SFA, and MUFAs deposition in fish muscle. The SREBP-1c activates downstream FAS including and SCD genes (Debose-Boyd and Ye, 2018). The AMPK is a key protein to phosphorylate SREBP-1c for inhibiting the nuclear translocation of these transcription factors responsible for the de novo synthesis of fatty acid and cholesterol (Bordoli et al., 2019). In the present study, SREBP-1c mRNA expression had a positive linear and quadratic response, whereas the AMPK mRNA expression had a negative linear and quadratic response with increasing dietary Ile level. Western blot result demonstrated that the p-AMPK level was dramatically decreased by dietary Ile. The correlative analysis also showed that the SREBP-1c was negatively linked with AMPK (r = 0.876, P = 0.01), whereas positively associated with FAS (r = 0.869, P < 0.05), ACC (r = 0.767, P = 0.095), and SCD (r = 0.706, P < 0.05) mRNA expression (Table 10). The results indicated the increased muscle lipid content by dietary Ile might be partly ascribed to elevated muscle lipid synthesis via AMPK-mediated SREBP-1c signaling pathway. The AMPK signaling can be regulated by AA (Dalle Pezze et al., 2016). The study on pigs demonstrated that dietary branched-chain AA deficiency activated AMPK of the skeletal muscle (Duan et al., 2017). The activation of AMPK can induce phosphorylation and activation of peroxisome proliferator-activated receptor γ co-activator 1x (PGC-1x), which enhances fatty acid oxidation in murine myotubes (Jäger et al., 2007). Therefore, a decreased fatty acid oxidation by AMPK might have triggered an enhanced lipid accumulation of fish muscle in the present study. The underlying mechanism however needs a further investigation.

The LPL exerts an important role in the removal of lipoprotein triglyceride from the circulation (Chen et al., 2021) and CPT1 is a rate-limiting enzyme of lipolysis and regulates fatty acid β-oxidation (Schlaepfer and Joshi, 2020). In the present study, muscle CPT1 activity and mRNA expression decreased in response to increasing
dietary Ile level, whereas muscle LPL activity and mRNA expression increased. These results were consistent with the pig's statement (Luo et al., 2019). The FATP1 and FABP4 regulate long-chain fatty acid uptake and transport (Blanchard et al., 2016). The PPARα binds the promoter region of CPT1 (Song et al., 2010; Wang et al., 2016; Zheng et al., 2014) and PPARγ has emerged as an important metabolic transcription regulator that promotes lipid accumulation via direct binding to the promoter region of adipogenic genes (Gavrilova et al., 2003; Oku and Umino, 2008; Zheng et al., 2014).

4.2. Dietary Ile improved muscle protein accumulation by down-regulating autophagy

Protein content is a key indicator of muscle quality (He et al., 2020). Fish muscle AA profiles could directly reflect the quality of protein (Yang et al., 2019). The present results showed muscle protein and total AA contents responded to the dietary Ile level in a positive quadratic manner. These findings paralleled those of hybrid catfish growth (Zhao et al., 2020c). These results suggest that improved dietary protein utilization by optimal Ile level may be one of the factors to improve AA profiles in fish muscle. The Ala, Asp, Gly, and Glu are regarded as flavor precursors and can react with soluble reducing sugars to form flavoring substances (Yang et al., 2019). The present results showed that dietary 12.5 g/kg Ile markedly improved total flavor AA, Ala, and Glu content and tended to increase Asp and Glu content in fish muscle. Similar observations have been reported in grass carp (Zhang et al., 2019). The EAA exert a crucial role on maintaining human health. The EAA content and ratio of EAA to

### Table 6

Amino acid profiles of muscle in hybrid catfish fed diets supplemented with graded levels of Ile for 8 weeks (g/100 g dry weight).\(^1\)

