

Research Article

Effects of Phosphatidic Acid on Mammalian Target of Rapamycin (mTOR) and Metabolic Pathways in Bivalve Mollusk *Sinonovacula constricta*

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It is of great significance for bivalve aquaculture to promote the growth through nutritional strategy. Phosphatidic acid (PA) is a potential growth-promoting nutraceutical that targets for mammalian target of rapamycin (mTOR) in vertebrates, but its role in invertebrates remains largely unknown. Here, the effects of PA on mTOR and metabolic pathways in bivalve mollusk *Sinonovacula constricta* were determined by intramuscular injection with di-18:2 PA and di-16:0 PA. PA (both di-18:2 and di-16:0 PA) was found to increase the glycogen concentration in the muscle of *S. constricta*. Di-16:0 PA decreased the triglyceride concentration from 0.143 ± 0.04 mmol/g protein to 0.040 ± 0.018 mmol/g protein. The concentration of Asp, Glu, Ala, Cys, Val, Met, Ile, Leu, Phe, Arg, and Pro was reduced by di-18:2 PA or/and di-16:0 PA. PA increased the mRNA level of mTOR and the phosphorylation levels of eIF4E binding protein 1 and p70S6 kinase 1. Furthermore, PA decreased the protein level of microtubule-associated protein 1 light chain 3 II/I and the mRNA level of AMP-activated protein kinase. The mRNA expressions of two key enzymes of glycolysis (pyruvate kinase and glucokinase) were also upregulated by both PA, while the mRNA level of glucose transporter 1 was increased by di-18:2 PA. Di-16:0 PA decreased the mRNA level of phosphoenolpyruvate carboxykinase. The mRNA levels of sterol responsive element binding protein, fatty acid synthase, and acetyl-CoA carboxylase were increased by both PA. The mRNA level of stearoyl-CoA desaturase was increased by di-18:2 PA. Both PA species increased the mRNA levels of key enzymes involved in tricarboxylic acid cycle (citrate synthase and NADP-isocitrate dehydrogenase). Our results indicated that PA activated mTOR signaling pathway, subsequently leading to the increase of anabolism and the inhibition of catabolism in *S. constricta*.

1. Introduction

Increasing or promoting growth is an important target for bivalve nutrition research. Growth in terms of biomass depends on the accumulation of proteins and lipids, and the redirection of nutrients from catabolism to anabolism [1]. A key regulator of growth from yeast to mammals is the mammalian target of rapamycin (mTOR), which promotes anabolic processes, including protein and lipid synthesis, and suppresses catabolic processes, such as autophagy [2]. First, mTOR stimulates protein synthesis mainly through eIF4E

binding protein 1 (4EBP1) and p70S6 kinase 1 (S6K1). The activation of 4EBP1 triggers 5'cap-dependent mRNA translation, while S6K1 enhances the translation efficiency of spliced mRNAs. Second, mTOR drives *de novo* lipid synthesis through the sterol responsive element binding protein (SREBP), which activates the transcription of genes involved in lipid biosynthesis. Third, mTOR also facilitates growth by promoting a shift in glucose metabolism from oxidative phosphorylation to glycolysis, which likely enhances the incorporation of nutrients into new biomass. Apart from these anabolic processes, mTOR also promotes cell growth by suppressing protein catabolism,

most notably autophagy. An important early step of autophagy is the activation of Unc-51-like kinases 1 (ULK1), a kinase that drives autophagosome formation [3]. mTOR phosphorylates ULK1, thereby disrupting the interaction between ULK1 and AMP activated protein kinase (AMPK, a known activator of autophagy) [4].

In bivalve, genes involved in protein synthesis are key regulators of growth [5, 6]. For example, the expression of ribosomal proteins shows linear relationships with growth rates in bivalve larvae (*Crassostrea gigas*) [5]. Previous studies have demonstrated that mTOR regulates autophagy in response to the environmental contaminants in mussels (*Mytilus galloprovincialis* Lam.) [7, 8]. Recently, we found that mTOR plays a conserved role in controlling protein synthesis, glucose metabolism, lipid synthesis, and autophagy in razor clam (*Sinonovacula constricta*) [9]. Thus, mTOR may be an attractive target for promoting bivalve growth.

