



Dietary Selenium Promotes Somatic Growth of Rainbow Trout (*Oncorhynchus mykiss*) by Accelerating the Hypertrophic Growth of White Muscle

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Abstract

As a nutritionally essential trace element, selenium (Se) is crucial for fish growth. However, the underlying mechanisms remain unclear. Fish somatic growth relies on the white muscle growth. This study aimed to explore the effects and underlying mechanisms of Se on fish white muscle growth using a juvenile rainbow trout (*Oncorhynchus mykiss*) model. Fish were fed a basal diet unsupplemented or supplemented with selenium yeast at nutritional dietary Se levels (2 and 4 mg/kg Se, respectively) for 30 days. Results showed that dietary Se supplementation significantly enhanced trout somatic growth. Histological and molecular analysis of trout white muscle tissues at the vent level showed that dietary Se supplementation elevated the total cross-sectional area of white muscle, mean diameter of white muscle fibers, protein content, nuclei number, and DNA content of individual muscle fiber, and suppressed the activities of calpain system and ubiquitin-proteasome pathway. Overall, this study demonstrated that dietary Se within the nutritional range inhibits calpain- and ubiquitin-mediated protein degradation and promotes the fusion of myoblasts into the existed muscle fibers to promote the hypertrophic growth of white muscle, thereby accelerating the somatic growth of rainbow trout. Our results provide a mechanistic insight into the regulatory role of Se in fish growth.

Keywords Selenium · Rainbow trout · White muscle growth · Hypertrophy · Proteolysis · Myogenesis

Introduction

Selenium (Se) is an essential trace element for fish [1, 2]. Its deficiency inevitably results in a variety of physiological dysfunctions in fish [3–6]. In intensive aquaculture systems, fish mainly obtain Se from the artificial feeds [1]. Optimal dietary Se level is crucial for fish to maintain the normal growth performance [1]. However, the underlying mechanisms are still unclear. White muscle, which represents 40–60% of fish body mass, is the largest tissue of fish [7, 8]. Numerous researches reported that white muscle growth makes a great

contribution to fish somatic growth [7]. Given that, we proposed a hypothesis whether dietary Se controls fish growth by regulating the white muscle growth.

Fish white muscle derives from the somites formed from the paraxial mesoderm in a stereotypic rostral to caudal progression, and its basic unit is muscle fiber which contains bundles of myofibrils [7]. Muscle formation, including development in embryonic phase and growth in postembryonic phase, is a complex dynamic process involving both the recruitment of new muscle fibers (hyperplasia) and the subsequent enlargement of existing fibers (hypertrophy) [7]. It is well known that two biological processes involve in the hyperplasia and hypertrophy of muscle fibers. Firstly, myoblasts which derive from stem cells undergo proliferation, differentiation, migration, and followed by fusion with each other to form new muscle fibers (hyperplasia), or fuse into the existing muscle fibers to enlarge their size (hypertrophy) [7]. Secondly, protein deposition, resulting from a rate of protein synthesis exceeds protein degradation, in the mature muscle fibers is another important approach for the hypertrophy of muscle fibers [7, 9]. In vertebrates, there is a huge difference in postembryonic growth pattern of skeletal muscle among species. In mammals, the postnatal muscle growth only

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involves the hypertrophy of muscle fibers while muscle hyperplasia ends soon after birth. Whereas in most fish, except small-size fish species such as zebrafish and medaka, skeletal muscle presents not only a hypertrophic growth but also a persistence of hyperplastic growth throughout the larval, juvenile, and even adult stages [10–12].

Rainbow trout (*Oncorhynchus mykiss*) is a commercially important fish species throughout the world and exhibits an indeterminate postembryonic growth pattern with a persistence of hyperplastic growth in skeletal muscle until around 40% of the maximum body size [13]. Postembryonic growth of trout white muscle has been well studied and proved to be sensitive to dietary nutritional strategies such as feed protein sources [14], dietary lipid content [15], availability of essential amino acids [16], vitamins [17], etc. However, limited attention has been paid to the effects of dietary Se on trout muscle growth.

Our previous study demonstrated that the optimal dietary Se supplemental level (based on Se-enriched yeast, Se-yeast) for rainbow trout growth is 4 mg/kg [18]. Based on the previous result, this study aimed to explore the effects of different supplemental levels of dietary Se (2 and 4 mg/kg as Se-yeast) on the mechanisms of white muscle growth in rainbow trout by using both cellular and molecular approaches.

Materials and Methods

The present experiment was carried out in accordance with the guidelines of The Scientific Ethic Committee of Huazhong Agricultural University, Wuhan, China.

Experimental Diet

Three experimental diets were prepared based on a basal diet unsupplemented or supplemented with 2 and 4 mg/kg Se as Se-yeast (Angel Yeast Co., Ltd., Hubei, China), and the measured total Se contents were 0.75, 2.60, and 4.68 mg/kg, respectively. Feed ingredients and proximate composition of the experimental diets are presented in supplementary Table 1, and diets were prepared as previously described [18].

Experimental Design and Feeding Trial

Juvenile immature rainbow trout (*O. mykiss*) were obtained from Enshi Guoxi Fishery Development Co., Ltd. (Hubei, China). After being transported to Huazhong Agricultural University, fish were kept in a flow-through rearing system supplied with clean water (0.5 l/min) under artificial photoperiods (12 h/12 h). Fish were fed basal diet for 2 weeks to acclimatize to laboratory conditions. Prior to the trial, 10 fish were randomly selected and the whole fish transverse sections with 0.3-cm width were cut at the vent level for histological characterization. Subsequently, a total of 630 fish (initial body

weight and length were 12.92 ± 2.29 g and 90.80 ± 1.75 mm, respectively) were individually weighed and divided into 9 plastic tanks (1000 l), with 70 fish in each tank. Experimental diets were randomly allocated in triplicate tanks. Fish were hand-fed to apparent satiation twice daily (09.00 and 16.00 h) for 30 days. During the experimental period, water temperature and dissolved oxygen were 18 ± 0.5 °C and 8.5 ± 0.2 mg/l, respectively.

At the end of the feeding trial, trout were fasted for 48 h. To follow growth and feed utilization, the final number, body weight, and body length of fish were measured. Three fish from each tank were randomly selected, anesthetized with tricaine methanesulfonate (100 mg/l water, Western Chemical, Inc., Ferndale, WA, USA) and killed by a sharp blow to the head. Two whole fish transverse sections with 0.3-cm width from the same fish were cut at the vent level for histological characterization and immunofluorescence assay, respectively. Three white muscle pellets (about 100 mg) under the dorsal fin and closed to the vertebra of each fish were collected, snap-frozen in liquid nitrogen, and then stored at -80 °C for enzymatic activity, mRNA, and protein analysis, respectively. The other dorsal muscles were minced and stored at -80 °C for the determination of muscle proximate composition and total Se content.