| Item | Dietary Ile levels, g/kg | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 17.5 | 20.0 |
|------|--------------------------|-----|-----|------|------|------|------|------|
| EAA  |                          |     |     |      |      |      |      |      |
| Lys  | 6.15 ± 0.17              | 6.28 | 6.39 | 6.64 | 6.86 | 7.11 | 7.36 | 7.62 |
| Met  | 1.78 ± 0.06\(^{ab}\)     | 2.00 | 2.24 | 2.37 | 2.55 | 2.67 | 2.80 | 2.95 |
| Thr  | 2.32 ± 0.04              | 2.51 | 2.72 | 2.85 | 3.07 | 3.23 | 3.40 | 3.56 |
| Arg  | 3.80 ± 0.04\(^{ab}\)     | 4.10 | 4.40 | 4.64 | 4.93 | 5.20 | 5.50 | 5.81 |
| Leu  | 5.38 ± 0.04              | 5.73 | 6.14 | 6.50 | 6.91 | 7.25 | 7.60 | 7.95 |
| His  | 1.54 ± 0.04              | 1.61 | 1.65 | 1.69 | 1.74 | 1.78 | 1.82 | 1.87 |
| Ile  | 2.98 ± 0.04\(^{ab}\)     | 3.12 | 3.19 | 3.27 | 3.35 | 3.41 | 3.48 | 3.55 |
| Phe  | 2.78 ± 0.04\(^{ab}\)     | 2.91 | 3.04 | 3.12 | 3.22 | 3.30 | 3.40 | 3.47 |
| Val  | 3.12 ± 0.04              | 3.24 | 3.35 | 3.45 | 3.56 | 3.68 | 3.80 | 3.91 |

### Table 7

The FAS, SCC, ACC, LPL, HSL, and CPT1 activities (U/L) in the muscle of hybrid catfish fed graded levels of Ile diets for 8 weeks.\(^1\)

| Item | Dietary Ile levels (g/kg) | Pr > F | ANOVA | Linear | Quadratic |
|------|--------------------------|--------|-------|--------|----------|
| EAA  |                          |        |       |        |          |
| Lys  | 6.09 ± 0.39              |        |       |        |          |
| Met  | 1.38 ± 0.06\(^{ab}\)     |        |       |        |          |
| Thr  | 3.34 ± 0.04              |        |       |        |          |
| Arg  | 4.23 ± 0.04\(^{ab}\)     |        |       |        |          |
| Leu  | 5.54 ± 0.06              |        |       |        |          |
| His  | 1.55 ± 0.06              |        |       |        |          |
| Ile  | 3.06 ± 0.03\(^{ab}\)     |        |       |        |          |
| Phe  | 2.70 ± 0.03\(^{ab}\)     |        |       |        |          |
| Val  | 3.20 ± 0.03              |        |       |        |          |

### Table 8

The FAS, ACC, SCC, LPL, HSL, and CPT1 activities (U/L) in the muscle of hybrid catfish fed graded levels of Ile diets for 8 weeks.\(^1\)

| Item | Dietary Ile levels (g/kg) | Pr > F | ANOVA | Linear | Quadratic |
|------|--------------------------|--------|-------|--------|----------|
| EAA  |                          |        |       |        |          |
| Lys  | 6.15 ± 0.17              |        |       |        |          |
| Met  | 1.78 ± 0.06\(^{ab}\)     |        |       |        |          |
| Thr  | 2.32 ± 0.04              |        |       |        |          |
| Arg  | 3.80 ± 0.04\(^{ab}\)     |        |       |        |          |
| Leu  | 5.38 ± 0.04              |        |       |        |          |
| His  | 1.54 ± 0.04              |        |       |        |          |
| Ile  | 2.98 ± 0.04\(^{ab}\)     |        |       |        |          |
| Phe  | 2.78 ± 0.04\(^{ab}\)     |        |       |        |          |
| Val  | 3.12 ± 0.04              |        |       |        |          |

### Footnotes

1. Values are means ± SEM (n = 18). Values within the same rows having different superscripts are significantly different (P < 0.05).