Phosphatidic acid (PA), a diacyl-glycerophospholipid, is a precursor of membrane phospholipid and triglyceride synthesis and can serve as a signaling lipid. PA is implicated in the control of cell cycle progression and cell growth [10]. Most significantly, PA can directly activate mTOR signaling via binding to the FKBP12-rapamycin binding (FRB) domain of mTOR [11]. It has been shown that exogenous PA from egg or soy lecithin can activate mTOR signaling and stimulate muscle protein synthesis in mammals [12, 13]. Feeding PA improves feed conversion ratio and increases live bird weight and breast fillet weight in broilers [14]. Exogenous PA improves the growth performance of turbot [15] and mechanically PA activates mTOR signaling, increases protein synthesis, lipogenesis, and glycolysis, and stimulates primary muscle cell proliferation in turbot [16]. Given that PA is a potential growth promoting supplement that can be incorporated in artificial diet of bivalves, this study is particularly interested in examining the effects of PA on mTOR and its metabolic pathways in bivalve species such as razor clam, which is an important mariculture bivalve that widely distributed in China, Japan, and Korea.

2. Materials and Methods

2.1. Animals and Experimental Treatments. The procedures for animal care and handling described herein were approved by Ningbo University's Institutional Animal Care and Use Committee (protocol# 2022-055). Adult *S. constricta* (shell length, 40–45 mm) collected from the intertidal zone at Sanmen Bay, Ningbo, China. The clams were maintained in an aerated aquarium (100 L) for 7-days starvation prior to the experiments. The seawater was filtered and maintained at 22–25°C, and the salinity was 20%–21%. A pilot experiment was conducted, and the dosage and timing of PA administration were determined per the phosphorylation levels of 4EBP1 (Thr^{36/47}). The clams that received intramuscularly with 200 μM di-18:2 PA (Cat No. L130340, Aladdin) after 6 hr showed a higher level of 4EBP1 phosphorylation (Figure S1). At the beginning of the experiment, one group of clams (30 clams) was uninjected while three

groups of clams were injected intramuscularly with, i.e., solvent control (0.02% dimethyl sulfoxide (DMSO); 30 clams, 50 μL/clam); 200 μM di-18:2 PA (200 μM di-18:2 PA resolved in DMSO; 30 clams, 50 μL/clam); 200 μM di-16:0 PA (Cat No. D274831, Aladdin, 200 μM di-16:0 PA resolved in DMSO; 30 clams, 50 μL/clam). After 6 hr of treatment, muscles were rapidly removed, pooled into tubes, chilled in liquid nitrogen, and stored at 80°C.

2.2. Biochemical Assays. Muscle glycogen concentration was tested according to the instructions of the Muscle Glycogen Assay Kit (Nanjing Jiancheng, China). In brief, lye (saline) was added to the muscle samples, boiled for 20 min, then cooled to glycogen hydrolysis solution. Glycogen test solution was prepared by adding appropriate amount of distilled water according to muscle weight. Distilled water, glycogen detection solution, and color developing solution were added into the tube to be tested, and then heated in boiling water for 5 min. After cooling, the absorbance at 620 nm was measured by an enzyme marker. Glycogen content was calculated based on the glycogen standard curve.

Triglyceride levels were measured using the TG kit (Nanjing Jiancheng, China) according to the manufacturer's instructions. In brief, samples were homogenized and followed by centrifugation at 2,500 rpm for 10 min, and the supernatant was taken to be measured. Then, 250 μL working solution was added to the supernatant and incubated at 37°C for 10 min. Triglyceride levels were determined using a microplate reader at 500 nm.

Amino acid content was determined using the L-8900 amino acid analyzer (Hitachi, Tokyo, Japan). In brief, samples were freeze-dried and hydrolyzed with HCL (6 mol/L) at 110°C for 24 hr. After cooling, the volume was fixed, dry, and then dissolved by 0.02 mol/L HCL. Finally, the amino acid content of the samples was determined by aqueous filtration membrane.

