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# Effect of dietary threonine on growth performance and muscle growth, protein synthesis and antioxidant-related signalling pathways of hybrid catfish *Pelteobagrus vachelli*♀ × *Leiocassis longirostris*♂

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## Abstract

The experiment was conducted to investigate the effects of dietary threonine (Thr) on growth performance and muscle growth, protein synthesis and antioxidant-related signalling pathways of hybrid catfish *Pelteobagrus vachelli*♀ × *Leiocassis longirostris*♂. A total of 1200 fish (14·19 (SE 0·13) g) were randomly distributed into six groups with four replicates each, fed six diets with graded level of Thr (9·5, 11·5, 13·5, 15·4, 17·4 and 19·3 g/kg diets) for 56 d. Results showed ($P < 0·05$) that dietary Thr (1) increased percentage weight gain, specific growth rate, feed efficiency and protein efficiency ratio; (2) up-regulated growth hormone (GH), insulin-like growth factor 1 (IGF-1), proliferating cell nuclear antigen, myogenic regulation factors (MyoD, Myf5, MyoG and Mrf4) and myosin heavy chain (MyHC) mRNA levels; (3) increased muscle protein content via regulating the protein kinase B/target of rapamycin signalling pathway and (4) decreased malondialdehyde and protein carbonyl contents, increased catalase, glutathione-S-transferase, glutathione reductase and GSH activities, up-regulated mRNA levels of antioxidant enzymes related to NFE2-related factor 2 and γ-glutamylcysteine ligase catalytic subunit. These results suggest that Thr has a potential role to improve muscle growth and protein synthesis, which might be due to the regulation of GH-IGF system, muscle growth-related gene, antioxidative capacity and protein synthesis-related signalling pathways. Based on the quadratic regression analysis of specific growth rate, the Thr requirement of hybrid catfish (14·19–25·77 g) was estimated to be 13·77 g/kg of the diet (33·40 g/kg of dietary protein).

**Key words:** **Threonine: Growth: Myogenic regulation: Protein synthesis: Muscle**

Muscle of teleost fish, comprising 60 % or more of the fish body, is the main edible portion and constitutes the most valuable part of the derived products[1]. Muscle growth, the main determinant of fish growth, is the result of both the recruitment of new muscle fibres (hyperplasia) and hypertrophy of existing muscle fibres[2]. In fish, muscle growth is known to be regulated by nutritional factors, especially amino acids[3–6]. As in animals[7,8], threonine (Thr) is an indispensable amino acid and it directly participates in protein synthesis as a substrate[9], critically influences the protein utilisation efficiency of fish and ultimately affects fish growth and health[10,11]. The previous studies mainly focused on the effects of Thr on intestinal mucin synthesis and health[11–14]. However, information concerning the effect of Thr on muscle growth is limited in fish.

Muscle growth by hyperplasia and hypertrophy is controlled by several genetic factors such as growth hormone (GH), insulin-like growth factors (IGF), myogenic regulatory factors (MRF) and myostatin[15]. The GH/IGF axis is considered the most important endocrine system regulating skeletal growth in fish[16,17].

**Abbreviations:** AKT, protein kinase B; CAT, catalase; 4E-BP, 4E-binding protein; GH, growth hormone; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; IGF-1, insulin-like growth factor 1; Keap1, Kelch like ECH associated protein 1; MDA, malondialdehyde; MRF, myogenic regulatory factors; Mrf4, myogenic regulatory factor 4; Myf5, myogenic factor 5; MyHC, myosin heavy chain; MyoD, myoblast determination protein; MyoG, myogenin; Nrf2, NFE2-related factor 2; PC, protein carbonyl; PCNA, proliferating cell nuclear antigen; PI3K, phosphoinositide 3-kinase; S6K, S6 kinase; SOD, superoxide dismutase; Thr, threonine; TOR, target of rapamycin.

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**Table 1.** Composition and nutrient content of diets

| Ingredients | Dietary Thr levels (g/kg) | | | | | |
|---|---|---|---|---|---|---|
| | 9·5 | 11·5 | 13·5 | 15·4 | 17·4 | 19·3 |
| Soyabean meal* | 80 | 80 | 80 | 80 | 80 | 80 |
| Rapeseed meal | 100 | 100 | 100 | 100 | 100 | 100 |
| Fishmeal | 160 | 160 | 160 | 160 | 160 | 160 |
| Maize protein meal | 330 | 330 | 330 | 330 | 330 | 330 |
| Maize starch | 207·7 | 207·0 | 206·2 | 205·5 | 204·8 | 204·0 |
| Soyabean oil | 40 | 40 | 40 | 40 | 40 | 40 |
| L-Lys-HCl (78 %) | 14 | 14 | 14 | 14 | 14 | 14 |
| L-Trp (98 %) | 2 | 2 | 2 | 2 | 2 | 2 |
| L-Thr (98 %) | 0 | 2 | 4 | 6 | 8 | 10 |
| L-Gly (98 %) | 6·3 | 5·0 | 3·8 | 2·5 | 1·2 | 0 |
| L-Arg (98 %) | 10 | 10 | 10 | 10 | 10 | 10 |
| $CaH_2PO_4$ | 20 | 20 | 20 | 20 | 20 | 20 |
| Choline chloride | 5 | 5 | 5 | 5 | 5 | 5 |
| Vitamin premix† | 10 | 10 | 10 | 10 | 10 | 10 |
| Mineral premix‡ | 15 | 15 | 15 | 15 | 15 | 15 |
| Nutrient content (%)§ | | | | | | |
| Crude protein | 41·44 | 41·72 | 42·25 | 41·82 | 41·64 | 41·53 |
| Crude lipid | 4·87 | 4·97 | 4·89 | 4·82 | 4·61 | 4·51 |
| Ash | 6·71 | 6·85 | 6·49 | 6·55 | 6·68 | 6·67 |
| Thr | 9·5 | 11·5 | 13·5 | 15·4 | 17·4 | 19·3 |

Thr, threonine.

* Soyabean meal (COFCO Oil Qinzhou Co. Ltd), rapeseed meal (Chengdu Huatai Grain and Oil Ltd), fishmeal (TASA steam dried fishmeal), maize gluten meal (Changchun Dacheng Industry Group), wheat meal (China National Cereals, Oils and Foodstuffs Corporation) and soyabean oil.

† Vitamin premix (IU or g/kg): retinyl acetate, 2 500 000 IU; cholecalciferol, 500 000 IU; $\alpha$-tocopherol, 6700 IU; thiamine, 10; riboflavin, 6; pyridoxine hydrochloride, 12; nicotinic acid, 40; D-calcium pantothenate, 15; biotin, 0·25; folic acid, 0·4; inositol, 200; cyanocobalamin 0·02; menadione, 4. All ingredients were diluted with maize starch to 1 kg.

‡ Mineral premix (g/kg): $FeC_6H_5O_7$, 4·57; $ZnSO_4{\cdot}7H_2O$, 9·43; $MnSO_4{\cdot}H_2O$, 4·14; $CuSO_4{\cdot}5H_2O$, 6·61; $MgSO_4{\cdot}7H_2O$, 238·97; KI, 1·10 g; $NaSeO_3$, 2·50 g; $CoCl_2{\cdot}6H_2O$, 1·36. All ingredients were diluted with $CaCO_3$ to 1 kg.

§ Crude protein, crude fat and ash were measured by using the Association of Official Analytical Chemists Methods. Thr concentrations were measured using HPLC (Agilent Technologies).

Several studies in fish have demonstrated that GH enhances somatic growth via muscle hypertrophy or hyperplasia[18–20]. The MRF, such as myoblast determination protein (MyoD), myogenic factor 5 (Myf5), myogenic regulatory factor 4 (Mrf4) and myogenin (MyoG), are critical for the determination and terminal differentiation of skeletal muscle[21]. MyoD and Myf5 regulate the activation and proliferation of satellite cells, whereas MyoG and Mrf4 act on cell differentiation[22]. Myostatin is a negative regulator of myogenesis, which inhibits myoblast cell proliferation and differentiation[23]. A number of studies have demonstrated that the GH differentially regulates the expression of MRF in fish muscle[17]. Despite increased understanding of the regulation of skeletal muscle growth by some of these factors, its regulation by nutrients remains poorly documented in fish. Studies on different aquatic species and pig already reported that dietary Thr significantly improved the growth rate[7,10,13,24]. However, actual role of Thr in regulating muscle growth in fish still needs to be clarified. Moreover, myogenic differentiation is a highly orchestrated sequential programme to generate mature skeletal muscle[25]. Studies in C2C12 cells show myogenic differentiation is regulated by reactive oxygen species[26–28]. Previous studies have demonstrated that dietary Thr deficiency increased reactive oxygen species, malondialdehyde (MDA) and protein carbonyl (PC) contents and decreased antioxidant-related enzyme activities via the regulation of the NFE2-related factor 2 (Nrf2) signalling pathway in the gills of juvenile grass carp *Ctenopharyngodon idellus*[29]. Whether Thr modulates myogenic differentiation by regulating antioxidant capacity via the Nrf2 signalling pathway in muscle needs to be investigated.

Skeletal muscle protein deposition greatly contributes to overall growth in fish[2]. The balance of protein synthesis and degradation is crucial to the protein deposition of skeletal muscle. A previous study has shown that nutrition can activate the IGF-1/phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signalling pathway and induce protein synthesis and accretion[30]. Target of rapamycin (TOR), a downstream component of the PI3K/AKT pathway, regulates protein synthesis via ribosomal S6 kinase (S6K) and the eukaryotic translation initiation factor 4E-binding protein (4E-BP) in fish[31,32]. Previous studies have demonstrated that Thr promotes protein synthesis of skeletal muscle in pigs and enterocyte via the regulation of TOR signalling pathway genes expression in fish[7,9]. These results supported a nutritional stimulatory role on muscle growth. Nevertheless, studies exploring the role of Thr on fish muscle protein synthesis as well as the signalling pathways involved are very scarce.

*Pelteobagrus vachelli*♀ × *Leiocassis longirostris*♂ is a hybrid catfish that has been widely cultured in China in recent years. To the best of our knowledge, there is no available information on the nutrition of hybrid catfish. The objective of the present study was to investigate the effects of dietary Thr on growth performance and muscle growth, protein synthesis and antioxidant-related signalling pathways of hybrid catfish. Furthermore, the dietary Thr requirement for hybrid catfish was evaluated.

## Materials and methods

### Experimental design and diets

The experimental diet formulations are shown in Table 1. Soyabean meal, rapeseed meal, fishmeal and maize protein meal were used as dietary protein sources. Maize starch and soyabean oil were used as dietary carbohydrates and lipid sources, respectively. Six experimental diets were prepared with graded levels of Thr, ranging from 9·5 (control), 11·5, 13·5, 15·4, 17·4 to 19·3 g/kg. The diets were formulated to contain about 41·2 % crude protein and 5·0 % crude lipid. All dry ingredients were ground through a sixty-mesh screen. The diets were prepared by mixing the dry ingredients with oil using a mixer. Then, each diet was extruded in a twin-screw extruder (MY-165) with a 2-mm die. The processing conditions were as follows: 100 rpm screw speed, 127°C temperature and 30–45 atm pressure. Floating extruded pellets were air-dried and stored at 4°C in plastic bags until being used.