Growth and Feed Utilization Analysis

The following variables were evaluated:

$$\text{Survival rate (SR, \%)} = N_t/N_o \times 100$$

$$\text{Weight gain (WG, \%)} = (W_t - W_o)/W_o \times 100$$

$$\text{Specific gain rate (SGR, \%day}^{-1}\text{)} = \ln(W_t - W_o)/(T_t - T_o) \times 100$$

$$\text{Feed intake (FI, \%day}^{-1}\text{)} = 100 \times \text{dry feed intake (g)} / [1/2 \times (W_t + W_o) \times (T_t - T_o)]$$

$$\text{Feed efficiency (FE)} = \text{live weight gain (g)} / \text{dry feed intake (g)}$$

where N_t is the number of fish at the end of the experiment, N_o is the number of fish at the beginning of the experiment, W_t is the final body weight of fish, W_o is the initial body weight of fish, T_t is the end of the experiment (day), and T_o is the beginning of the experiment (day).

Histological Characterization

After fixed in paraformaldehyde solution (4%, w/v) and dehydrated with ethanol series, whole fish transverse sections located at the vent level were embedded in paraffin, cut transversely into 10- μ m thick sections, stained with hematoxylin and eosin [19], and scanned with a Panoramic 250 Flash digital microscope (3D HISTECH, Budapest, Hungary). The total cross-sectional area of white muscle, individual cross-sectional area of white muscle fibers, equivalent mean diameter of white fibers, and the total number of white muscle fibers

Table 1 Growth performance, nutrient utilization and somatic parameter of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days

Parameters	Dietary Se supplementation (mg/kg diet)		
	0	2	4
Initial body weight (g)	12.9 ± 0.014	12.9 ± 0.007	12.9 ± 0.012
Initial body length (mm)	91.3 ± 1.15	91.3 ± 1.53	90.7 ± 2.31
Final body weight (g)	52.8 ± 0.28 ^a	57.7 ± 0.29 ^b	62.1 ± 0.73 ^c
Final body length (mm)	145 ± 2.55 ^a	149 ± 2.27 ^{ab}	154 ± 2.17 ^b
WG (%)	309 ± 2.13 ^a	346 ± 2.29 ^b	381 ± 5.76 ^c
SGR (% day ⁻¹)	4.69 ± 0.02 ^a	4.99 ± 0.02 ^b	5.23 ± 0.04 ^c
FI (% day ⁻¹)	3.92 ± 0.13	3.95 ± 0.05	3.95 ± 0.19
FE	1.09 ± 0.03	1.09 ± 0.01	1.11 ± 0.05
SR (%)	100	100	100

WG, weight gain; SGR, specific gain rate; FI, feed intake; FE, feed efficiency; SR, survival rate

Values are represented as mean ± SD ($n = 3$ tanks per treatment). In each line, different superscript letters indicate significant differences among treatments ($P < 0.05$)

were measured following the method of Alami-Durante et al. [16]. The density of white muscle fibers was calculated according to Wang et al. [19]. The relative contribution of hyperplasia and hypertrophy to white muscle growth throughout the whole feeding trial was calculated as previously reported [20].

Proximate Composition and Total Se Content Analysis

Moisture, crude protein, crude lipid, and ash were analyzed as previously described [21]. Total Se content was measured as described by Fontagné-Dicharry et al. [22].

Extraction of Total RNA and DNA

Total RNA and DNA were extracted from 100-mg muscle samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After extraction, RNA and DNA pellets were resuspended in 40- μ L nuclease-free water and the concentrations were determined by NanoDrop ND-1000 (NanoDrop Technologies®, Wilmington, DE, USA). RNA samples were run on a 1.5% agarose gel and visualized on a Gel Doc EQ imaging system (BIO-RAD Laboratories, California, USA). Densitometric measurements of the 28S and 18S ribosomal RNA were performed with Image J software. Relative RNA/DNA ratio and ribosomal RNA (28S and 18S) were counted and normalized to fish fed the basal diet. Relative DNA/muscle fiber was counted as the ratio of DNA content divided by muscle fiber density and normalized to the data in fish fed the basal diet.

Quantitative Real-Time PCR (qPCR)

Complementary DNA synthesis and qPCR were conducted as previously described [18]. Specific primers for target genes are presented in supplementary Table 2. The relative quantification of the target gene was performed using the mathematical model described by Pfaffl [23] and normalized to the geometric mean of the best combination of eukaryotic translation elongation factor 1 alpha (*EF1 α*) and actin beta (*β -actin*) [24].

Western Blot Analysis

Muscle samples were homogenized and lysated in radio immunoprecipitation assay buffer, which was supplemented with protease inhibitor and phosphatase inhibitor, according to the manufacturer's instructions (Beyotime Biotechnology, Nanjing, China). Lysates were centrifuged (12,000g, 10 min, 4 °C) and the supernatant was removed for protein quantification by a BCA Protein Assay Kit (P0012S, Beyotime Biotechnology, Nanjing, China). Lysates (20 μ g of protein) were separated by SDS-PAGE, transferred to 0.45- μ m nitrocellulose membrane (Millipore Co., Bradford, MA, USA) for western blot analysis. Primary antibodies against ribosomal protein S6 (S6, Cat. no. 2217), phospho-S6 (p-S6 Ser^{235/Ser236}, Cat. no. 4856), eukaryotic initiation factor 4E binding protein (4E-BP1, Cat. no. 9452), phospho-4E-BP1 (p-4E-BP1 Thr^{37/46}, Cat. no. 9459), eukaryotic initiation factor 2 α -subunit (eIF2 α , Cat. no. 9722), and phospho-eIF2 α (p-eIF2 α Ser⁵¹, Cat. no. 9721) were purchased from Cell Signaling Technology (CST) Inc. which have been previously validated in rainbow trout [25, 26]. For primary antibodies against eukaryotic elongation factor 2 (eEF2, Cat. no. 2332, CST), phospho-eEF2 (p-eEF2 Thr⁵⁶, Cat. no. 2331, CST), and β -Tubulin (Cat. no. AC008, ABclonal, Wuhan, China), the amino acid sequences were monitored in SIGENAE database (<http://www.sigenaec.org>) to check for a good conservation of the antigen sequence. Subsequently, membranes were washed and incubated with an IRDye Infrared secondary antibody (LI-COR Inc. Biotechnology, Lincoln, NE, USA). Bands were visualized by infrared fluorescence using the Odyssey imaging system (LI-COR Inc. Biotechnology, Lincoln, NE, USA) and were quantified by Odyssey Infrared imaging system software (version 5.2; LI-COR Inc. Biotechnology, Lincoln, NE, USA). Results were normalized to the data in fish fed the basal diet.