2.3. Real-Time RT-PCR. Muscle RNA was obtained using TRIzol (Invitrogen, USA) per the manufacturer's recommendations. Total RNA concentration and purity were analyzed using a NanoDrop[®] ND-1000 (NanoDrop, USA), and 1 μg of cDNA was synthesized with a commercial PrimeScript[™] RT Reagent Kit (Takara, Japan) following the manufacturer's instructions. Real-time RT-PCR was conducted with gene specific primers (Table 1). Real-time RT-PCR was carried out in a 20 μL reaction system containing 1 μL of first-strand cDNA, 10 μL of SYBR Premix Ex Taq (TliRNase Plus) (TaKaRa, Japan), 5 pmol each of forward and reverse primers, and 5 μL of nuclease-free water. Cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 60°C for 20 s, and extensions at 72°C for 20 s, followed by melting curve analysis of the PCR product. The relative gene expression levels were analyzed by the 2^{-ΔΔCT} method [17], and data were normalized to the expression of β-actin.

2.4. Western Blotting. The muscle samples were lysed in RIPA buffer (Cowin Biotech, China) with protease (Cowin

TABLE 1: Primers for real-time RT-PCR.

Target gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
mTOR	CAGCGGTCTGGTAATGTGAAGG	TAGCAACTTAGGTGACACATACTGG
AMPK	ACCACAGGCACCTCAGTAAACA	AGGATGCGTGCGTGAAGTTA
PK	TCGTGTAATGGCAATAATCG	GTAGAAGCATCGTTCAAGTC
GK	GTTCCGCCGTTTATGCTTGG	CAAGTCCAGGGCGAGAAAGT
GLUT1	CGTTATCCTCGTCGCTTCCA	CCACCATTGCTTCTGTTGGC
G6P	CCCTCGTCTGTCTGGCATT	CAGCATCCCTGTACACAGCA
PEPCK	GGGAGGACAAGAAGGGAGT	ATTGTATCCCATGAAAGGTCTC
SREBP	GCTCCTACTCTGTTATCCGATTGTG	TCCTGAGACTGGCGAGATCCT
FAS	CTATGTGCGAGGCCCATGGTACT	CCGAGTGACCCATGTTTCGAT
ACC	TGGATGGCAATGTTGATGA	GGCACTGATGGTAGAGAAG
SCD	CACCGCATCCCCGAAAAATC	AGGCGCAAATTATGGTTGCC
CS	CAGTTCAGTGCTGCCATA	CAAGTTACGGTAGATGATAGAC
NADP	ATGTTGCTAAGGATGTTACC	TTAGGAGATGGACTGTTCTT
β -Actin	CCATCTACGAAGGTTACGCC	TCGTAGTGAAGGAGTAGCCTCTTT

Biotech, China) and phosphatase inhibitors (Thermo Fisher Scientific, USA). Protein concentrations were determined using a BCA Protein Assay Kit (Sangon Biotech, China). Lysates (10 μ g of total protein per lane) were separated by denaturing SDS-PAGE, transferred onto a PVDF membrane and blocked with 5% nonfat milk. Membranes were incubated with antibodies (4°C overnight) against β -actin (Cat No. EM21002, HuaBio, 1:10,000), phosphor-4EBP1 (Thr^{37/46}) (Cat No. 236B4, Cell Signal Technology, 1:1,000), phosphor-p70S6K (Thr³⁸⁹) (Cat No. 28735-1-AP, Proteintech, 1:2,000), LC3A/B (Cat No. 4108, Cell Signal Technology, 1:3,000) and visualized with the corresponding secondary antibody: anti-mouse or antirabbit (Beyotime Biotechnology, China). The target protein was measured by a chemiluminescence imager (ChemiScope Capture Image System).