### Fish management and feeding

The feeding trial was conducted at Experiment Station of Ya'an, Sichuan Agricultural University, China. Hybrid catfish were obtained from Rongsen Corporation. Fish were adapted to the experimental environment for 4 weeks. A total of 1200 fish with an average initial weight of 14·19 ± 0·13 g were randomly distributed into twenty-four concrete tanks ($200 \times 100 \times 105$ cm$^3$), resulting in fifty fish in each tank. Fish were fed with their respective diets to satiation level two times (08.00 and 18.00 hours) per d for 8 weeks. Each of the diet was fed to four replicates of fish. The daily feed supplied was recorded, and the uneaten feed was collected 30 min after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate feed intake. The water temperature and pH were maintained at 25°C ± 3·0°C and 7·0 ± 0·5, respectively. The rate of water flow was adjusted to dissolved $O_2$ >5·0 mg/l. During the experimental period, fish were reared under natural light conditions. All experimental protocols were approved by Animal Care Advisory Committee of Sichuan Agricultural University.

### Samples collection

At the termination of feeding trial, benzocaine solution (50 mg/l) was used to anaesthetise fish after fasting for 24 h. For each tank, total fish were taken for measuring the number and body weight, then fish were killed by a sharp blow to the head according to Lisbeth *et al.*[33]. After slaughtering, the pituitary, liver and muscle samples from the left side of six fish each tank were quickly obtained and frozen in liquid $N_2$ and then stored at −80°C for RNA extraction and Western blot analysis. Muscle samples from the right side of the same fish were obtained for biochemical analysis.

### Biochemical analysis

Approximate compositions of diets and fish muscle were analysed according to the standard methods of the Association of Official Analytical Chemists. Crude protein (N × 6·25) was determined by the Kjeldahl method after an acid digestion was performed. Crude lipid was obtained by the diethyl ether-extraction method using the Soxhlet method. Muscle samples were homogenised in ice-cold physiological saline solution (10 volumes, w/v) and centrifuged at 6000 ***g*** and 4°C for 20 min. The supernatant was collected for enzyme activity analysis. Protein content was determined by the Bradford method[34]. Contents of MDA, PC and GSH were assayed as described by Yonar *et al.*[35]. Catalase (CAT) activity was determined by measuring the decomposition of hydrogen peroxide[36]. Activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were assayed as described by Zhang *et al.*[37]. Glutathione-S-transferase (GST) activity was measured by monitoring the reduction of GSH concentration[38], and glutathione reductase (GR) activity was measured by the consumption of NADPH during the production of GSH according to Loar *et al.*[39]. Anti-superoxide anion and anti-hydroxy radical activities were measured as described by Cheng *et al.*[40].

### Real-time quantitative PCR

The procedures of RNA isolation, reverse transcription and quantitative real-time PCR were similar to the previous study[41]. Total RNA was extracted from the muscle using an RNAiso Plus kit (TaKaRa) according to the manufacturer's instruction and followed by DNAse I treatment. RNA purity and integrity were assessed by spectrophotometric (A260:280 nm ratio) analysis and agarose gel (1 %) electrophoresis. Subsequently, 2 µl of total RNA were used to synthesise cDNA using the PrimeScript® RT reagent kit with gDNA Eraser (TaKaRa). Specific primers for IGF-1, IGF-2, AKT, MyoD, MyoG, myostatin, GPx, GST and Kelch like ECH associated protein 1 (Keap1) were designed using published sequences of yellow catfish (Table 2). Specific primers for IGF-1 receptor, PI3K, TOR, 4E-BP, S6K1, proliferating cell nuclear antigen (PCNA), Myf5, Mrf4, myosin heavy chain (MyHC), CuZnSOD, CAT, γ-glutamylcysteine ligase catalytic subunit and Nrf2 were designed according to sequences of hybrid catfish cloned in our laboratory (Table 2). Real-time PCR analysis was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad). Target gene mRNA concentration was normalised to that of reference genes (β-actin and 18S rRNA). Target and reference genes' amplification efficiency was calculated according to specific gene standard curves generated from 10-fold serial dilutions. Results were calculated using the $2^{-\Delta\Delta CT}$ method after verifying that the primers amplified with an efficiency of approximately 100 % as described by Livak *et al.*[42].

### Protein extraction and Western blot analysis

Fish muscle tissues were homogenised with a glass Tenbroeck tissue grinder (Kimble Chase) on ice and lysed in RIPA with 1 mM phenylmethanesulfonyl fluoride (a protease inhibitor; Amresco) and 1 mM sodium β-glycerophosphate (Beyotime). A bicinchoninic acid protein assay kit (Pierce) was used to determine the protein concentration. In all, 20 µg of protein extractions were separated by 12 % SDS–polyacrylamide gel and then transferred to a polyvinyldifluoride membrane

**Table 2.** Primer sequences and optimal annealing temperatures (OAT, °C) of genes selected for analysis by real-time PCR

| Name | Sequences | OAT | Accession number |
|---|---|---|---|
| IGF-1-QF | CAGCCAAGTCTGGTGGTAAAGC | 56·8 | KX434878 |
| IGF-1-QR | CTACATCCGATAGTTCCTCCCC | | |
| IGF-2-QF | GTGGAGGAATGCTGTTTTCGGAG | 61·4 | JN378897 |
| IGF-2-QR | AACTTTCTGGAGCGGAGGATGG | | |
| IGF-1R-QF | CCTCAATCCAAGCAAGCCTAT | 56·6 | MK440318 |
| IGF-1R-QR | TCCCCAATCTATCACTGTTCC | | |
| PI3K-QF | GTGGACCATCAACAGCAACCT | 62·6 | MK440320 |
| PI3K-QR | GGACAGACAAAGACGAGCAGC | | |
| AKT-QF | ACACGACCGCTTGTGCTTC | 61·7 | KX131157.1 |
| AKT-QR | TCCGTCCGTTATGCCCTCT | | |
| TOR-QF | TCCCTTGCCCAGACCTACA | 58·2 | MG773199 |
| TOR-QR | CATTATCGTCCCTCAGCGG | | |
| 4E-BP-QF | ACGCCACCCAGTTGCCTA | 62·6 | MG773207 |
| 4E-BP-QR | GGATGCTTTTGCTGCCGAC | | |
| S6K1-QF | GCAAACTGAATCTCCCACCC | 61·7 | MG773195 |
| S6K1-QR | AGGCTTGAAAGGCGGCTC | | |
| PCNA-QF | GTTGATGGACTTGGATGTGGA | 60·1 | MK281343 |
| PCNA-QR | CGTTGCTGGTTTGGGAGA | | |
| myf5-QF | CTCCAGTCCTTCATCATCCACC | 64·9 | MK253547 |
| myf5-QR | CACTCGCACTCTGACCTTCGT | | |
| MyoD-QF | CCTAATCAGAGGCTTCCCA | 55·5 | HM363525 |
| MyoD-QR | TCACCGCTGTATTGTTCCA | | |
| MyoG-QF | TACTTTTTCCCCGAACAGC | 57·6 | HQ246723 |
| MyoG-QR | TCCAGTCCTACATTGCCAGA | | |
| Mrf4-QF | CAGACTGTCAGAGGACGGGG | 52·8 | MK281342 |
| Mrf4-QR | CAGCCTTCTCTTTGGTGGGA | | |
| MyHC-QF | GCAATGAAGGAGAACTATG | 60·0 | MK440319 |
| MyHC-QR | TCACACTTTCCTCAGCGT | | |
| MSTN-QF | ACGCCACTACCGAGACCG | 64·6 | DQ767967 |
| MSTN-QR | CTCAATACCCCAGTTTGTTTCC | | |
| CuZnSOD-QF | ATCTGGGTAATGTGACTGCCGA | 60·4 | KX455916 |
| CuZnSOD-QR | TTCATCATCTCCGCCCTTGC | | |
| CAT-QF | ACACCGATGAGGGAAACTGG | 58 | KX455919 |
| CAT-QR | GTGGATGAAGGACGGGAACA | | |
| GPx-QF | GTGACGACTCTGTGTCCTTG | 61 | KY312111 |
| GPx-QR | AACCTTCTGCTGTATCTCTTGA | | |
| GST-QF | TCTACCCTTTACACCTGCTGAC | 62·6 | Ku *et al.* (2014) |
| GST-QR | GATGGCTGGGATTGCTTTC | | |
| GCLC-QF | GACAAACGGAGGAAGGAGG | 58·2 | KX455918 |
| GCLC-QR | TCATCAGGAAAGAAGAGGGACT | | |
| Nrf2-QF | CGGAACAAGATGGAGAAGCC | 64 | KX455917 |
| Nrf2-QR | ACAGGGAGGAATGGAGGGA | | |
| Keap1a-QF | GCATCCTCTTCACCTGTCT | 61·7 | MG773201 |
| Keap1a-QR | CGTGTAGGCGAACTCTATC | | |
| $\beta$-Actin-QF | CCTAAAGCCAACAGGGAAAA | 59 | EU161066 |
| $\beta$-Actin-QR | ATGGGGCAGAGCATAACC | | |
| 18S-QF | CCTGAGAAACGGCTACCACATCC | 57·1 | KP938527 |
| 18S-QR | AGCAACTTTAATATACGCTATTGGAG | | |

IGF-1, insulin-like growth factor 1; Q, quantitative PCR primer; F, forward; R, reverse; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; TOR, target of rapamycin; PCNA, proliferating cell nuclear antigen; Myf5, myogenic factor 5; MyoD, myoblast determination protein; Mrf4, myogenic regulatory factor 4; MyHC, myosin heavy chain; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; GCLC, $\gamma$-glutamylcysteine ligase catalytic subunit; Nrf2, NFE2-related factor 2; Keap1a, Kelch like ECH associated protein 1a.

(Millipore, Inc.) using a wet Trans-Blot System (Bio-Rad). After blocking with TRIS-buffered saline Tween 20 (TBS/T) containing 5 % bovine serum albumin for 2 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. Antibodies directed against AKT, phospho-AKT (Ser473), TOR and phospho-TOR (Ser2448) were purchased from Cell Signaling Technology Inc. The polyvinyldifluoride membranes were washed with TBS/T three times for 10 min each, incubated with second antibodies for 1 h at room temperature and then washed with TBS/T three times. Clarity Western-enhanced chemiluminescence substrate (Bio-Rad) was used to visualise signals. The Gel-Pro Analyser (Media Cybernetics) was used to quantify protein expression, and the ratio of target proteins expression was normalised to $\beta$-actin.