Calpain Activity Analysis

Calpain activity of muscle samples was determined using a calpain activity assay kit according to the manufacturer's instructions (Biovision, Mountain View, CA, USA). Results were normalized to the data in fish fed the basal diet.

Immunofluorescence Assay

Immunofluorescence of whole fish transverse sections at the vent level was performed as previously described [27]. Briefly, muscle cryosections were fixed with 1% paraformaldehyde. After washing, sections were blocked using a PBS solution containing 1% heat-inactivated donkey serum, 1% bovine serum albumin, and 0.025% Tween 20. Sections were incubated with appropriate primary antibodies against forkhead box class O (FoxO) family member transcription factors 1 and 3a at 4 °C overnight, washed with PBS, and then incubated with fluorescent labeled secondary antibodies (Invitrogen Alexa-Fluor). After a 5-min wash with PBS, the sections were incubated with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI), and slides were mounted and sealed using Shandon Immu-Mount (Thermo Scientific, MA, USA) and glass coverslips. Fluorescent images were acquired with a Panoramic 250 Flash digital microscope (3D HISTECH, Budapest, Hungary).

Statistical Analysis

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Shapiro-Wilks and Levene's tests were performed to test the normal distribution and homogeneity of the variances, respectively. One-way analysis of variance (ANOVA) followed by Duncan's test were carried out for comparing different treatments when normal variables were analyzed. Data with non-normal distribution were subjected to the nonparametric statistical analysis on ranks (Kruskal-Wallis) followed by Dunn-Bonferroni post hoc test. Linear regression analysis was performed to plot dietary Se levels against fish growth and total cross-sectional area of white muscle. Correlations between data were examined using Pearson's correlation test. For all statistical analysis, the level of significance was set at $P < 0.05$.

Results

Growth Performance and Feed Utilization

Fish well accepted the experimental diets and no death was observed throughout the 30-day feeding trial (Table 1). Fish final body size (weight and length) and growth rates (WG and SGR) were significantly increased by dietary Se supplementation (Table 1, $P < 0.05$), and followed the dose responses to the elevated dietary Se levels (final body weight: $R^2 = 0.93$, $P < 0.0001$; final body length: $R^2 = 0.75$, $P = 0.0025$; WG: $R^2 = 0.94$, $P < 0.0001$; SGR: $R^2 = 0.94$, $P < 0.0001$; supplementary Fig. 1). No significant differences were found in feed intake and feed efficiency among groups (Table 1).

White Muscle Growth

The total cross-sectional area of trout white muscle at the vent level was significantly increased by dietary Se supplementation ($P < 0.05$) and followed a dose response to dietary Se level ($R^2 = 0.5226$, $P < 0.0001$; Fig. 1a, b). Additionally, it presented the significantly positive correlations with fish final body size (final body weight, $r = 0.7010$, $P < 0.0001$; final body length, $r = 0.4976$, $P = 0.0083$; Fig. 1c) and somatic growth rate (WG, $r = 0.9320$, $P = 0.0003$; SGR, $r = 0.9309$, $P = 0.0003$; Fig. 1d). Transverse sections of white muscle fibers at the vent level are presented in Fig. 1e. Fish fed Se supplemented diets showed higher percentages of large fibers (diameter $> 55 \mu\text{m}$) and lower percentages of small fibers (diameter $< 40 \mu\text{m}$) than those fed the basal diet (Fig. 1f, g). The total number of white muscle fibers was only significantly increased by diet supplemented with 4 mg/kg Se ($P < 0.05$, Fig. 1h), while the significantly larger mean diameter (Fig. 1i) and lower density (Fig. 1j) of white muscle fibers were observed in fish fed diets supplemented with both 2 and 4 mg/kg Se ($P < 0.05$). Although trout white muscle in all groups presented a high percentage of hyperplastic growth (contributes 53.94–56.56%) throughout the whole feeding trial period, an interesting phenomenon was observed that dietary Se supplementation resulted in a significantly declined percentage of hyperplastic growth and a significantly increased percentage of hypertrophic growth of rainbow trout white muscle ($P < 0.05$, Fig. 1k).

Proximate Composition and Total se Content of White Muscle

Diets supplemented with 2 and 4 mg/kg Se significantly increased the crude protein content and total Se content of white muscle in juvenile rainbow trout ($P < 0.05$), while exerted no significant effects on the content of moisture, crude lipid and ash (Table 2).

Protein Synthesis in White Muscle

Factors involve in the processes of protein synthesis including RNA/DNA, quantity of ribosomal RNA (28 S and 16 S), phosphorylation of ribosomal protein S6 on Ser^{235/236}, 4E-BP1 on Thr^{37/46}, eIF2 α on Ser⁵¹, and eEF2 on Thr⁵⁶ exhibited no significant differences among groups (Fig. 2).

Protein Degradation in White Muscle

At the transcription level, diets supplemented with both 2 and 4 mg/kg Se significantly decreased the expression of 6 genes related to autophagy-lysosome pathway, 1 gene related to calpain system, and 1 gene related to ubiquitin-proteasome pathway (Fig. 3a, $P < 0.05$). The autophagy-lysosome