2.5. Statistical Analysis. All data were expressed as mean \pm SEMs. The density of protein bands was quantified by Image J software (NIH, USA). Difference between control and PA treatment group was analyzed by *t*-tests using SPSS 16.0 for Windows (SPSS Inc, USA), and *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. PA Influenced Glycogen, Triglyceride, and Amino Acid Concentrations in Muscle. The glycogen concentration in the muscle of razor clam was significantly increased by PA (Figure 1). The glycogen concentration was increased from 9 ± 1.12 to 15.6 ± 1.59 mg/g or 14.2 ± 2.30 mg/g after clam injected intramuscularly with 200 μ M di-18:2 PA or di-16:0 PA for 6 hr. Di-16:0 PA significantly decreased the triglyceride concentration from 0.143 ± 0.04 mmol/g protein to 0.040 ± 0.018 mmol/g protein, while di-18:2 PA did not affect the triglyceride concentration (Figure 2). The concentration of Asp, Glu, Val, and Arg in the muscle of clam was significantly reduced by di-18:2 PA and di-16:0 PA (Table 2). The concentration of Ala, Cys, Met, Ile, and Pro was reduced by di-18:2 PA, while the concentration of Leu and Phe was reduced by di-16:0 PA (Table 2).

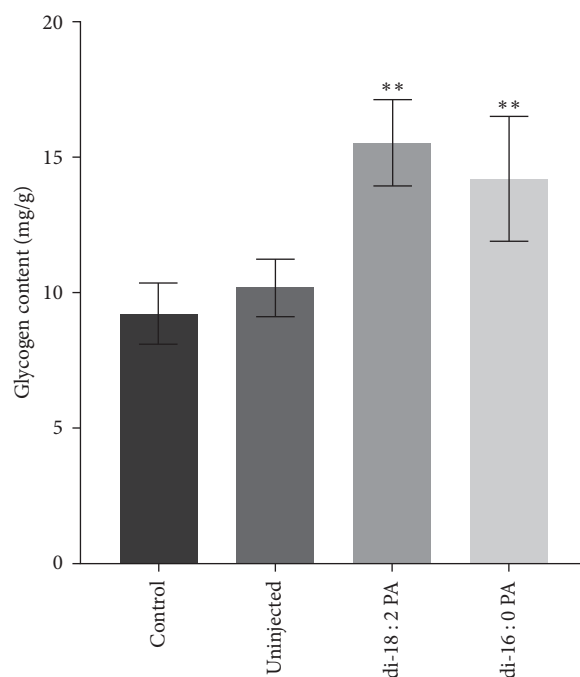


FIGURE 1: Effect of phosphatidic acid on the glycogen concentration in the muscle of *Simonovacula constricta*. Values are means \pm SEMs ($n=6$). Difference between control and PA treatment group was analyzed by *t*-tests (* $P<0.05$, ** $P<0.01$).

3.2. PA Activated the mTOR Signaling Pathway. The mRNA level of mTOR was significantly upregulated by di-18:2 PA and di-16:0 PA (Figure 3(a)). The phosphorylation levels of 4EBP1 and p70S6K were significantly increased by di-18:2 PA and di-16:0 PA, but to a lesser extent than di-18:2 PA (Figures 3(b) and 3(c)).

3.3. PA Suppressed Autophagy. The protein level of microtubule-associated protein 1 light chain 3 II/I (LC3-II/I) was significantly reduced by di-18:2 PA, and by di-16:0 PA, but to a lesser extent than di-18:2 PA (Figure 4(a)). The mRNA level of AMPK was significantly decreased by di-18:2 PA, but not by di-16:0 PA (Figure 4b).

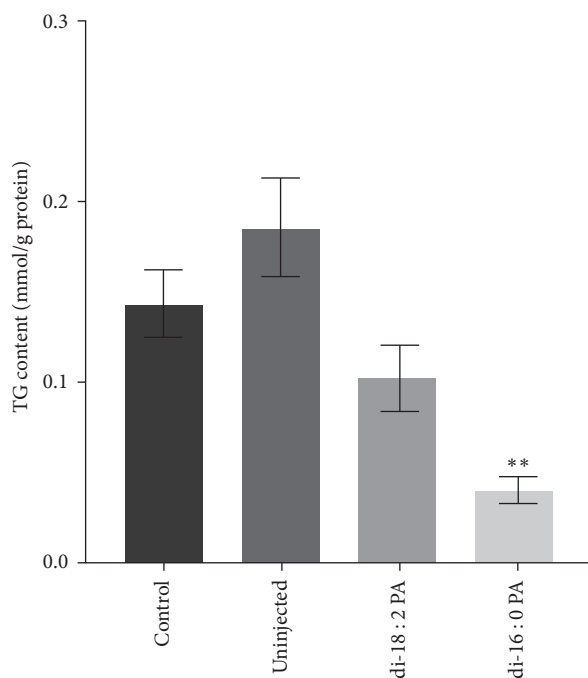


FIGURE 2: Effect of phosphatidic acid on the triglyceride concentration in the muscle of *Sinonovacula constricta*. Values are means \pm SEMs ($n = 6$). Difference between control and PA treatment group was analyzed by t -tests (* $P < 0.05$, ** $P < 0.01$). TG, triglyceride.