### Statistical analysis

Results were presented as mean values with their standard errors. All data being tested for normality of distribution using the Shapiro–Wilk test and homogeneity of variance using

**Table 3.** Initial body weight (IBW, g/fish), final body weight (FBW, g/fish), percentage weight gain (PWG, %), specific growth rate (SGR, %/d), feed intake (FI, g/fish), feed efficiency (FE) and protein efficiency ratio (PER) of hybrid catfish fed diets with graded levels of Thr (g/kg) for 56 d
(Mean values with their standard errors of three replicates, while quadratic regression was run with the triplicate data points)

| Thr... | 9·5 | | 11·5 | | 13·5 | | 15·4 | | 17·4 | | 19·3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| IBW | 14·22 | 0·05 | 14·18 | 0·09 | 14·16 | 0·06 | 14·26 | 0·08 | 14·11 | 0·05 | 14·21 | 0·07 |
| FBW | 25·31$^{a,b}$ | 0·73 | 26·26$^{b}$ | 0·55 | 26·93$^{b,c}$ | 0·29 | 26·96$^{c}$ | 0·23 | 24·97$^{a,b}$ | 0·57 | 24·20$^{a}$ | 0·42 |
| PWG* | 78·05$^{a,b}$ | 4·74 | 85·19$^{b,c}$ | 3·66 | 90·27$^{c}$ | 2·43 | 89·15$^{c}$ | 2·48 | 76·94$^{a,b}$ | 4·00 | 70·31$^{a}$ | 2·39 |
| SGR† | 1·03$^{a,b}$ | 0·05 | 1·10$^{b,c}$ | 0·04 | 1·15$^{c}$ | 0·02 | 1·14$^{c}$ | 0·02 | 1·02$^{a,b}$ | 0·04 | 0·95$^{a}$ | 0·03 |
| FI | 16·84$^{a}$ | 0·27 | 17·50$^{a,b}$ | 0·48 | 18·65$^{b}$ | 0·76 | 17·02$^{a,b}$ | 0·73 | 16·34$^{a}$ | 0·44 | 15·88$^{a}$ | 0·36 |
| FE‡ | 65·93$^{a,b}$ | 4·20 | 68·95$^{a,b}$ | 1·20 | 68·97$^{a,b}$ | 3·98 | 75·00$^{b}$ | 3·26 | 66·60$^{a,b}$ | 4·12 | 63·07$^{a}$ | 3·12 |
| PER§ | 1·63$^{a,b}$ | 0·10 | 1·77$^{a,b}$ | 0·03 | 1·71$^{a,b}$ | 0·10 | 1·79$^{b}$ | 0·08 | 1·59$^{a,b}$ | 0·10 | 1·50$^{a}$ | 0·07 |
| Regression | | | | | | | | | | | | |
| $Y_{FBW} = -0{\cdot}090X^2 + 2{\cdot}460X + 10{\cdot}080$ | | | | | | | $X = 13{\cdot}60$ | | $R^2$ 0·903 | | $P < 0{\cdot}05$ | |
| $Y_{PWG} = -0{\cdot}645X^2 + 17{\cdot}640X - 31{\cdot}450$ | | | | | | | $X = 13{\cdot}68$ | | $R^2$ 0·939 | | $P < 0{\cdot}05$ | |
| $Y_{SGR} = -0{\cdot}006X^2 + 0{\cdot}176X - 0{\cdot}065$ | | | | | | | $X = 13{\cdot}77$ | | $R^2$ 0·945 | | $P < 0{\cdot}05$ | |
| $Y_{FI} = -0{\cdot}061X^2 + 1{\cdot}608X + 7{\cdot}171$ | | | | | | | $X = 13{\cdot}22$ | | $R^2$ 0·730 | | $P = 0{\cdot}14$ | |
| $Y_{PER} = -0{\cdot}008X^2 + 0{\cdot}214X + 0{\cdot}327$ | | | | | | | $X = 13{\cdot}40$ | | $R^2$ 0·841 | | $P = 0{\cdot}06$ | |

$^{a,b,c}$ Mean values within a row with unlike superscript letters are significantly different ($P < 0{\cdot}05$).
* PWG = weight gain (g)/initial weight (g) × 100.
† SGR = (ln FBW − ln IBW)/d × 100.
‡ FE = weight gain (g)/feed intake (g) × 100.
§ PER = weight gain (g)/protein intake (g).

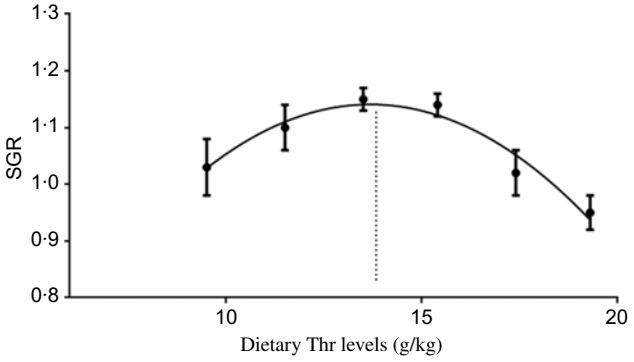


**Fig. 1.** Quadratic regression analysis of specific growth rate (SGR) for hybrid catfish fed diets containing graded levels of Thr for 56 d. $Y = -0{\cdot}0064X^2 + 0{\cdot}1763X - 0{\cdot}0647$; $R^2$ 0·9446; $X = 13{\cdot}77$.

Levene's test then were subjected to a one-way ANOVA. Differences between the treatment means were determined using Duncan's multiple-range test at a $P < 0{\cdot}05$ level of significance. Pearson correlation coefficient analysis was conducted using the Bivariate Correlation program. Statistical analyses were done using SPSS 13.0 (SPSS Inc.). Dietary Thr requirement of hybrid catfish was estimated by the quadratic regression method.

## Results

### Growth performance and muscle composition

All experimental diets were well accepted by the fish. The dietary Thr did not have a significant effect on the survival rate (>97 %) of hybrid catfish. Table 3 shows growth and feed utilisation parameters of hybrid catfish fed diets with graded levels of Thr. Compared with the control group, final body weight, feed intake, feed efficiency and protein efficiency ratio were increased with Thr level increasing up to 15·4, 13·5, 15·4 and 15·4 g/kg diet, respectively ($P < 0{\cdot}05$). Fish fed diets containing 13·5 and 15·4 g/Thr per kg diet had higher PWG and specific growth rate than those fish fed the control diet ($P < 0{\cdot}05$). Based on the quadratic regression analysis of specific growth rate, the dietary Thr requirement of hybrid catfish was estimated to be 13·77 g/kg of the diet, corresponding to 33·40 g/kg of dietary protein (Fig. 1). Fish fed diet containing 13·5 g/Thr per kg had higher muscle protein content than those fish fed the control diet (Table 4, $P < 0{\cdot}05$). The Thr treatment had no effects on muscle moisture, lipid and ash contents (Table 4, $P > 0{\cdot}05$).

### Muscle growth-related gene mRNA expression

The relative gene mRNA expressions of GH, IGF-1, IGF-2 and IGF-1 receptor are displayed in Fig. 2. Fish fed diets containing 11·5, 13·5 and 15·4 g/Thr per kg had higher GH mRNA level in pituitary than those fish fed the other diet groups ($P < 0{\cdot}05$). Compared with the control group, liver IGF-1 mRNA level was higher in fish fed 13·5 and 15·4 g/Thr per kg diets ($P < 0{\cdot}05$). However, dietary Thr did not significantly affect IGF-2 and IGF-1 receptor mRNA levels in fish liver ($P > 0.05$). As shown in Fig. 3, fish fed diet containing 17·4 g/Thr per kg had higher mRNA levels of PCNA and Myf5 in muscle than those fish fed the control diet ($P < 0{\cdot}05$). In comparison with the control group, muscle mRNA levels of MyoD and MyHC were higher in fish fed 11·5 and 13·5 g/Thr per kg diets ($P < 0{\cdot}05$). Fish fed 13·5 g/Thr per kg diet had higher MyoG mRNA level in muscle than those fish fed other diet groups ($P < 0{\cdot}05$). Dietary Thr level (>9·5 g/Thr per kg diet) significantly up-regulated Mrf4 mRNA expression. Muscle myostatin mRNA levels were higher in fish fed the control diet ($P < 0{\cdot}05$), and no significant differences were found among other groups ($P > 0{\cdot}05$).

**Table 4.** Muscle composition of hybrid catfish fed diets with graded levels of Thr (g/kg) for 56 d (Mean values with their standard errors)

| Thr... | 9·5 | | 11·5 | | 13·5 | | 15·4 | | 17·4 | | 19·3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Moisture | 78·52 | 0·86 | 78·50 | 0·21 | 77·84 | 1·03 | 78·62 | 1·36 | 79·22 | 1·11 | 78·52 | 0·85 |
| Protein | 17·05$^{a}$ | 0·27 | 17·82$^{a,b}$ | 0·35 | 18·93$^{b}$ | 0·54 | 18·24$^{a,b}$ | 1·07 | 18·00$^{a,b}$ | 0·11 | 17·61$^{a,b}$ | 0·70 |
| Lipid | 2·64 | 0·06 | 2·79 | 0·00 | 2·90 | 0·11 | 2·87 | 0·23 | 2·86 | 0·12 | 2·72 | 0·23 |
| Ash | 1·19 | 0·02 | 1·25 | 0·04 | 1·26 | 0·05 | 1·20 | 0·10 | 1·25 | 0·08 | 1·22 | 0·04 |
| Regression | | | | | | | | | | | | |
| $Y_{Protein} = -0{\cdot}042X^2 + 1{\cdot}206X + 9{\cdot}746$ | | | | | | | $X = 14{\cdot}36$ | | $R^2$ 0·709 | | $P = 0{\cdot}157$ | |

$^{a,b}$ Mean values within a row with unlike superscript letters are significantly different ($P < 0{\cdot}05$).

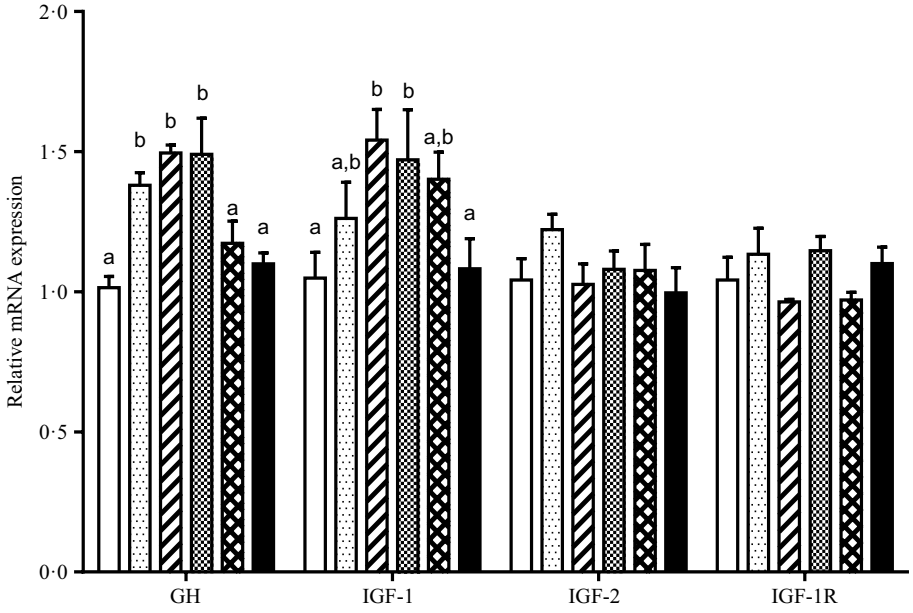


**Fig. 2.** Effects of dietary Thr on growth hormone (GH) in pituitary, insulin-like growth factor 1 (IGF-1) and IGF-2 in liver, and IGF-1 receptor (IGF-1R) in muscle gene expressions of hybrid catfish. Values are means with their standard errors, of three replicates, with six fish in each replicate. $^{a,b}$ Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). (▭), 9·5; (▭), 11·5; (▭), 13·5; (▭), 15·4; (▭), 17·4; (▭), 19·3 g Thr/kg.