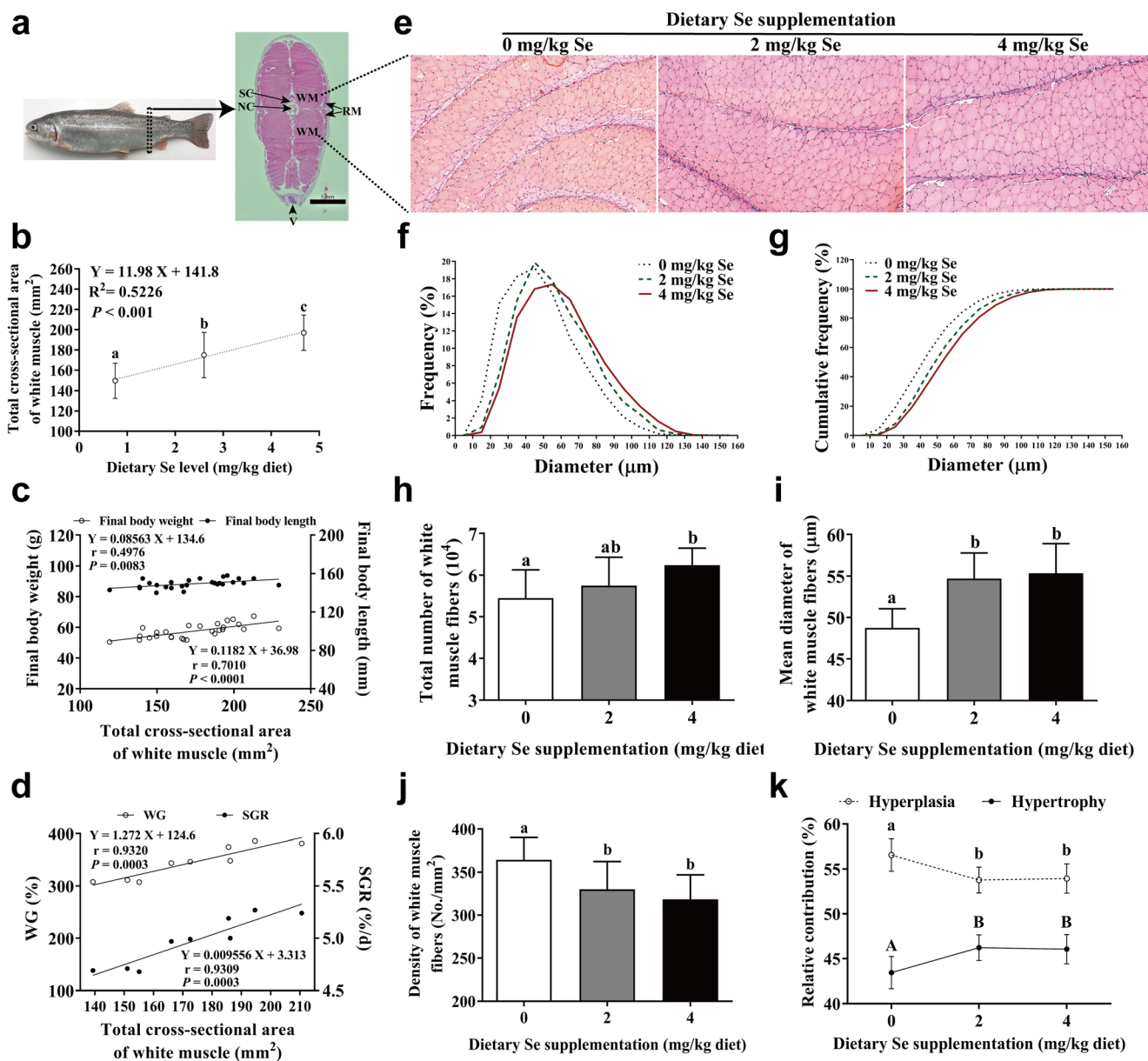


Fig. 1 White muscle growth in juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days. (a) Fish transverse sections at the vent level were stained with hematoxylin and eosin (SC: spinal cord; NC: notochord; WM: white muscle; RM: red muscle; V: vent). (b) Total cross sectional area of white muscle at the vent level ($n = 9$ fish per treatment). Pearson correlation analysis between the total cross sectional area of white muscle with (c) fish body size and (d) somatic growth rates

($n = 3$ tanks per treatment). (e) Images of transverse sections of muscle fibers for the analysis of (f) frequency, (g) cumulative frequency, (h) total number, (i) mean diameter and (j) density of white muscle fibers ($n = 9$ fish per treatment). (k) Relative contribution of hyperplasia and hypertrophy to white muscle growth throughout the feeding trial ($n = 9$ fish per treatment). Values are means \pm SDs. Different superscript letters (a, b, c or A, B, C) indicate significant differences among treatments ($P < 0.05$)

pathway-related genes are autophagy-related 4b and 12-like (*Atg4b* and *Atg12l*), microtubule-associated light chain 3B (*LC3B*), gamma-aminobutyric acid type A receptor-associated protein-like 1 (*Gabarapl1*), and cathepsin D and L (*CtsD* and *CtsL*). The calpain system-related gene is calpain 1 (*CAPN1*). The ubiquitin-proteasome pathway-related gene is muscle atrophy F-box 32 (*Fbx32*). Additionally, diets supplemented with both 2 and 4 mg/kg Se significantly increased

the expression of the long and short isoforms of calpastatin (*CAST-L* and *CAST-S*) (Fig. 3a, $P < 0.05$). The expression of cathepsin B (*CtsB*) and muscle atrophy F-box 25 (*Fbx25*) were only declined in fish fed diet supplemented with 4 mg/kg Se compared with the basal diet (Fig. 3a, $P < 0.05$). At the protein level, white muscle calpain activity (Fig. 3b) was significantly decreased by diet supplementation with 4 mg/kg Se while the abundance of ubiquitinated proteins

Table 2 Proximate composition (g/kg of wet weight) and total Se content (mg/kg of wet weight) of white muscle from juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days

Parameters	Dietary Se supplementation (mg/kg diet)		
	0	2	4
Moisture	758 ± 15.5	755 ± 14.5	751 ± 10.8
Crude protein	176 ± 8.62 ^a	186 ± 8.62 ^b	189 ± 6.58 ^b
Crude lipid	53.4 ± 11.8	58.5 ± 15.9	52.0 ± 15.4
Ash	12.6 ± 2.01	12.9 ± 0.84	13.4 ± 0.78
Total Se content	1.16 ± 0.09 ^a	1.53 ± 0.15 ^b	1.80 ± 0.26 ^c

Values are represented as mean ± SD ($n=9$ fish per treatment). In each line, different superscript letters indicate significant differences among treatments ($P < 0.05$)

(Fig. 3d) was significantly declined by diets supplemented with both 2 and 4 mg/kg Se ($P < 0.05$). However, the LC3-II/LC3-I ratio, a biomarker for activity of autophagy [28], presented no significant difference among groups (Fig. 3c). Immunofluorescence assay showed that dietary Se supplementation exerted no effects on the nuclear localization of FoxO1 (Fig. 3e), while decreased the nuclear localization of FoxO3a (Fig. 3f) in white muscle of juvenile rainbow trout ($P < 0.05$).

Expression of Genes Involving in Muscle Structure and Myogenesis

The ratio of nuclei/muscle fiber (Fig. 4a) and relative DNA content/muscle fiber (Fig. 4b) were significantly higher in fish fed diet supplemented with 4 mg/kg Se than those fed the basal diet ($P < 0.05$). Gene expression analysis showed that diets supplemented with 2 and 4 mg/kg Se significantly up-regulated the expression of genes coding fast myosin heavy chain (fMyHC), paired-homeobox transcription factor (Pax) 7a and 7b, and myocyte enhancer factor (MEF2c) ($P < 0.05$). Besides, the expression of gene coding myogenin (MyoG) was significantly increased by diet supplemented with 4 mg/kg Se (Fig. 4c, $P < 0.05$). No significant differences were observed in the expression of genes coding fast myosin light chain 2 (fMyLC2), proliferative cell nuclear antigen (PCNA), myocyte enhancer factor 2a (MEF2a), myoblast determination protein (MyoD), myogenic regulatory factor 4 (MRF4), myogenic factor 5 (Myf5), and myostatin (MSTN) 1a and 1b among groups (Fig. 4c).