2. Significance probability associated with the F-statistic.
analyzed by orthogonal polynomial contrasts; Quadratic

Table 8

| Item                  | Dietary Ile levels, g/kg | Pr > F | ANOVA  | Linear  | Quadratic |
|-----------------------|--------------------------|--------|--------|---------|-----------|
|                       | 5.0          | 7.5   | 10.0  | 12.5  | 15.0  | 17.5 | 20.0 |
| FAS 1.00 ± 0.05\(^a\) | 1.22 ± 0.11\(^b\) | 2.35 ± 0.36\(^d\) | 2.64 ± 0.35\(^e\) | 2.42 ± 0.35\(^a\) | 2.05 ± 0.19\(^e\) | 1.90 ± 0.05\(^d\) | 0.00 | 0.00 | 0.00 |
| SCD 1.01 ± 0.13\(^e\) | 1.42 ± 0.36\(^b\) | 3.25 ± 0.32\(^b\) | 2.83 ± 0.39\(^c\) | 1.71 ± 0.23\(^b\) | 1.48 ± 0.06\(^a\) | 1.30 ± 0.21\(^b\) | 0.00 | 0.60 | 0.00 |
| ACC 1.09 ± 0.14\(^a\) | 1.18 ± 0.21\(^b\) | 1.46 ± 0.08\(^b\) | 2.30 ± 0.10\(^c\) | 1.99 ± 0.29\(^b\) | 1.52 ± 0.24\(^b\) | 1.32 ± 0.08\(^c\) | 0.00 | 0.05 | 0.00 |
| LPL 1.01 ± 0.06\(^a\) | 1.24 ± 0.12\(^a\) | 1.51 ± 0.01\(^b\) | 1.60 ± 0.08\(^c\) | 1.36 ± 0.12\(^d\) | 1.11 ± 0.02\(^e\) | 1.04 ± 0.09\(^c\) | 0.00 | 0.46 | 0.00 |
| HSL 1.20 ± 0.27\(^b\) | 1.02 ± 0.12\(^a\) | 1.09 ± 0.04\(^c\) | 0.63 ± 0.10\(^b\) | 0.67 ± 0.08\(^b\) | 1.02 ± 0.12\(^d\) | 1.34 ± 0.07\(^d\) | 0.00 | 0.99 | 0.00 |
| CPT1 1.01 ± 0.10\(^a\) | 0.92 ± 0.14\(^b\) | 0.83 ± 0.05\(^c\) | 0.79 ± 0.08\(^d\) | 0.94 ± 0.04\(^a\) | 0.98 ± 0.10\(^c\) | 1.12 ± 0.12\(^e\) | 0.00 | 0.00 | 0.00 |
| FABP4 1.01 ± 0.17\(^b\) | 1.13 ± 0.11\(^a\) | 2.02 ± 0.23\(^d\) | 1.89 ± 0.13\(^c\) | 1.59 ± 0.21\(^d\) | 1.33 ± 0.05\(^a\) | 1.25 ± 0.19\(^d\) | 0.00 | 0.02 | 0.00 |
| SREBP-1c 1.00 ± 0.07\(^b\) | 1.20 ± 0.13\(^a\) | 1.48 ± 0.11\(^e\) | 2.10 ± 0.12\(^d\) | 1.53 ± 0.18\(^d\) | 1.36 ± 0.06\(^d\) | 1.34 ± 0.04\(^d\) | 0.00 | 0.00 | 0.00 |
| Pr 1.06 ± 0.13\(^b\) | 0.96 ± 0.05\(^c\) | 0.73 ± 0.07\(^a\) | 0.56 ± 0.04\(^b\) | 0.70 ± 0.04\(^c\) | 1.07 ± 0.12\(^d\) | 1.16 ± 0.13\(^c\) | 0.00 | 0.24 | 0.00 |
| PPARα 1.03 ± 0.09\(^b\) | 0.88 ± 0.10\(^a\) | 0.83 ± 0.04\(^e\) | 0.61 ± 0.05\(^b\) | 0.58 ± 0.03\(^d\) | 0.48 ± 0.02\(^b\) | 0.38 ± 0.04\(^e\) | 0.00 | 0.00 | 0.50 |
| SREBP-1c 1.00 ± 0.02\(^d\) | 1.28 ± 0.13\(^c\) | 2.47 ± 0.24\(^d\) | 2.54 ± 0.21\(^d\) | 1.85 ± 0.22\(^d\) | 1.99 ± 0.19\(^d\) | 1.85 ± 0.15\(^e\) | 0.00 | 0.00 | 0.00 |

FAS – fatty acid synthase; SCD – stearoyl-CoA desaturase; ACC – acetyl-CoA carboxylase; LPL – lipoprotein lipase; HSL – hormone sensitive lipase; CPT1 – carnitine palmitoyl transferase 1; FABP4 – fatty acid binding protein 4; FATP1 – fatty acid transport protein 1; PPARα – peroxisome proliferator-activated receptor α; PPARγ – peroxisome proliferator-activated receptor γ; SREBP-1c – sterol regulating element binding protein-1c.

1 The relative mRNA expression levels were calculated using the arithmetic formula 2^(-ΔΔCT). The β-actin and 18 S rRNA were used as housekeeping genes. Values are means ± SEM (n = 18). Values within the same rows having different superscripts are significantly different (P < 0.05).

2 Significance probability associated with the F-statistic.

Fig. 1. Effects of dietary Ile on crude lipid (A) and protein (B) contents of hybrid catfish muscle. Values are means ± SEM (n = 18) and different letter denotes significant difference (P < 0.05).