### *Protein synthesis-related gene mRNA and protein expression in muscle*

As shown in Fig. 4, fish fed 13·5 and 15·4 g/Thr per kg diets had higher PI3K mRNA level than those fish fed the control diet ($P < 0{\cdot}05$). Dietary Thr level (>9·5 g/Thr per kg diet) significantly up-regulated AKT mRNA expression. The TOR mRNA level was higher in fish fed 11·5 and 13·5 g/Thr per kg diets than those fish fed other diet groups ($P < 0{\cdot}05$). Inversely, the 4E-BP mRNA level was lower in fish fed Thr at 13·5 g/kg diet ($P < 0{\cdot}05$). The S6K1 mRNA level was gradually increased with increasing dietary Thr levels up to 17·4 g/kg diet and decreased thereafter ($P < 0{\cdot}05$). As shown in Fig. 5, compared with the control group, fish fed diets containing 13·5 and 19·3 g/Thr per kg increased the level of phospho-AKT:total AKT ($P < 0{\cdot}05$). The level of phospho-TOR:total TOR was the highest for fish fed 13·5 g/Thr per kg diet, then followed by 19·3 g/Thr per kg diet, and the lowest for fish fed the control diet ($P < 0{\cdot}05$).

### *Antioxidant-related parameters in muscle*

As shown in Table 5. The contents of MDA and PC were decreased with the increasing dietary Thr level up to 13·5 g/kg diet and then increased with further increasing of dietary Thr levels ($P < 0{\cdot}05$). Fish fed 13·5 g/Thr per kg diet had higher CAT activity than those fish fed 19·3 g/Thr per kg diet ($P < 0{\cdot}05$). Compared with the control group, GST activity was increased in fish fed 11·5, 13·5 and 15·4 g/Thr per kg diets ($P < 0{\cdot}05$). The activity of GR was a maximum for fish fed 15·4 g/Thr per kg diet and minimal for fish fed 19·3 g/Thr per kg diet ($P < 0{\cdot}05$). The GSH content was increased with the increasing dietary Thr level up to 15·4 g/Thr per kg diet ($P < 0{\cdot}05$) and plateaued thereafter ($P > 0{\cdot}05$). No significant difference in SOD, GPx, anti-superoxide anion and anti-hydroxy radical activities among treatments was detected ($P > 0{\cdot}05$).

As shown in Figs. 6 and 7. Fish fed diet containing 19·3 g/Thr per kg had lower level of CuZnSOD mRNA than those fish fed

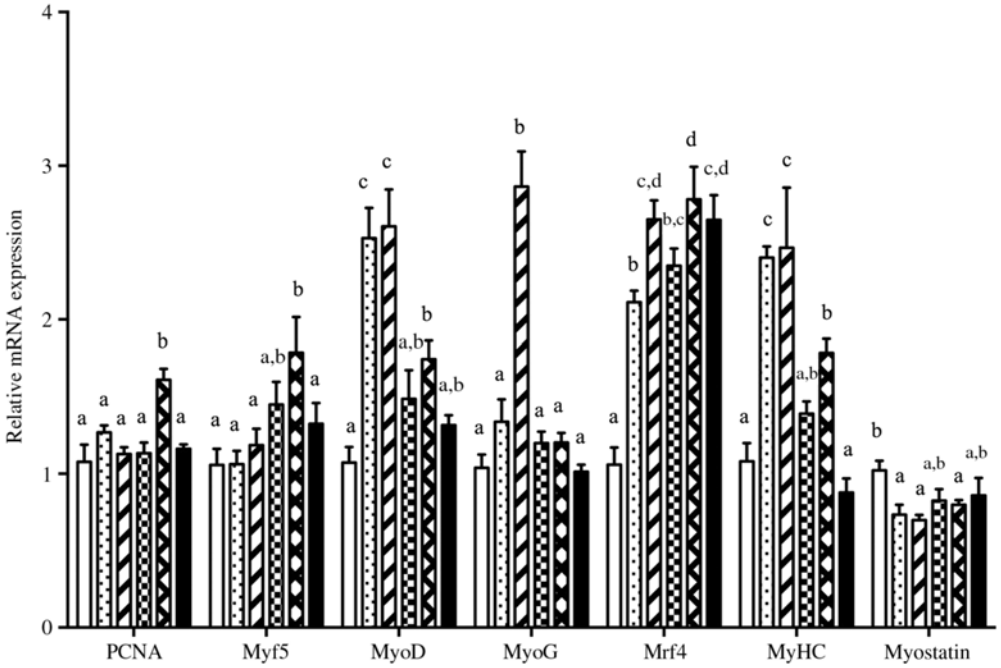


**Fig. 3.** Effects of dietary Thr on proliferating cell nuclear antigen (PCNA), myogenic factor 5 (Myf5), myoblast determination protein (MyoD), myogenin (MyoG), myogenic regulatory factor 4 (Mrf4), myosin heavy chain (MyHC) and myostatin gene expressions in the muscle of hybrid catfish. Values are means with their standard errors, of three replicates, with six fish in each replicate. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). (▭), 9·5; (▭), 11·5; (▭), 13·5; (▭), 15·4; (▭), 17·4; (▭), 19·3 g Thr/kg.

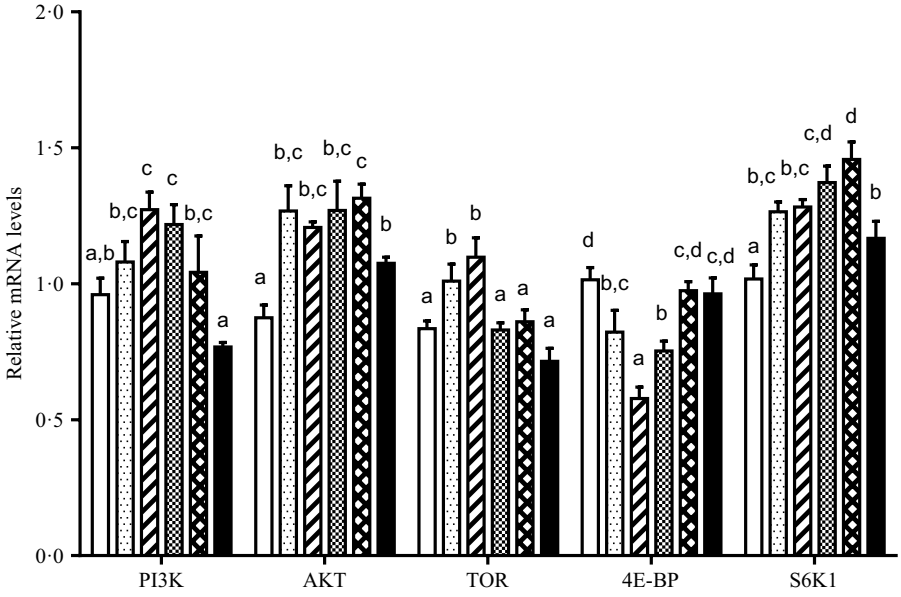


**Fig. 4.** Effects of dietary Thr on phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), target of rapamycin (TOR), 4E-binding protein (4E-BP) and S6 kinase 1 (S6K1) gene expressions in muscle of hybrid catfish. Values are means with their standard errors, of three replicates, with six fish in each replicate. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). (▭), 9·5; (▭), 11·5; (▭), 13·5; (▭), 15·4; (▭), 17·4; (▭), 19·3 g Thr/kg.

diets with other Thr levels ($P < 0{\cdot}05$). Significantly lower level of CAT mRNA was found in fish fed the control and 19·3 g/kg Thr diets. The GST, GPx and γ-glutamylcysteine ligase catalytic mRNA levels gradually increased with increasing Thr levels up to 15·4, 15·4 and 13·5 g/kg diet, respectively, and decreased thereafter ($P < 0{\cdot}05$). The Nrf2 mRNA level was the maximum for fish fed the diet of 13·5 g/Thr per kg and was the minimum for fish fed the diet of 19·3 g/Thr per kg ($P < 0{\cdot}05$). Interestingly, the Keap1 mRNA level showed the opposite of trend with respect to Nrf2 mRNA level ($P < 0{\cdot}05$).

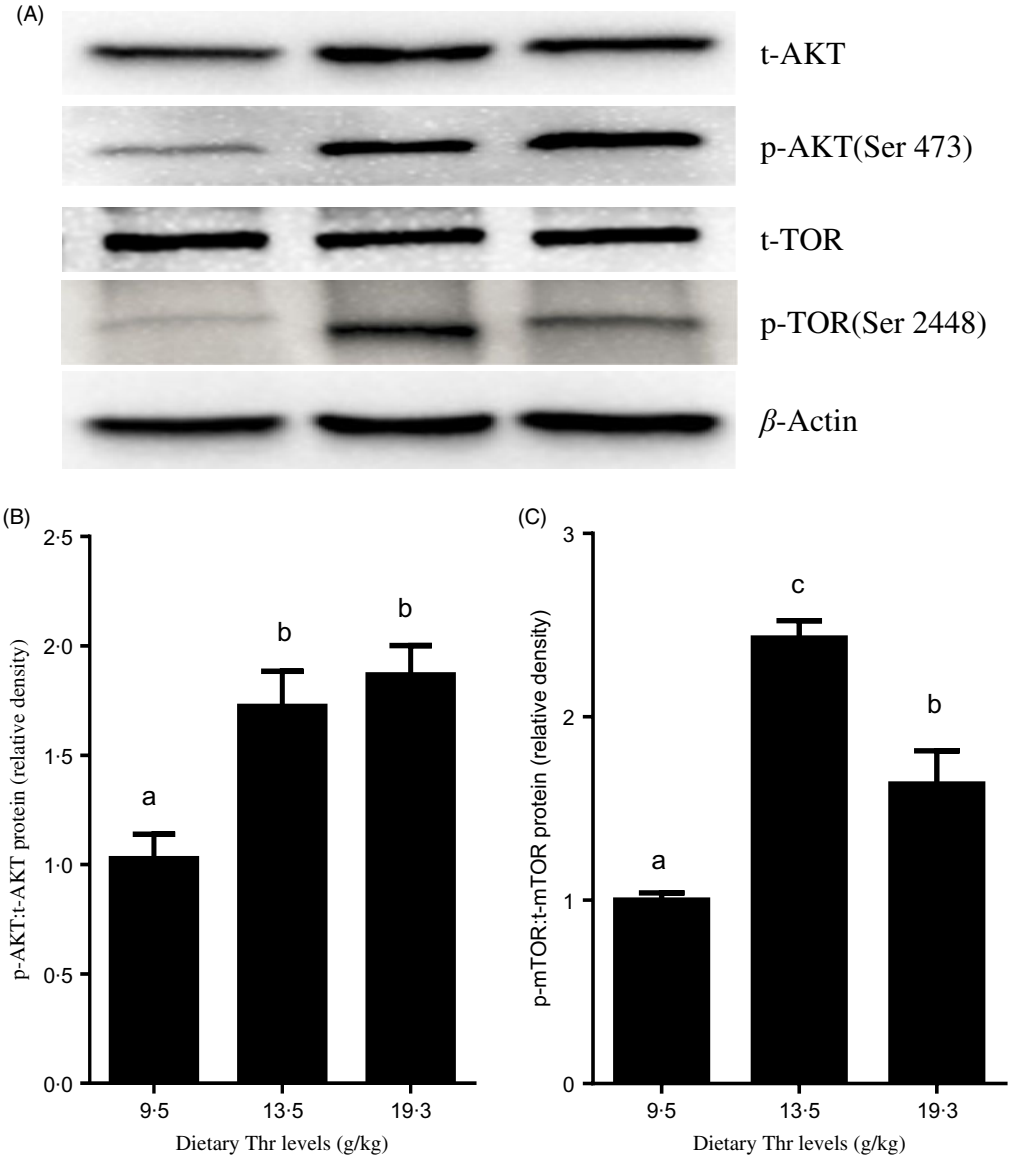


**Fig. 5.** Effects of dietary Thr on the protein kinase B (AKT)/target of rapamycin (TOR) signalling pathway in the muscle of hybrid catfish. The total AKT (t-AKT) and phospho-AKT (p-AKT) (A and B) and total TOR (t-TOR) and phospho-TOR (p-TOR) (A and C) protein levels were determined by Western blot analysis. Equal loading was monitored with anti-*β*-actin antibody. Values are means with their standard errors, of three replicates, with three individuals in each replicate. [a,b,c] Mean values with unlike letters were significantly different ($P < 0·05$).