Expression of Selenoprotein Genes in White Muscle

Expression of 11 selenoprotein genes coding glutathione peroxidase (GPX) 1a, 1b2, and 4b; selenoprotein (Sel) K, P, T1-

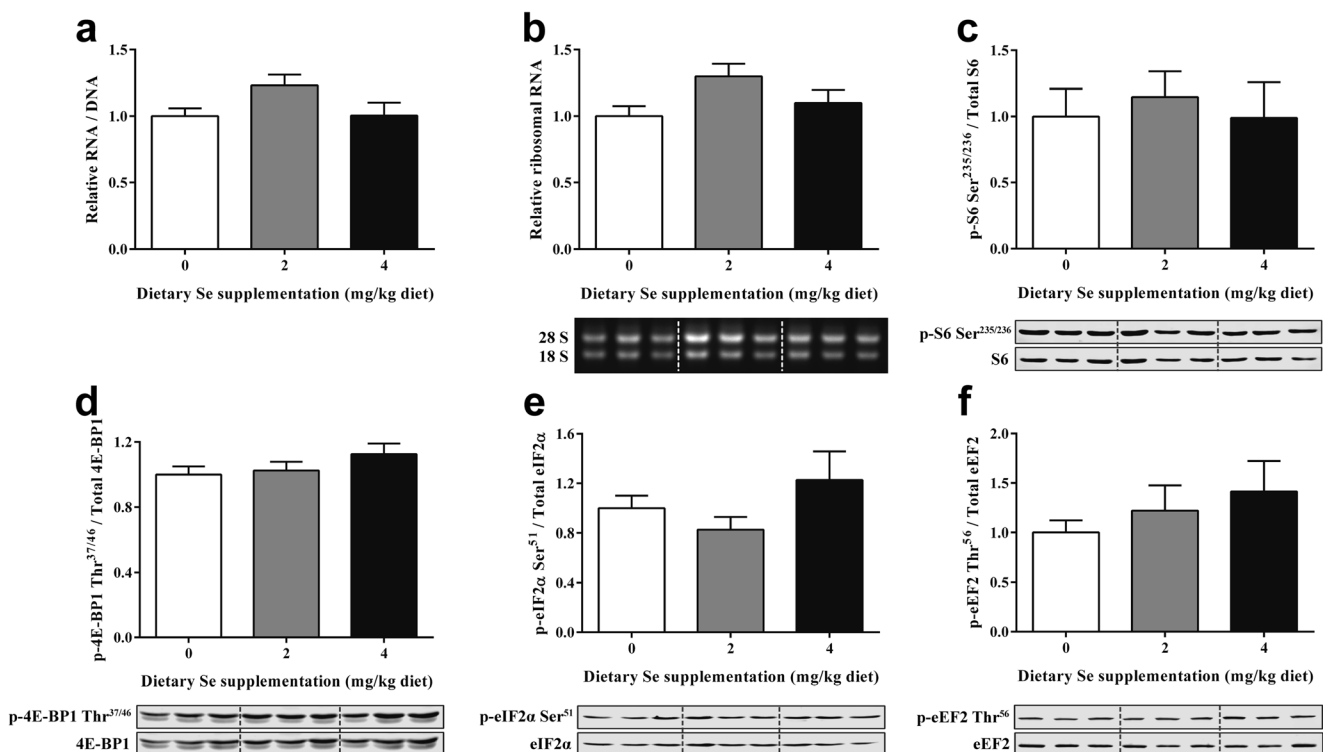


Fig. 2 Protein synthesis indexes in terms of (a) relative RNA/DNA ratio, (b) relative ribosomal RNA, and phosphorylation (p-) of (c) ribosomal protein S6 (S6) on Ser^{235/236}, (d) eukaryotic translation initiation factor 4E binding protein (4E-BP1) on Thr^{37/46}, (e) eukaryotic translation initiation factor 2 α -subunit (eIF2 α) on Ser⁵¹, and (f) eukaryotic

translation elongation factor 2 (eEF2) on Thr⁵⁶ in the white muscle of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days. Values are represented as mean ± SEM ($n=9$ fish per treatment)