Fig. 2. Effects of dietary Ile on AMPK mRNA expression (A), and AMPK and p-AMPK protein levels (B) of hybrid catfish muscle. Values are means ± SEM (n = 18) and different letter denotes significant difference (P < 0.05). AMPK – adenosine 5’-monophosphate-activated protein kinase; ANOVA – the variance analyzed by one-way ANOVA; Linear – linear trend analyzed by orthogonal polynomial contrasts; Quadratic – quadratic trend analyzed by orthogonal polynomial contrasts.

NEAA reflect nutritional value of muscle protein. The present study showed that EAA content and ratio of EAA to NEAA were increased by dietary Ile. These results suggested that dietary Ile could improve fish muscle protein nutritional value. Muscle protein deposition is determined by protein synthesis and degradation rates. Autophagy-lysosome system is an important proteolytic system involved in intracellular protein degradation in fish (Seiliez et al., 2010; Wang et al., 2018). During macro autophagy, a part of the cytoplasm is firstly surrounded by an isolation membrane called phagophore to form autophagosome, then fused with lysosomes to form autolysosomes, and the isolated internal substances are finally degraded. The ULK1 can sense the level of intracellular nutrients to initiate early
Table 9
Effects of dietary ile on ULK1, Becn1, Atg4b, Atg5, Atg7, LC3B, and SQSTM1 mRNA expressions in muscle of hybrid catfish fed graded levels of ile diets for 8 weeks.¹

| Item                | Dietary ile levels (g/kg) | Pr > F² | ANOVA | Linear | Quadratic |
|---------------------|--------------------------|---------|--------|--------|-----------|
| ULK1                | 5.0                      | 0.001   | 0.04   | 0.14   |           |
|                     | 7.5                      | 0.00    | 0.01   | 0.05   |           |
|                     | 10.0                     | 0.05    | 0.05   | 0.01   |           |
|                     | 12.5                     | 0.05    | 0.05   | 0.01   |           |
|                     | 15.0                     | 0.05    | 0.05   | 0.01   |           |
|                     | 17.5                     | 0.05    | 0.05   | 0.01   |           |
|                     | 20.0                     | 0.05    | 0.05   | 0.01   |           |

ULK1 = uncoordinated 51-like kinase 1; Becn1 = beclin 1; Atg4b = autophagy-related protein 4 b; Atg5 = autophagy-related protein 5; Atg7 = autophagy-related protein 7; LC3B = autophagy marker light chain 3 B; SQSTM1 = sequestosome 1.

¹ The relative mRNA expression levels were calculated using the arithmetic formula $2^{-ΔΔCT}$. The β-actin and 18 S rRNA were used as housekeeping genes. Values are means ± SEM (n = 18). Values within the same row having different superscripts are significantly different (P < 0.05).

² Significance probability associated with the F-statistic.

Fig. 3. The ULK1 (A) and SQSTM1 (B) protein levels were determined by Western blot analysis. Equal loading was monitored with anti-β-actin antibody. Values are means ± SEM (n = 18) and different letter denotes significant difference (P < 0.05). ULK1 = uncoordinated 51-like kinase 1; SQSTM1 = sequestosome 1; ANOVA = the variance analyzed by one-way ANOVA; Linear = linear trend analyzed by orthogonal polynomial contrasts; Quadratic = quadratic trend analyzed by orthogonal polynomial contrasts.

Table 10
Regressions of FBW, PWG, and muscle protein, lipid, SFA, PUFA, FAA, EAA, and TAA contents in hybrid catfish fed graded levels of ile diets for 8 weeks.

| Item      | Equation                                  | R²      | P-value | Optimal dietary ile levels, g/kg |
|-----------|-------------------------------------------|---------|---------|----------------------------------|
| FBW¹      | $Y = -0.258x^2 + 6.283x + 28.97$          | 0.75    | 0.00    | 12.20                            |
| PWG¹      | $Y = -0.007x^2 + 0.169x - 0.0414$          | 0.83    | 0.00    | 12.43                            |
| Protein   | $Y = -0.024x^2 + 0.606x + 14.611$          | 0.69    | 0.00    | 12.63                            |
| Lipid     | $Y = -0.013x^2 + 0.358x + 4.449$          | 0.86    | 0.00    | 13.77                            |
| SFA       | $Y = -0.012x^2 + 0.330x + 23.214$         | 0.62    | 0.05    | 13.75                            |
| PUFA      | $Y = -0.040x^2 - 0.916x + 41.436$         | 0.71    | 0.00    | 11.45                            |
| FAA       | $Y = -0.016x^2 + 0.336x + 24.448$         | 0.69    | 0.00    | 10.50                            |
| FAA       | $Y = -0.019x^2 + 0.476x + 23.013$         | 0.52    | 0.05    | 12.53                            |
| TAA       | $Y = -0.052x^2 + 1.270x + 59.406$         | 0.79    | 0.00    | 12.21                            |

FBW = final body weight; PWG = percent weight gain; SFA = saturated fatty acid; PUFA = polyunsaturated fatty acid; FAA = flavor amino acid; EAA = essential amino acids; TAA = total amino acid.