## Discussion

As an essential amino acid, dietary Thr level had a clear effect on growth and feed utilisation of hybrid catfish. The dietary Thr requirement of hybrid catfish (14·19–25·77 g) was estimated to be 13·77 g/kg of the diet, corresponding to 33·40 g/kg of dietary protein (Fig. 1). This value (g/kg of dietary protein) was close to that (36·10 g/kg of dietary protein) reported in grass carp[13], and Japanese flounder *Paralichthys olivaceus* (32·20 g/kg of dietary protein)[43], lower than that reported in Jian carp *Cyprinus carpio* (51·30 g/kg of dietary protein)[9], and blunt snout bream *Megalobrama amblycephala* (46·20 g/kg of dietary protein)[44]. These discrepancies might be ascribed to the differences in genetics of species, selection of dietary protein source, growth environment or growth stages. Correlation analysis showed that PWG was positively related to feed intake ($r$ +0·835, $P = 0·039$, Table 6) and feed efficiency ($r$ +0·865, $P = 0·026$, Table 6), suggesting that the improved growth by Thr was partly due to increased feed intake and feed utilisation, which was also observed in Jian carp[9]. Fish weight gain is primarily attributed to the accretion of protein and fat[45]. Muscle protein synthesis in teleostean fish contributes to 50 % of fish growth[16]. The present study showed that Thr significantly enhanced hybrid catfish muscle protein content, indicating that Thr has beneficial effects on protein synthesis and muscle growth, which were in accordance with the results for young pigs, rats and broiler chickens[7,46,47].

The lower PWG observed in fish fed 9·5 g/Thr per kg diet or 19·3 g/Thr per kg diet compared with that of fish fed 13·5 g/Thr per kg diet indicated that both excess and insufficiency of Thr in diets could induce a decrease in growth performance of hybrid

**Table 5.** Malondialdehyde (MDA, nmol/mg protein), protein carbonyl (PC, nmol/mg protein) and GSH (mmol/g tissue) contents and superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), anti-superoxide anion (ASA) and anti-hydroxyl radical (AHR) activities (U/mg protein) in the muscle of hybrid catfish fed with graded levels of Thr (g/kg) for 56 d
(Mean values with their standard errors of three replicates with six fish in each replicate)

| Thr... | 9·5 | | 11·5 | | 13·5 | | 15·4 | | 17·4 | | 19·3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| MDA | 0·72$^{b}$ | 0·02 | 0·63$^{a,b}$ | 0·02 | 0·56$^{a}$ | 0·04 | 0·57$^{a}$ | 0·03 | 0·63$^{a,b}$ | 0·03 | 0·70$^{b}$ | 0·03 |
| PC | 1·31$^{b,c}$ | 0·13 | 1·17$^{a,b,c}$ | 0·10 | 0·9$^{a}$ | 0·01 | 1·05$^{a,b}$ | 0·13 | 1·18$^{a,b,c}$ | 0·06 | 1·41$^{c}$ | 0·08 |
| SOD | 38·12 | 1·20 | 40·08 | 3·63 | 38·42 | 3·36 | 37·46 | 1·26 | 39·60 | 2·19 | 36·27 | 2·43 |
| CAT | 0·67$^{a,b}$ | 0·01 | 0·71$^{a,b}$ | 0·08 | 0·88$^{b}$ | 0·07 | 0·85$^{b}$ | 0·08 | 0·77$^{b}$ | 0·08 | 0·52$^{a}$ | 0·06 |
| GST | 9·47$^{b}$ | 0·39 | 12·57$^{c}$ | 0·75 | 11·6$^{c}$ | 0·72 | 11·4$^{c}$ | 0·18 | 6·10$^{a}$ | 0·23 | 6·16$^{a}$ | 0·43 |
| GPx | 15·92 | 0·99 | 15·28 | 1·29 | 14·56 | 0·95 | 14·21 | 0·74 | 15·06 | 1·24 | 13·40 | 1·57 |
| GR | 2·86$^{a}$ | 0·18 | 3·09$^{a}$ | 0·22 | 4·43$^{b}$ | 0·73 | 5·09$^{b}$ | 0·23 | 4·60$^{b}$ | 0·57 | 2·37$^{a}$ | 0·04 |
| GSH | 133·13$^{a}$ | 17·06 | 138·57$^{a}$ | 5·31 | 154·09$^{a,b}$ | 5·21 | 177·14$^{b}$ | 17·9 | 181·78$^{b}$ | 5·01 | 175·95$^{b}$ | 7·87 |
| ASA | 46·43 | 1·99 | 46·38 | 2·97 | 47·31 | 4·58 | 51·96 | 3·29 | 48·01 | 5·22 | 45·06 | 5·12 |
| AHR | 93·65 | 1·82 | 91·11 | 5·38 | 81·71 | 3·75 | 87·64 | 7·66 | 88·79 | 7·13 | 85·12 | 9·23 |
| Regression | | | | | | | | | | | | |
| $Y_{MDA} = 0{\cdot}006X^2 - 0{\cdot}178X + 1{\cdot}859$ | | | | | | | $X = 14{\cdot}60$ | | $R^2$ 0·980 | | $P < 0{\cdot}01$ | |
| $Y_{PC} = 0{\cdot}016X^2 - 0{\cdot}453X + 4{\cdot}181$ | | | | | | | $X = 14{\cdot}16$ | | $R^2$ 0·902 | | $P < 0{\cdot}05$ | |
| $Y_{CAT} = -0{\cdot}011X^2 + 0{\cdot}318X - 1{\cdot}369$ | | | | | | | $X = 14{\cdot}45$ | | $R^2$ 0·878 | | $P < 0{\cdot}05$ | |
| $Y_{GST} = -0{\cdot}154X^2 + 3{\cdot}911X - 13{\cdot}092$ | | | | | | | $X = 12{\cdot}70$ | | $R^2$ 0·789 | | $P = 0{\cdot}097$ | |
| $Y_{GR} = -0{\cdot}090X^2 + 2{\cdot}640X - 14{\cdot}566$ | | | | | | | $X = 14{\cdot}67$ | | $R^2$ 0·755 | | $P = 0{\cdot}121$ | |
| $Y_{GSH} = -0{\cdot}443X^2 + 18{\cdot}112X - 4{\cdot}066$ | | | | | | | $X = 20{\cdot}44$ | | $R^2$ 0·908 | | $P < 0{\cdot}05$ | |

$^{a,b,c}$ Mean values within a row with unlike superscript letters are significantly different ($P < 0{\cdot}05$).

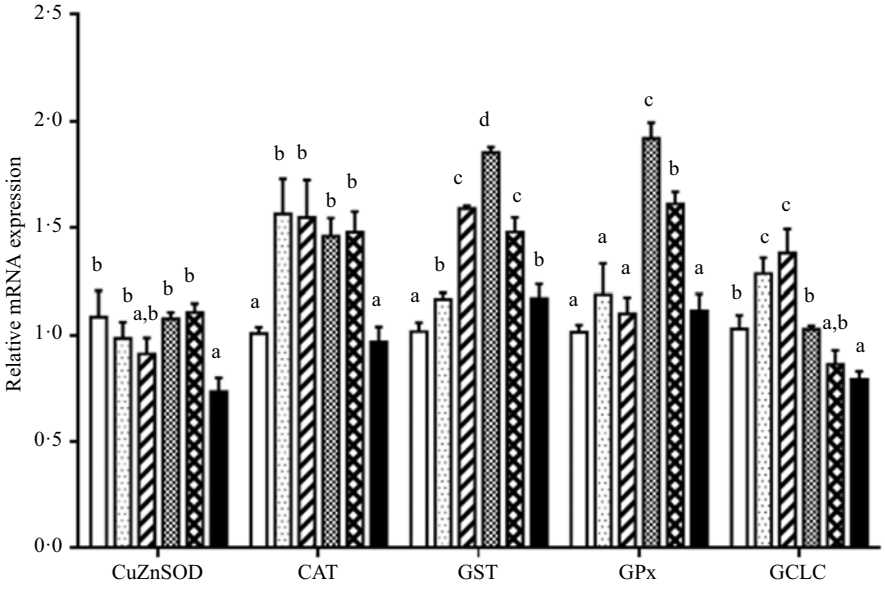


**Fig. 6.** Effects of dietary Thr on copper,zinc-superoxide dismutase (CuZnSOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and γ-glutamylcysteine ligase catalytic subunit (GCLC) gene expressions in the muscle of hybrid catfish. Values are means with their standard errors, of three replicates, with six fish in each replicate. $^{a,b,c,d}$ Mean values with unlike letters were significantly different ($P < 0{\cdot}05$). (▭), 9·5; (▭), 11·5; (▭), 13·5; (▭), 15·4; (▭), 17·4; (▭), 19·3 g Thr/kg.

catfish, being in agreement with the results reported in Jian carp[9], grass carp[13], Japanese flounder[43] and blunt snout bream[44]. Fish growth is regulated by the GH/IGF axis[48]. Several of the components of the GH and IGF system described in mammals have been isolated, characterised and assessed in fish[49–53]. In the present study, relative expressions of GH and IGF-1 gene were up-regulated by Thr. Similarly, studies in growing pigs and rats have demonstrated that a low-Thr diet significantly reduced plasma IGF-1 levels[54,55]. The previous study also observed dose dependency of IGF-1 mRNA expression to Thr concentrations in cultured pig hepatocytes[56]. Correlation analysis showed that PWG was positively correlated with mRNA levels of GH ($r$ +0·902, $P = 0{\cdot}014$) and IGF-I ($r$ +0·765, $P = 0{\cdot}076$), which partially explained the variations in PWG values among different experimental treatments. This further demonstrated that dietary Thr levels can affect the expressions of pituitary GH and hepatic IGF-1 to alter fish growth in hybrid catfish.