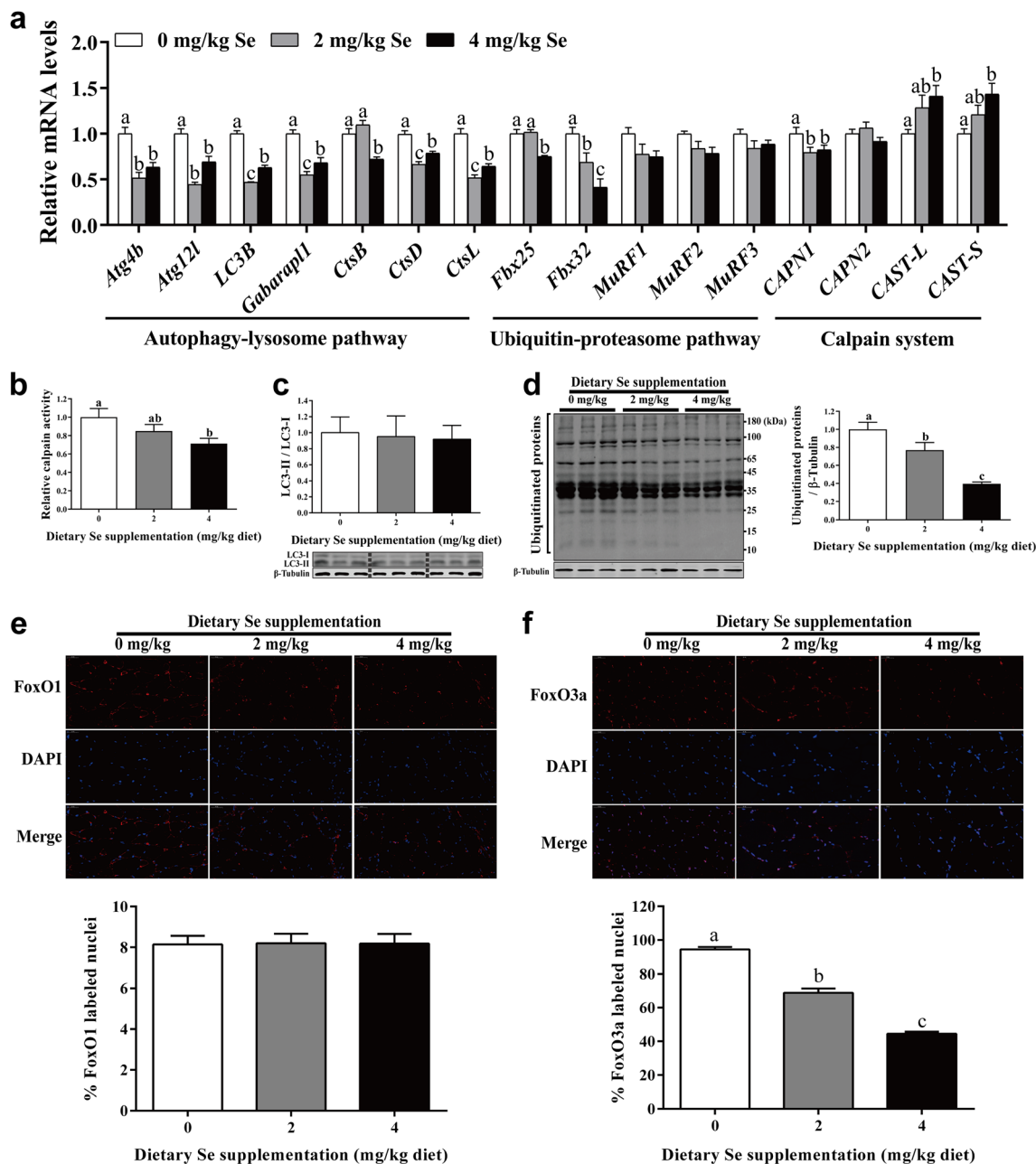


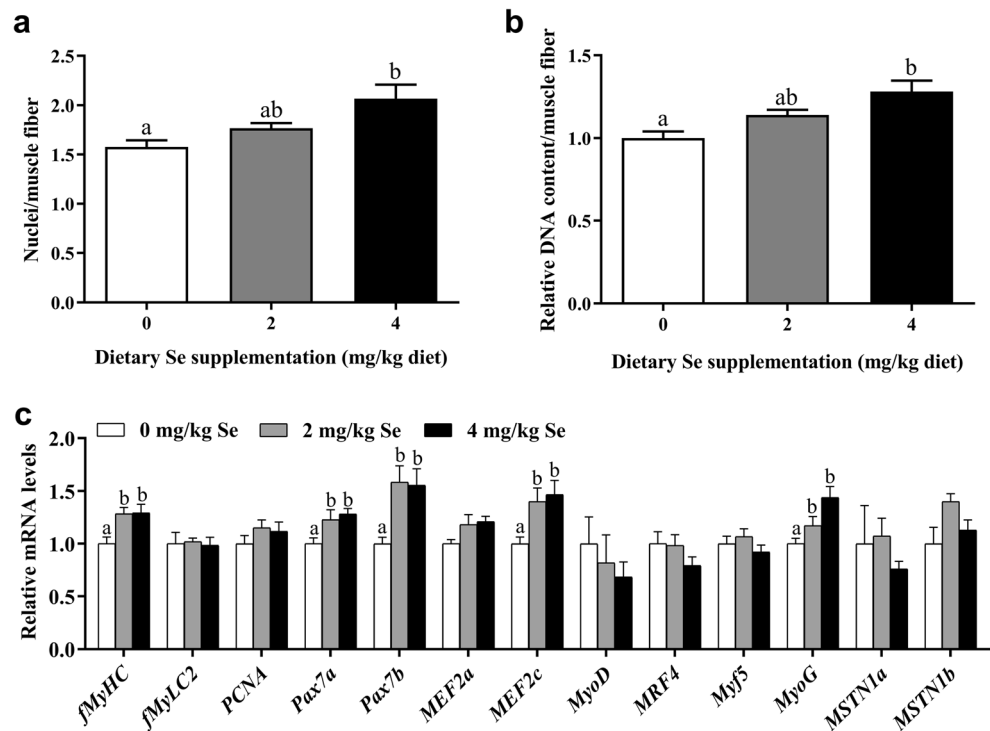
Fig. 3 Protein degradation indexes in terms of (a) expression of genes related to autophagy-lysosome pathway, ubiquitin-proteasome pathway and calpain system, (b) LC3-II/LC3-I ratio, (c) relative calpain activity, (d) abundance of ubiquitinated proteins, nuclear localization of (e) FoxO1 and (f) FoxO3a in the white muscle of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days. Values are represented as mean \pm SEM ($n = 9$ fish per treatment). Different superscript letters indicate

significant differences among treatments ($P < 0.05$). *Atg4b*, autophagy-related 4b; *Atg12l*, autophagy-related 12-like; *LC3B*, microtubule-associated light chain 3B; *Gabarapl1*, gamma-aminobutyric acid type A receptor-associated protein-like 1; *Cts*, cathepsin; *CAPN 1*, catalytic subunits of μ -calpain; *CAPN 2*, catalytic subunits of m-calpain; *CAST-L*, calpastatin long isoform; *CAST-S*, calpastatin short isoform; *Fbx*, F-box protein; *MuRF*, muscle RING finger; β -Tubulin, tubulin beta; FoxO, forkhead box class O family member protein

like, T2-like, U, and W-like; thioredoxin reductase 3b (TrxR3b); and methionine sulfoxide reductase B 1A-like (MsrB1A), which have been proved sensitive to dietary Se level by our previous research [18], have been analyzed. Among these selenoprotein genes, *GPX4b*, *SelK*, *SelP*, *SelU*, and *SelWl* were significantly upregulated by dietary Se

supplementation ($P < 0.05$, Fig. 5). Correlation analysis showed that the expression of *SelK* and *SelWl* were significantly positively correlated to the WG, SGR, total cross-sectional area of white muscle, relative contribution of hypertrophy and muscle protein content, and negative correlations with the relative contribution of hyperplasia, calpain activity,

Fig. 4 Quantitative data showing the (a) ratio of nuclei/muscle fiber, (b) relative DNA content/muscle fiber, and (c) expression of genes related to muscle structure and myogenesis in the white muscle of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days. Values are represented as mean \pm SEM ($n = 9$ fish per treatment). Different superscript letters indicate significant differences among treatments ($P < 0.05$). *fMyHC*, fast myosin heavy chain; *fMyLC2*, fast myosin light chain 2; *PCNA*, proliferative cell nuclear antigen; *Pax7*, paired-homeobox transcription factor 7; *MEF2*, myocyte enhancer factor 2; *MyoD*, myoblast determination protein; *MRF4*, myogenic regulatory factor 4; *Myf5*, myogenic factor 5; *MyoG*, myogenin; *MSTN*, myostatin



abundance of ubiquitinated proteins, FoxO3a-labeled nuclei, nuclei/muscle fiber, and relative DNA content/muscle fiber (Table 3).