¹ Regressions of FBW and PWG according to the published results in Zhao et al. (2021).

Table 11
Correlation analysis of parameters in the muscle of hybrid catfish.

| Independent parameters | Dependent parameters | Correlation coefficients | P-value |
|------------------------|----------------------|--------------------------|---------|
| SREBP-1c               | AMPK                 | -0.876                   | < 0.01  |
|                       | FAS                  | 0.869                    | < 0.05  |
|                       | ACC                  | 0.767                    | < 0.10  |
|                       | SCDF                 | 0.706                    | < 0.05  |
|                       | CPT1                 | 0.916                    | < 0.01  |
| PPARα                 |                      |                          |         |

SREBP-1c = sterol regulating element binding protein-1c; PPARα = peroxisome proliferator-activated receptor α; AMPK = adenosine 5'-monophosphate-activated protein kinase; FAS = fatty acid synthase; ACC = acetyl-CoA carboxylase; SCDF = stearoyl-CoA desaturase; CPT1 = carnitine palmitoyl transferase 1.
events of autophagy process (King et al., 2021). Becn1, an important autophagy-promoting protein, regulates the early stages of autophagosome formation (Kaur and Changotra, 2020). The other autophagy-related proteins (Atg9a, Atg4b, Atg5, and Atg7) are known to engage in autophagosome formation (Hocking et al., 2012). The LC3-II is closely related to the membrane of autophagosomes (Rouschop et al., 2016). The SQSTM1, an important LC3 interactor under autophagy stimulation, degrades normally during autophagy and accumulated during autophagy damage (Poon et al., 2021). The present study found that dietary Ile deficiency up-regulated ULK1, Becn1, Atg9a, Atg4b, Atg5, Atg7, and LC3B mRNA expressions, indicating that autophagy was induced. Western blot results also demonstrated Ile markedly decreased ULK1 protein level and increased SQSTM1 protein level. TOR activity is also one of the most important autophagy regulators (Yina et al., 2020). An active TOR promotes phosphorylation of ULK1 on Ser 757 to inhibit autophagy (Cao et al., 2021). A previous study also demonstrated dietary 12.5 g/kg Ile markedly decreased ULK1 protein level and accumulated during autophagy damage (Poon et al., 2021). The protein degradation via the autophagy-lysosome system, respectively. Based on the quadratic regression analysis for muscle protein, lipid, SFA, PUFA, FAA, EAA, and TAA contents, Ile requirement of hybrid catfish (33 to 27 g) was respectively estimated to be 12.63, 13.77, 13.75, 11.45, 10.50, 12.53 and 12.21 g/kg diet. These levels – except PUFA, FAA, and TAA – are in majority higher than 12.43 g/kg diet base on percent weight gain (Zhou et al., 2021).

5. Conclusion

The present study demonstrated that dietary Ile modified fish muscle fatty acid and AA profiles, by regulating lipid metabolism via AMPK-mediated SREBP-1c and PPARz signaling pathway, and protein degradation via the autophagy-lysosome system, respectively. Based on the quadratic regression analysis for muscle protein, lipid, SFA, PUFA, FAA, EAA, and TAA contents, Ile requirement of hybrid catfish (33 to 72 g) was respectively estimated to be 12.63, 13.77, 13.75, 11.45, 10.50, 12.53 and 12.21 g/kg diet, respectively. These results provided evidence that fish muscle nutritional value was effectively improved by dietary supplementation of Ile.

Author contributions

Ye Zhao, Xia Dong, and Chang-Rui Guo: Formal analysis, Investigation, Writing-original draft, Writing-review & editing. Lin Feng, Yang Liu, and Wei-Dan Jiang: Formal analysis, Investigation. Pei Wu, Xiao-Li Huang, and De-Fang Chen: Methodology, Resources. Wei Luo: Resources. Xiao-Qiu Zhou and Jun Jiang: Conceptualization, Formal analysis, Investigation, Writing-review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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