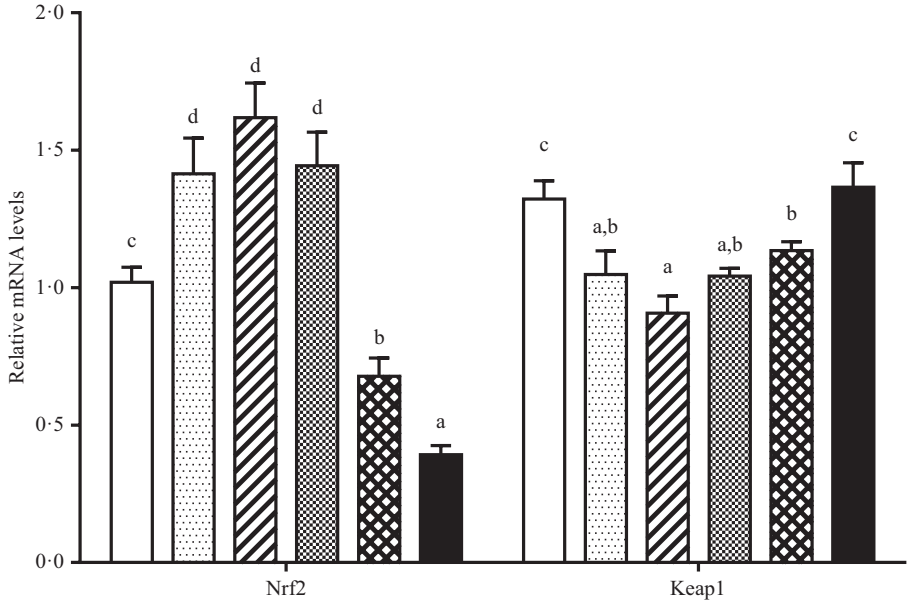


**Fig. 7.** Relative mRNA expressions of NFE2-related factor 2 (Nrf2) and Kelch like ECH associated protein 1 (Keap1) in the muscle of hybrid catfish fed diets containing graded levels of Thr for 56 d. Values are means with their standard errors, of three replicates, with six fish in each replicate. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0·05$). (▭), 9·5; (▭), 11·5; (▭), 13·5; (▭), 15·4; (▭), 17·4; (▭), 19·3 g Thr/kg.

In fish, the majority of growth is invested in accretion of muscle tissue, since muscle may account for more than half of the fish's body mass. As mammals, the GH/IGF axis is the most important endocrine system regulating muscle growth in fish[17]. GH exerts its action directly via binding to the GH receptor in its target tissues such as muscle or indirectly via local GH-induced IGF-1 production. The present results showed that dietary Thr had GH-stimulating effects and increased IGF-1 mRNA level. Correlation analysis showed that GH was positively correlated with mRNA level of IGF-1 ($r$ +0·841, $P = 0·036$), which suggested that the up-regulated IGF-1 mRNA expression by Thr was partly due to increased GH expression. Fish muscle growth is complicated and precisely controlled processes, including proliferation and differentiation of myoblasts[57]. IGF-1 stimulates both proliferation and differentiation of myoblasts[58] to promote muscle growth in fish[59,60]. PCNA is a DNA polymerase δ associated peptide that is synthesised early in the G1 and S-phase of the cell cycle, which expression is correlated with DNA synthesis[61,62]. Expression of PCNA in association with MyoD in myogenic cells is a marker of myogenic progenitor cell activation[63]. During myogenesis, the MyoD and Myf5 are required for the initial specification of the myogenic lineage, while MyoG and Mrf4 are activated during myoblast differentiation and cell fusion[64]. MyHC plays important roles in fish muscle growth via hyperplasia and hypertrophy of muscle fibres[65]. The present results for the first time show that dietary Thr up-regulated PCNA, Myf5, MyoD, Mrf4, MyoG and MyHC mRNA expressions. Correlation analysis indicated that the IGF-1 was positively correlated with MyoD ($r$ 0·604, $P = 0·204$), MyoG ($r$ 0·658, $P = 0·155$) and MyHC ($r$ 0·656, $P = 0·157$) mRNA levels, indicating that dietary Thr increasing the muscle growth in fish might be partly related to up-regulated IGF-1 transcription. Myostatin acts as a negative regulator, which inhibits satellite cell proliferation during muscle development and growth[66]. In the present study, the myostatin mRNA level followed an opposite pattern to MyoD mRNA level. Correlation analysis indicated that the myostatin was negatively correlated with GH ($r$ −0·767, $P = 0·075$) and IGF-I ($r$ −0·726, $P = 0·102$) mRNA expressions, suggesting that the dietary Thr decreasing myostatin might partly be associated with the increased GH and IGF-I mRNA levels in fish muscle. Similar results were found in rainbow trout and gilthead sea bream *Sparus aurata*[67,68]. All the above results indicate that there may be a relationship between the improvement of the MRF and the enhanced IGF-1 mRNA level by Thr. However, more studies are required to elucidate a more detailed mode in which Thr regulated muscle growth-related gene expression in fish.

In addition to MRF, the PI3K/AKT pathway plays crucial roles in fish muscle protein synthesis and myoblast differentiation and hypertrophy[30,69]. AKT phosphorylation is an important marker of the activation of the PI3K/AKT pathway and muscle growth[70]. Despite their significance however, their regulation by nutrients remains poorly understood in fish. The present study demonstrated for the first time dietary Thr supplementation resulted in up-regulation of PI3K and AKT mRNA expressions. The phospho-AKT:total AKT ratio was increased by dietary Thr, indicating that dietary Thr increased muscle protein synthesis via the PI3K/AKT signalling pathway. TOR, a downstream component of the PI3K/AKT pathway, promotes cellular growth by stimulating protein synthesis via 4E-BP and S6K in fish[31]. The present data showed that dietary Thr supplementation increased muscle TOR and S6K1 mRNA levels and the phosphorylation of TOR. This result was in good agreement with reports on rainbow trout *Oncorhynchus mykiss* and gilthead sea

**Table 6.** Correlation analysis of parameters in the muscle of hybrid catfish

| Independent parameters | Dependent parameters | Correlation coefficients | *P* |
|---|---|---|---|
| PWG | FI | 0·835 | 0·039 |
| | FE | 0·865 | 0·026 |
| | GH mRNA | 0·902 | 0·014 |
| | IGF-1 mRNA | 0·765 | 0·076 |
| GH mRNA | IGF-1 mRNA | 0·841 | 0·036 |
| | MyoD mRNA | 0·705 | 0·118 |
| | MyoG mRNA | 0·630 | 0·180 |
| | MyHC mRNA | 0·682 | 0·136 |
| | myostatin mRNA | −0·767 | 0·075 |
| IGF-1 mRNA | MyoD mRNA | 0·604 | 0·204 |
| | MyoG mRNA | 0·658 | 0·155 |
| | Mrf4 mRNA | 0·583 | 0·225 |
| | MyHC mRNA | 0·656 | 0·157 |
| | Myostatin mRNA | −0·726 | 0·102 |
| | PI3K mRNA | 0·882 | 0·020 |
| | AKT mRNA | 0·770 | 0·073 |
| | TOR mRNA | 0·586 | 0·221 |
| | 4E-BP mRNA | −0·780 | 0·067 |
| | S6K1 mRNA | 0·782 | 0·066 |
| Protein content | GH mRNA | 0·847 | 0·033 |
| | IGF1 mRNA | 0·922 | 0·009 |
| | MyoD mRNA | 0·706 | 0·117 |
| | MyoG mRNA | 0·823 | 0·044 |
| | Mrf4 mRNA | 0·695 | 0·125 |
| | MyHC mRNA | 0·670 | 0·145 |
| | Myostatin mRNA | −0·830 | 0·041 |
| | PI3K mRNA | 0·764 | 0·077 |
| | AKT mRNA | 0·681 | 0·137 |
| | TOR mRNA | 0·633 | 0·177 |
| | 4E-BP mRNA | −0·892 | 0·017 |
| | S6K1 mRNA | 0·633 | 0·178 |
| AKT mRNA | MyoD mRNA | 0·609 | 0·200 |
| | Mrf4 mRNA | 0·759 | 0·080 |
| | MyHC mRNA | 0·611 | 0·198 |
| TOR mRNA | MyoD mRNA | 0·900 | 0·014 |
| | MyoG mRNA | 0·828 | 0·042 |
| | MyHC mRNA | 0·942 | 0·005 |
| 4E-BP mRNA | MyoD mRNA | −0·729 | 0·100 |
| | MyoG mRNA | −0·847 | 0·033 |
| | MyHC mRNA | −0·677 | 0·139 |
| S6K1 mRNA | PCNA mRNA | 0·699 | 0·122 |
| | Myf5 mRNA | 0·767 | 0·075 |
| | Mrf4 mRNA | 0·758 | 0·080 |
| MDA | MyoD mRNA | −0·656 | 0·157 |
| | MyoG mRNA | −0·753 | 0·081 |
| | MyHC mRNA | −0·675 | 0·141 |
| PC | MyoD mRNA | −0·687 | 0·131 |
| | MyoG mRNA | −0·807 | 0·052 |
| | MyHC mRNA | −0·747 | 0·088 |
| GSH | Myf5 mRNA | 0·879 | 0·021 |
| | Mrf4 mRNA | 0·772 | 0·072 |
| Nrf2 mRNA | CAT | 0·786 | 0·064 |
| | GST | 0·948 | 0·004 |
| | GR | 0·486 | 0·329 |
| Keap1 mRNA | CAT | −0·887 | 0·018 |
| | GST | −0·678 | 0·139 |
| | GR | −0·722 | 0·105 |

PWG, percentage weight gain; FI, feed intake, FE, feed efficiency; IGF-1, insulin-like growth factor 1; GH, growth hormone; MyoD, myoblast determination protein; MyoG, myogenin; MyHC, myosin heavy chain; TOR, target of rapamycin; PCNA, proliferating cell nuclear antigen; Myf5, myogenic factor 5; Mrf4, myogenic regulatory factor 4; MDA, malondialdehyde; PC, protein carbonyl; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; GCLC, γ-glutamylcysteine ligase catalytic subunit; Nrf2, NFE2-related factor 2; Keap1, Kelch like ECH associated protein 1.

bream myocytes, which reported that Thr could stimulate TOR phosphorylation and gene expressions of its downstream effectors[71,72]. The correlation analysis also indicated that muscle protein content was positively correlated with PI3K ($r$ 0·764, $P=0·077$), AKT ($r$ 0·681, $P=0·137$), TOR ($r$ 0·633, $P=0·177$) and S6K1 ($r$ 0·633, $P=0·178$) mRNA levels. A negative correlation was observed between muscle protein content and 4E-BP ($r$ −0·892, $P=0·017$) mRNA level. These results suggested that dietary Thr increasing muscle protein content might be partly related to elevate muscle protein synthesis via the PI3K/AKT/TOR signalling pathway. Meanwhile, the present results also showed IGF-I was positively correlated with mRNA expressions of PI3K ($r$ 0·882, $P=0·020$), AKT ($r$ 0·770, $P=0·073$) and S6K1 ($r$ 0·782, $P=0·066$), which suggested that dietary Thr promotes muscle protein synthesis by activating the PI3K/AKT/TOR signalling pathway via IGF-I. Similar results were observed in muscle of rainbow trout and fine flounder[30,69]. Moreover, muscle protein mass is regulated primarily through alterations in protein synthesis[73]. TOR is essential for satellite cell function and skeletal muscle regeneration through controlling the expression of myogenic genes[74]. Rion *et al.* reported mTOR controls embryonic and adult myogenesis via mTORC1[75]. As a result, the rate of whole-body growth can be delineated from an assessment of muscle protein synthesis. The present study showed that a positive correlation was observed between the AKT, TOR and S6K1 and muscle growth-related gene mRNA levels (Table 6). These findings suggest that, as in mammals, the Thr could activate the PI3K/AKT/TOR signalling pathways via IGF-I and contribute to muscle protein synthesis and growth in fish.