Discussion

Optimal dietary Se level is crucial for fish growth [1]. Our previous study reported that rainbow trout reached the maximum growth rate when fed diet supplemented with 4 mg/kg Se as Se-yeast [18]. Based on this result, juvenile rainbow trout in the present study were fed a basal diet unsupplemented or supplemented with gradient levels of Se-yeast within nutritional level (≤ 4 mg/kg Se). Results showed that trout growth was enhanced by diets supplemented with both 2 and 4 mg/kg Se and followed a liner response to the increasing dietary Se level.

It is well known that fish somatic growth is largely determined by white muscle growth [7]. Thus, we further investigated the effects of dietary Se on white muscle growth in rainbow trout to attempt to give an insight into the regulatory mechanisms of Se in trout somatic growth.

The increase of total cross-sectional area of white muscle has been regarded as an indicator of fish muscle growth [15, 16]. Our results showed that the total cross-sectional area of trout white muscle at the vent level was increased by diets supplemented with both 2 and 4 mg/kg Se and followed a liner response to the increasing dietary Se level, reflecting an enhancement of nutritional levels of dietary Se on white muscle growth in rainbow trout. It is reported that the total cross-sectional area of fish white muscle increased at almost the same rate as fish somatic growth [29]. In the present study, the significantly positive correlations between the total cross-

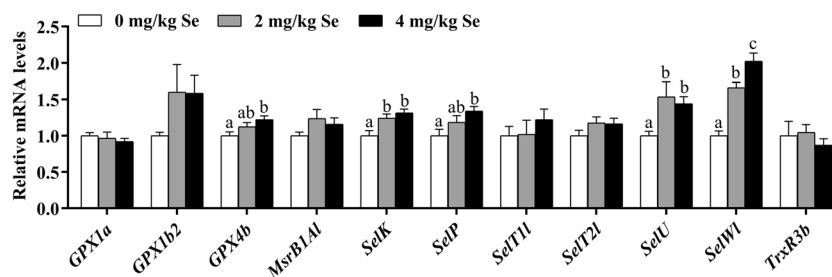


Fig. 5 Relative mRNA levels of selenoprotein genes in the white muscle of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days. Values are represented as mean \pm SEM ($n = 9$ fish per treatment). Different superscript letters indicate significant differences

among treatments ($P < 0.05$). *GPX*, glutathione peroxidase; *MsrB1A1*, methionine sulfoxide reductase B 1A-like; *Sel*, selenoprotein; *SelT1*, selenoproteins T1 like; *SelT2*, selenoproteins T2 like; *SelW1*, selenoproteins W like; *TrxR3b*, thioredoxin reductase 3

Table 3 Pearson correlation coefficients between the expression of selenoprotein genes in muscle and growth performance as well as muscle growth of juvenile rainbow trout (*Oncorhynchus mykiss*) fed a basal diet supplemented with or without 2 and 4 mg/kg Se (as Se-yeast) for 30 days

	<i>GPX4b</i>	<i>SelK</i>	<i>SelP</i>	<i>SelU</i>	<i>SelW-like</i>
WGR	0.878**	0.847**	0.810**	0.669*	0.963**
SGR	0.876**	0.851**	0.809**	0.683*	0.966**
Total cross-sectional area of white muscle	0.377	0.394*	0.332	0.187	0.627**
Relative contribution of hyperplasia	-0.318	-0.534**	-0.355	-0.386*	-0.576**
Relative contribution of hypertrophy	0.318	0.534**	0.355	0.386*	0.576**
Protein content	0.351	0.544**	0.415*	0.380	0.685**
Calpain activity	-0.424*	-0.496**	-0.417*	-0.250	-0.657**
Ubiquitinated proteins	-0.281	-0.390*	-0.326	-0.142	-0.626**
FoxO3a labeled nuclei	-0.856**	-0.8338**	-0.768*	-0.660	-0.960**
Nuclei/muscle fiber	0.334	0.387*	0.410*	0.169	0.538**
Relative DNA content/muscle fiber	0.329	0.333*	0.122	0.332	0.631**

WGR, weight gain rate; SGR, specific gain rate

Asterisks indicate significant correlation: * $P < 0.05$, ** $P < 0.01$

sectional area of white muscle and trout final body size (weight and length) as well as the somatic growth rate (including WG and SGR) have also been observed in trout fed increasing levels of dietary Se. These results strongly support our hypothesis that the improvement of somatic growth of rainbow trout by dietary Se is contributed to the promoted white muscle growth.

Postembryonic growth of fish muscle involves the hyperplasia and hypertrophy of muscle fibers [7]. Hyperplasia has been proved to be the major contributor (more than 50%) to muscle growth in rainbow trout with the body weight ranges from 0.1 to 200 g [30]. Be in line with the previous report, in the present study, trout white muscle presented a relatively higher percentage (53.94–56.56%) of hyperplastic growth than hypertrophic growth throughout the whole feeding trial. The total number and mean diameter of muscle fibers have been widely used as the indicators for hyperplasia and hypertrophy of muscle fibers, respectively [15, 16]. In the present study, the increased mean diameter of white muscle fibers was observed in trout fed diets supplemented with both 2 and 4 mg/kg Se while the increased total number of white muscle fibers was only observed in trout fed diet supplemented with 4 mg/kg Se. It suggests that, in trout white muscle, hypertrophy of muscle fiber is more sensitive to dietary Se than hyperplasia. This phenomenon could also be proved by the elevated contribution of hypertrophy and the declined contribution of hyperplasia to the growth of white muscle in trout fed diets supplemented with both 2 and 4 mg/kg Se. These results reveal that dietary Se primarily promotes the hypertrophy of muscle fibers to promote white muscle growth in rainbow trout.

Protein is the predominant component of muscle dry biomass; its deposition in muscle has been demonstrated an important approach for the hypertrophy of muscle fibers [7, 9]. It is reported that diets supplemented with 2–4 mg/kg Se from Se-yeast led to an increase of protein content in rainbow trout

muscle [21, 31]. Similar to the previous researches, the significantly higher muscle protein content has also been observed in trout fed diets supplemented with 2 and 4 mg/kg Se than those fed the basal diet, suggesting an enhancement of dietary Se on protein deposition in trout white muscle. Protein deposition depends on the balance of protein synthesis and degradation [7, 9]. Thus, protein synthesis and degradation in trout white muscle have been further analyzed.