In fish, PC and MDA contents are widely used as markers for protein oxidation and lipid peroxidation, respectively[76]. Increasing evidence indicates that the efficiency of myogenic differentiation is reduced by oxidative damage[27,77,78]. In the present study, both PC and MDA contents in fish muscle were decreased by Thr. The correlation analysis indicated that muscle PC and MDA contents were negatively correlated with MyoD ($r_{PC}=-0·687$, $P=0·131$; $r_{MDA}=-0·656$, $P=0·157$), MyoG ($r_{PC}=-0·807$, $P=0·052$; $r_{MDA}=-0·753$, $P=0·081$) and MyHC ($r_{PC}=-0·747$, $P=0·088$; $r_{MDA}=-0·675$, $P=0·141$) mRNA levels. These results suggested that Thr may improve muscle growth via suppressing oxidative damage in fish. However, no information is available to date about the effect of Thr on oxidative damage in fish muscle. Recent studies from our laboratory showed that lipid peroxidation and protein oxidation in intestine and gill could be effectively prevented by Thr in grass carp[24,29]. Fish antioxidant systems are composed of antioxidant enzymes (SOD, CAT, GPx, GST and GR) and non-enzymatic compounds (GSH)[79,80]. The present study showed that CAT, GST and GR activities in muscle were increased by dietary Thr. Similar results are found in the intestine of grass carp[24]. Antioxidant enzyme activities were closely related to their mRNA levels in fish[76]. The present study indicated that dietary Thr increased mRNA levels of CAT and GST in muscle, which showed the same trend to their enzyme activities. The cellular

pool of GSH is maintained by the activity status of the enzyme γ-glutamylcysteine ligase catalytic subunit[81]. The present study showed dietary Thr also up-regulated the muscle γ-glutamylcysteine ligase catalytic mRNA levels. Nrf2 is a key regulator to transcriptions of antioxidant enzymes, which could be prevented by blinding to Keap1 from translocation into the nucleus in fish[82,83]. When activated, Nrf2 is bound to antioxidant transcription elements in the promoter regions of phase 2 detoxification enzyme genes and certain antioxidant genes[82,84]. Keap1 is identified as a Nrf2-binding protein that prevents Nrf2 translocation to the nucleus and promotes the ubiquitination-proteasomal degradation of Nrf2[85]. In the present study, dietary Thr supplementation elevated Nrf2 mRNA expression and blocked the increase in Keap1 mRNA expression in muscle of hybrid catfish. Similar results were found in gills of grass carp fed with diets containing graded dietary Thr levels[29]. Correlation analysis indicated that CAT ($r$ 0·786, $P = 0·064$) and GST ($r$ 0·948, $P = 0·004$) activities were positively correlated with Nrf2 mRNA level, whereas muscle Keap1 mRNA level was negatively correlated with CAT ($r$ −0·887, $P = 0·018$), GST ($r$ −0·678, $P = 0·139$) and GR ($r$ −0·722, $P = 0·105$) activities. These results suggested that increased antioxidant enzyme activities by Thr might partly attribute to the Nrf2/Keap1 signalling in fish muscle. Thus, dietary Thr could improve muscle growth (myogenic differentiation) via suppressing oxidative damage through regulating the Nrf2/Keap1 signalling pathway to elevate antioxidative capacity.

In summary, the present work showed that dietary Thr improved the growth of hybrid catfish. For the first time, we found that dietary Thr up-regulated muscle growth-related gene (GH, IGF-1, PCNA, Myf5, MyoD, MyoG, Mrf4 and MyHC) expression, improved muscle protein content via the AKT/TOR signalling pathway. Furthermore, Thr supplementation improved muscle antioxidant capacity via regulating the Nrf2/Keap1 signalling pathway, reducing oxidative damage and promoted muscle growth. Based on the quadratic regression analysis of specific growth rate, the dietary Thr requirement of hybrid catfish (14·19–25·77 g) was estimated to be 13·77 g/kg of the diet, corresponding to 33·40 g/kg of dietary protein.

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Y. Z. and Q. J. conducted the trial, performed the RT-PCR experiments and wrote the manuscript. J. J. and X.-Q. Z. contributed to the design of the study. L. F. and W.-D. J. assisted in the manuscript preparation. S.-X. X. and J. Z. assisted with all data analysis. Y. L. and P. W. assisted with the trail.

There are no conflicts of interest.

## References

1. Periago MJ, Ayala MD, López-Albors O, *et al.* (2005) Muscle cellularity and flesh quality of wild and farmed sea bass, *Dicentrarchus labrax* L. *Aquaculture* **249**, 175–188.
2. Greer-Walker M (1970) Growth and development of the skeletal muscle fibres of the cod (*Gadus morhua* L.). *ICES J Mar Sci* **33**, 228–244.
3. Zhao Y, Li J, Yin L, *et al.* (2019) Effects of dietary glutamate supplementation on flesh quality, antioxidant defense and gene expression related to lipid metabolism and myogenic regulation in Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture* **502**, 212–222.
4. Michelato M, Zaminhan M, Boscolo WR, *et al.* (2016) Dietary histidine requirement of Nile tilapia juveniles based on growth performance, expression of muscle-growth-related genes and haematological responses. *Aquaculture* **467**, 63–70.
5. Alamidurante H, Bazin D, Cluzeaud M, *et al.* (2018) Effect of dietary methionine level on muscle growth mechanisms in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **483**, 273–285.
6. Canada P, Engrola S, Mira S, *et al.* (2018) Larval dietary protein complexity affects regulation of muscle growth and expression of DNA methyltransferases in Senegalese sole. *Aquaculture* **491**, 28–38.
7. Wang X, Qiao S, Yin Y, *et al.* (2007) A deficiency or excess of dietary threonine reduces protein synthesis in jejunum and skeletal muscle of young pigs. *J Nutr* **137**, 1442–1446.
8. Hamard A, Sève B & Floc'h NL (2009) A moderate threonine deficiency differently affects protein metabolism in tissues of early-weaned piglets. *Comp Biochem Physiol A Mol Integr Physiol* **152**, 491–497.
9. Feng L, Peng Y, Wu P, *et al.* (2013) Threonine affects intestinal function, protein synthesis and gene expression of TOR in Jian carp (*Cyprinus carpio* var. Jian). *PLOS ONE* **8**, e69974.
10. Habte-Tsion H, Ge X, Liu B, *et al.* (2015) A deficiency or an excess of dietary threonine level affects weight gain, enzyme activity, immune response and immune-related gene expression in juvenile blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immun* **42**, 439–446.
11. Dong YW, Jiang WD, Liu Y, *et al.* (2017) Threonine deficiency decreased intestinal immunity and aggravated inflammation associated with NF-κB and target of rapamycin signalling pathways in juvenile grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. *Brit J Nutr* **118**, 92–108.
12. Habte-Tsion H, Ren M, Liu B, *et al.* (2016) Threonine modulates immune response, antioxidant status and gene expressions of antioxidant enzymes and antioxidant-immune-cytokine-related signaling molecules in juvenile blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immun* **51**, 189–199.
13. Gao Y, Yang H, Liu Y, *et al.* (2014) Effects of graded levels of threonine on growth performance, biochemical parameters and intestine morphology of juvenile grass carp *Ctenopharyngodon idella*. *Aquaculture* **424**, 113–119.
14. Faure M, Moënnoz D, Montigon F, *et al.* (2005) Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. *J Nutr* **135**, 486–491.
15. Johnston IA, Lee H, MacQueen DJ, *et al.* (2009) Embryonic temperature affects muscle fibre recruitment in adult zebrafish: genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. *J Exp Biol* **212**, 1781–1793.

16. Mommsen TP (2001) Paradigms of growth in fish. *Comp Biochem Physiol B Biochem Mol Biol* **129**, 207–219.
17. Fuentes EN, Valdés JA, Molina A, *et al.* (2013) Regulation of skeletal muscle growth in fish by the growth hormone-insulin-like growth factor system. *Gen Comp Endocr* **192**, 136–148.
18. Hill JA, Kiessling A & Devlin RH (2000) Coho salmon (*Oncorhynchus kisutch*) transgenic for a growth hormone gene const construct exhibit increased rates of muscle hyperplasia and detectable levels of differential gene expression. *Can J Fish Aquat Sci* **57**, 939–950.
19. Kuradomi RY, Figueiredo MA, Lanes CF, *et al.* (2011) GH overexpression causes muscle hypertrophy independent from local IGF-I in a zebrafish transgenic model. *Transgenic Res* **20**, 513–521.
20. Serrana DGDL, Devlin RH & Johnston IA (2015) RNAseq analysis of fast skeletal muscle in restriction-fed transgenic coho salmon (*Oncorhynchus kisutch*): an experimental model uncoupling the growth hormone and nutritional signals regulating growth. *BMC Genomics* **16**, 564.
21. Berkes CA & Tapscott SJ (2005) MyoD and the transcriptional control of myogenesis. *Semin Cell Dev Biol* **16**, 585–595.
22. Watabe S (2001) 2-Myogenic regulatory factors. *Fish Physiol* **18**, 19–41.
23. Rescan PY (2005) Muscle growth patterns and regulation during fish ontogeny. *Gen Comp Endocr* **142**, 111–116.
24. Hong Y, Jiang W, Kuang S, *et al.* (2015) Growth, digestive and absorptive capacity and antioxidant status in intestine and hepatopancreas of sub-adult grass carp *Ctenopharyngodonidella* fed graded levels of dietary threonine. *J Anim Sci Biotechnol* **6**, 34.
25. Jang Y & Baik EJ (2013) JAK-STAT pathway and myogenic differentiation. *Jakstat* **2**, e23282.
26. Hansen JM, Klass M, Harris C, *et al.* (2007) A reducing redox environment promotes C2C12 myogenesis: implications for regeneration in aged muscle. *Cell Biol Int* **31**, 546–553.
27. Barbieri E & Sestili P (2012) Reactive oxygen species in skeletal muscle signaling. *J Signal Transduct* **2012**, 982794.
28. Sagliocchi S, Cicatiello AG, Di Cicco E, *et al.* (2019) The thyroid hormone activating enzyme, type 2 deiodinase, induces myogenic differentiation by regulating mitochondrial metabolism and reducing oxidative stress. *Redox Biol* **24**, 101228.
29. Dong YW, Feng L, Jiang WD, *et al.* (2017) Dietary threonine deficiency depressed the disease resistance, immune and physical barriers in the gills of juvenile grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*. *Fish Shellfish Immun* **72**, 161.
30. Fuentes EN, Björnsson BT, Valdés JA, *et al.* (2011) IGF-I/PI3K/AKT and IGF-I/MAPK/ERK pathways *in vivo* in skeletal muscle are regulated by nutrition and contribute to somatic growth in the fine flounder. *Am J Physiol Regul Integr Comp Physiol* **300**, R1532–R1542.
31. Trevino JG, George SA, Hughes SJ, *et al.* (2008) An *in vivo* and *in vitro* assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol* **295**, 329–335.
32. Lansard M, Panserat S, Plagnes-Juan E, *et al.* (2010) Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* **39**, 801–810.
33. Lisbeth H, Tran Minh P, Torbj Rn T, *et al.* (2012) Effects of preslaughter stress on proteolytic enzyme activities and muscle quality of farmed Atlantic cod (*Gadus morhua*). *Food Chem* **134**, 1399–1408.
34. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
35. Yonar ME, Yonar SM & Silici S (2011) Protective effect of propolis against oxidative stress and immunosuppression induced by oxytetracycline in rainbow trout (*Oncorhynchus mykiss*, W.). *Fish Shellfish Immun* **31**, 318–325.
36. Aebi H (1984) Catalase *in vitro*. *Method Enzymol* **105**, 121–126.
37. Zhang X, Zhu Y, Cai L, *et al.* (2008) Effects of fasting on the meat quality and antioxidant defenses of market-size farmed large yellow croaker (*Pseudosciaena crocea*). *Aquaculture* **280**, 136–139.
38. Lushchak VI, Lushchak LP, Mota AA, *et al.* (2001) Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *Am J Physiol Regul Integr Comp Physiol* **280**, R100–R107.
39. Lora J, Alonso FJ, Segura JA, *et al.* (2004) Antisense glutaminase inhibition decreases glutathione antioxidant capacity and increases apoptosis in Ehrlich ascitic tumour cells. *FEBS J* **271**, 4298–4306.
40. Cheng H, Lin T, Yu K, *et al.* (2003) Antioxidant and free radical scavenging activities of *Terminalia chebula*. *Biol Pharm Bull* **26**, 1331–1335.
41. Jiang J, Wu X, Zhou X, *et al.* (2016) Effects of dietary curcumin supplementation on growth performance, intestinal digestive enzyme activities and antioxidant capacity of crucian carp *Carassius auratus*. *Aquaculture* **463**, 174–180.
42. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
43. Alam MS, Teshima S, Koshio S, *et al.* (2003) Optimum dietary threonine level for juvenile Japanese flounder *Paralichthys olivaceus*. *Asian Fisheries Science* **16**, 175–184.
44. Habte-Tsion H, Liu B, Ren M, *et al.* (2015) Dietary threonine requirement of juvenile blunt snout bream (*Megalobrama amblycephala*). *Aquaculture* **437**, 304–311.
45. Bureau DP, Azevedo PA, Tapia-Salazar M, *et al.* (2000) Pattern and cost of growth and nutrient deposition in fish and shrimp: potential implications and applications. *Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola* **19**, 111–140.
46. Sidransky H & Verney E (1967) Decreased protein synthesis in the skeletal muscle of rats force-fed a threonine-devoid diet. *BBA-Nucl Acids Prot Synth* **138**, 426–429.
47. Ciftci I & Ceylan N (2004) Effects of dietary threonine and crude protein on growth performance, carcase and meat composition of broiler chickens. *Br Poult Sci* **45**, 280–289.
48. Picha M, J Turano M, Tipsmark C, *et al.* (2008) Regulation of endocrine and paracrine sources of Igfs and Gh receptor during compensatory growth in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). *J Endocrinol* **199**, 81–94.
49. Mckay SJ, Trautner J, Smith MJ, *et al.* (2004) Evolution of duplicated growth hormone genes in autotetraploid salmonid fishes. *Genome* **47**, 714–723.
50. Baowei J, Xigui H, Bun CC, *et al.* (2006) The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. *J Mol Endocrinol* **36**, 23–40.
51. Saera-Vila A, Calduch-Giner J & Pérez-Sánchez J (2005) Duplication of growth hormone receptor (GHR) in fish genome: gene organization and transcriptional regulation of GHR type I and II in gilthead sea bream (*Sparus aurata*). *Gen Comp Endocr* **142**, 193–203.
52. Yuan X, Jiang X, Pu J, *et al.* (2011) Functional conservation and divergence of duplicated insulin-like growth factor 2 genes in grass carp (*Ctenopharyngodon idellus*). *Gene* **470**, 46–52.

53. Zou S, Kamei H, Modi Z, *et al.* (2009) Zebrafish IGF genes: gene duplication, conservation and divergence, and novel roles in midline and notochord development. *PLoS ONE* **4**, e7026.
54. Katsumata M, Kawakami S, Kaji Y, *et al.* (2016) Circulating levels of insulin-like growth factor-1 and associated binding proteins in plasma and mRNA expression in tissues of growing pigs on a low threonine diet. *Anim Sci* **79**, 101–109.
55. Takenaka A, Oki N, Takahashi S, *et al.* (2000) Dietary restriction of single essential amino acids reduces plasma insulin-like growth factor-I (IGF-I) but does not affect plasma IGF-binding protein-1 in rats. *J Nutr* **130**, 2910–2914.
56. Brameld JM, Gilmour RS & Buttery PJ (1999) Glucose and amino acids interact with hormones to control expression of insulin-like growth factor-I and growth hormone receptor mRNA in cultured pig hepatocytes. *J Nutr* **129**, 1298–1306.
57. Johnston IA (1999) Muscle development and growth: potential implications for flesh quality in fish. *Aquaculture* **177**, 99–115.
58. Coolican SA, Samuel DS, Ewton DZ, *et al.* (1997) The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J Biol Chem* **272**, 6653–6662.
59. Rius-Francino M, Acerete L, Jiménez-Amilburu V, *et al.* (2011) Differential effects on proliferation of GH and IGFs in sea bream (*Sparus aurata*) cultured myocytes. *Gen Comp Endocr* **172**, 44–49.
60. Montserrat N, Capilla E, Navarro I, *et al.* (2012) Metabolic effects of insulin and IGFs on gilthead sea bream (*Sparus aurata*) muscle cells. *Front Endocrinol* **3**, 55.
61. Bravo R, Frank R, Blundell PA, *et al.* (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. *Nature* **326**, 515–517.
62. Baserga R (1991) Growth regulation of the PCNA gene. *J Cell Sci* **98**, 433.
63. Yablonka-Reuveni Z & Rivera AJ (1994) Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol* **164**, 588–603.
64. Cornelison DDW, Olwin BB, Rudnicki MA, *et al.* (2000) MyoD$^{-/-}$ satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Dev Biol* **224**, 122–137.
65. Biga PR & Goetz FW (2006) Zebrafish and giant danio as models for muscle growth: determinate vs. indeterminate growth as determined by morphometric analysis. *Am J Physiol Regul Integr Comp Physiol* **291**, R1327–R1337.
66. Seiliez I, Sabin N & Gabillard JC (2012) Myostatin inhibits proliferation but not differentiation of trout myoblasts. *Mol Cell Endocrinol* **351**, 220–226.
67. Vélez EJ, Perelló M, Azizi S, *et al.* (2018) Recombinant bovine growth hormone (rBGH) enhances somatic growth by regulating the GH-IGF axis in fingerlings of gilthead sea bream (*Sparus aurata*). *Gen Comp Endocr* **257**, 192–202.
68. Gahr SA, Vallejo RL, Weber GM, *et al.* (2008) Effects of short-term growth hormone treatment on liver and muscle transcriptomes in rainbow trout (*Oncorhynchus mykiss*). *Physiol Genomics* **32**, 380–392.
69. Montserrat N, Sánchez-Gurmaches J, Serrana DGDL, *et al.* (2007) IGF-I binding and receptor signal transduction in primary cell culture of muscle cells of gilthead sea bream: changes throughout *in vitro* development. *Cell Tissue Res* **330**, 503–513.
70. Rommel C, Bodine SC, Clarke BA, *et al.* (2001) Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* **3**, 1009–1013.
71. Vélez EJ, Lutfi E, Jiménez-Amilburu V, *et al.* (2014) IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. *Gen Comp Endocr* **205**, 296–304.
72. Seiliez I, Gabillard JC, Skiba-Cassy S, *et al.* (2008) An *in vivo* and *in vitro* assessment of TOR signaling cascade in rainbow trout(*Oncorhynchus mykiss*). *Am J Physiol* **295**, 329–335.
73. Millward DJ, Garlick PJ, Nnanyelugo DO, *et al.* (1976) The relative importance of muscle protein synthesis and breakdown in the regulation of muscle mass. *Biochem J* **156**, 185–188.
74. Zhang P, Liang X, Shan T, *et al.* (2015) mTOR is necessary for proper satellite cell activity and skeletal muscle regeneration. *Biochem Biophys Res Commun* **463**, 102–108.
75. Rion N, Castets P, Lin S, *et al.* (2019) mTOR controls embryonic and adult myogenesis via mTORC1. *Development* **146**, dev172460.
76. Jiang J, Wu X, Zhou X, *et al.* (2016) Glutamate ameliorates copper-induced oxidative injury by regulating antioxidant defences in fish intestine. *Br J Nutr* **116**, 70–79.
77. Ardite E, Barbera JA, Roca J, *et al.* (2004) Glutathione depletion impairs myogenic differentiation of murine skeletal muscle C2C12 cells through sustained NF-κB activation. *Am J Pathol* **165**, 719–728.
78. Kozakowska M, Pietraszek-Gremplewicz K, Jozkowicz A, *et al.* (2015) The role of oxidative stress in skeletal muscle injury and regeneration: focus on antioxidant enzymes. *J Muscle Res Cell M* **36**, 377–393.
79. Martínez-Álvarez RM, Morales AE & Sanz A (2005) Antioxidant defenses in fish: biotic and abiotic factors. *Rev Fish Biol Fisher* **15**, 75–88.
80. Srikanth K, Pereira E, Duarte AC, *et al.* (2013) Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish – a review. *Environ Sci Pollut R* **20**, 2133–2149.
81. Hirzel E, Lindinger PW, Maseneni S, *et al.* (2013) Differential modulation of ROS signals and other mitochondrial parameters by the antioxidants MitoQ, resveratrol and curcumin in human adipocytes. *J Recept Sig Transd* **33**, 304–312.
82. Ma Q (2013) Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol* **53**, 401–426.
83. Giuliani ME & Regoli F (2014) Identification of the Nrf2–Keap1 pathway in the European eel *Anguilla anguilla*: role for a transcriptional regulation of antioxidant genes in aquatic organisms. *Aquat Toxicol* **150**, 117–123.
84. Jiang W, Liu Y, Jiang J, *et al.* (2015) Copper exposure induces toxicity to the antioxidant system via the destruction of Nrf2/ARE signaling and caspase-3-regulated DNA damage in fish muscle: amelioration by myo-inositol. *Aquat Toxicol* **159**, 245–255.
85. Kaspar JW, Niture SK & Jaiswal AK (2009) Nrf2: INrf2 (Keap1) signaling in oxidative stress. *Free Radical Bio Med* **47**, 1304–1309.