Protein synthesis is a complex process of messenger RNA translation on the ribosomes [32]. It can be divided into multiple steps including the translation initiation, elongation, and termination [33]. The activation of translation initiation and elongation (translational activity) is crucial for protein synthesis [32]. In eukaryotic cells, translation initiation is primarily under the control of target of rapamycin complex 1 (TORC1) and eIF2 α -eIF2B pathways [34]. TORC1 is activated by a variety of hormones and nutrients, etc., thereby phosphorylating the downstream 4E-BP1 to drive the 5'-cap-dependent translation or phosphorylating ribosomal protein S6 to enhance translation initiation efficiency [34]. eIF2 α is phosphorylated by eIF2 α kinases under conditions of stress or starvation. Phosphorylation of eIF2 α suppresses the catalysis of eIF2B on the conversion of eIF2-GDP to eIF2-GTP, which is crucial for the round of translation initiation [34]. Eukaryotic translation elongation is regulated by various eukaryotic translation elongation factors (eEFs) [34]. Among them, eEF2 mediates the translocation of the ribosome to the next codon during translation [32]. Phosphorylation of eEF2 by its sole kinases dissociates it from ribosomes and inhibits the translation elongation [32]. In the present study, dietary Se supplementation exerted no influences on the phosphorylation of 4E-BP1, S6, eIF2 α , and eEF2, indicating no influences of dietary Se on translation initiation and elongation in rainbow trout white muscle. In addition to the translational activity, protein synthesis also relies on the ribosome biogenesis, a

determinant of translational capacity [35]. The ratio of RNA/DNA and the abundance of ribosomal RNA are considered accurate indexes of translational capacity and have been used for the evaluation of muscle protein synthesis [30, 36]. In this study, the translational capacity in rainbow trout white muscle was also not affected by dietary Se proved by the similar ratio of RNA/DNA and abundance of ribosomal RNA among groups. Collectively, dietary Se has no effects on protein synthesis in rainbow trout white muscle.

Protein degradation in fish muscle relies on three major proteolytic systems: calpain system, autophagy-lysosome pathway, and ubiquitin-proteasome pathway [7, 9]. Calpain cascade involves CAPN 1, CAPN 2 and their inhibitors, CAST-L, and CAST-S [37]. In the present study, although the expression of *CAPN 1* significantly declined in trout fed diets supplemented with both 2 and 4 mg/kg Se, the expression of *CAST-L* and *CAST-S* only increased in trout fed diet supplemented with 4 mg/kg Se. The combined effect of the declined expression of *CAPN 1* and the elevated expression of *CAST-L* and *CAST-S* led to a significantly decreased calpain activity in trout fed diet supplemented with 4 mg/kg Se. Protein degradation through ubiquitin-proteasome pathway relies on the selective attachment of ubiquitin molecules to the protein substrate by E3 ubiquitin ligases. Among these E3 ubiquitin ligases, Fbx32 (also called atrogenin-1) and MuRF1 have been studied in depth and shown to play the key roles in the control of skeletal muscle mass [38]. Our results showed that the expression of *MuRF1* exhibited no significant difference among groups, whereas, the expression of *Fbx32* significantly declined in trout fed diets supplemented with both 2 and 4 mg/kg Se accompanied by a significant decrease of ubiquitinated proteins. Transcription of *Fbx32* requires the nuclear localization of the FoxOs [38]. In the present study, the nuclear localization of FoxO3a significantly declined in trout fed diets supplemented with both 2 and 4 mg/kg Se, which could be a reason for the decrease of *Fbx32* expression. In autophagy-lysosome pathway, dietary Se significantly downregulated the expression of numerous autophagy-related genes; however, it exerted no significant influences on LC3-II/LC3-I ratio, a biomarker for activity of autophagy [28]. Collectively, these results suggest that dietary Se exerts a negative influence on protein degradation in trout white muscle by suppressing the calpain system and ubiquitin-proteasome pathway.

In addition to the protein deposition, fusion of myoblast into the mature muscle fibers is another important way for the hypertrophy of muscle fibers [7]. Fusion of myoblast into the existed muscle fibers leads to an increased nuclei number of the muscle fiber [7]. In the present study, the elevated nuclei/muscle fiber and DNA content/muscle fiber in white muscle of trout fed diet supplemented with 4 mg/kg Se indicate an acceleration of dietary Se on the fusion of myoblast into the mature muscle fibers. Myoblasts derive from muscle

stem cells and undergo proliferation, differentiation, and migration before fusion into the existing muscle fibers [7]. These processes are under the control of a suite of gene products including myogenic regulatory factors (MRFs), Paxs and MEFs, etc. [39]. Our results showed that diets supplemented with 2 and 4 mg/kg Se upregulated the expression of *Pax7a*, *Pax7b*, *MyoG*, and *MEF2c*. *Pax7* has been demonstrated highly expressed in both quiescent and activated muscle satellite cells [39, 40]. The elevated expression of *Pax7a* and *Pax7b* might indicate an improvement of dietary Se on the proliferation of muscle satellite cells in trout white muscle. *MyoG* and *MEF2c* have been reported crucial for the differentiation of myoblasts [40, 41]. The elevated expression of *MyoG* and *MEF2c* in rainbow trout white muscle indicates a positive influence of dietary Se on the differentiation of myoblasts, which could also be evidenced by the upregulated expression of *fMyHC*, a marker for the sarcomeric assembly in the late stages of myoblast differentiation [40]. The promoted proliferation of muscle satellite cells and differentiation of myoblasts might be the possible explanations for the promoted fusion of myoblast into the mature muscle fibers.

Se performs its biological functions mainly by incorporation into selenoproteins [42]. In the present study, dietary Se supplementation increased the expression of *GPX4b*, *SelK*, *SelP*, *SelU*, and *SelW* in trout white muscle. Correlation analysis showed that the expression of *SelK* and *SelW* were significantly correlated to all the indexes related to trout somatic growth, white muscle growth, white muscle protein deposition, and fusion of myoblasts into the existed muscle fibers. It was similar to our previous observation that expression of *SelK* and *SelW* exhibited the higher and more significant correlations with the rainbow trout growth than other selenoprotein genes [18]. These results reveal that *SelK* and *SelW* might be the key selenoproteins involving in the regulation of trout muscle growth. *SelW* has been proved to highly express in muscle tissues and important to maintain the metabolic stability in muscle [43]. Although limited information is available for the functions of *SelK* in animal muscle, the aberrant expression of *SelK* has been reported to lead to the dysfunction in chicken muscles [44] and the apoptosis of chicken myoblasts [45].

Conclusion

This study illustrated that dietary Se within the nutritional range promotes the hypertrophic growth of trout white muscle by upregulating the expression of selenoproteins, in particular *SelK* and *SelW*, which (1) inhibit the calpain system and ubiquitin-proteasome pathway-mediated protein degradation and (2) promote the fusion of myoblasts into existed muscle fibers in trout muscle. Finally, the enhanced hypertrophic growth of white muscle leads to the improvement of trout

somatic growth. The present results reveal a possible regulatory mechanism of dietary Se on fish growth, and could help to better understand the physiological functions of Se in fish.

Availability of Data and Material The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval This study was approved by The Scientific Ethic Committee of Huazhong Agricultural University (no. HZAUF1-2018-017).

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