TABLE 2: Effect of phosphatidic acid on the amino acid concentration (%) in the muscle of *Sinonovacula constricta*.

Amino acid	Control	Uninjected	di-18:2 PA	di-16:0 PA
Asp	65.92 \pm 0.41	65.51 \pm 0.62	63.08 \pm 1.24*	62.72 \pm 0.48*
Thr	31.98 \pm 0.52	31.64 \pm 0.29	30.33 \pm 0.60	30.80 \pm 0.41
Ser	31.33 \pm 0.62	31.19 \pm 0.67	31.65 \pm 0.55	31.37 \pm 0.46
Glu	96.56 \pm 0.66	96.30 \pm 0.45	91.87 \pm 0.76*	91.91 \pm 0.57*
Gly	38.66 \pm 0.93	37.03 \pm 1.38	38.30 \pm 1.82	36.74 \pm 1.63
Ala	123.60 \pm 1.30	124.80 \pm 2.21	116.40 \pm 2.04*	120.60 \pm 2.16
Cys	5.18 \pm 0.16	5.43 \pm 0.28	4.29 \pm 0.34*	5.20 \pm 0.15
Val	31.06 \pm 0.25	30.82 \pm 0.25	29.01 \pm 0.23*	29.50 \pm 0.21*
Met	17.16 \pm 0.24	17.33 \pm 0.29	14.42 \pm 1.36*	17.12 \pm 0.13
Ile	32.39 \pm 0.33	32.25 \pm 0.08	30.47 \pm 0.32*	30.95 \pm 0.18
Leu	66.74 \pm 0.43	65.95 \pm 0.26	55.66 \pm 6.85	64.45 \pm 0.45*
Tyr	19.33 \pm 0.31	19.36 \pm 0.47	16.05 \pm 1.73	19.08 \pm 0.29
Phe	22.32 \pm 0.19	22.38 \pm 0.31	19.49 \pm 1.79	21.55 \pm 0.14*
Lys	51.58 \pm 0.53	51.65 \pm 0.28	50.39 \pm 0.58	51.14 \pm 0.44
His	14.14 \pm 0.12	14.29 \pm 0.15	13.76 \pm 0.16	13.93 \pm 0.11
Arg	55.31 \pm 1.26	53.56 \pm 1.63	51.05 \pm 0.89*	50.98 \pm 0.92*
Pro	8.02 \pm 0.06	7.95 \pm 0.15	7.54 \pm 0.01*	8.054 \pm 0.09

Values are means \pm SEMs ($n = 3$). * $P < 0.05$.

3.4. PA-Modulated Glucose Metabolism, Lipid Synthesis, and Tricarboxylic Acid Cycle. The mRNA levels of pyruvate kinase (PK) and glucokinase (GK) were significantly upregulated by di-18:2 PA and di-16:0 PA (Figures 5(a) and 5(b)). The mRNA level of glucose transporter 1 (GLUT1) was significantly increased by di-18:2 PA, while di-16:0 PA did not influence GLUT1 mRNA abundance (Figure 5(c)). Di-16:0 PA significantly decreased the mRNA level of phosphoenolpyruvate

carboxykinase (PEPCK) compared with control, while di-18:2 PA did not affect the mRNA level of PEPCK (Figure 5(d)). There was no effect of PA on the mRNA level of glucose-6-phosphatase (G6Pase) mRNA (Figure 5(e)).

The mRNA levels of SREBP, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) were significantly increased by di-18:2 PA and by di-16:0 PA (Figure 6(a)–6(c)). The mRNA level of stearoyl-CoA desaturase (SCD) was significantly

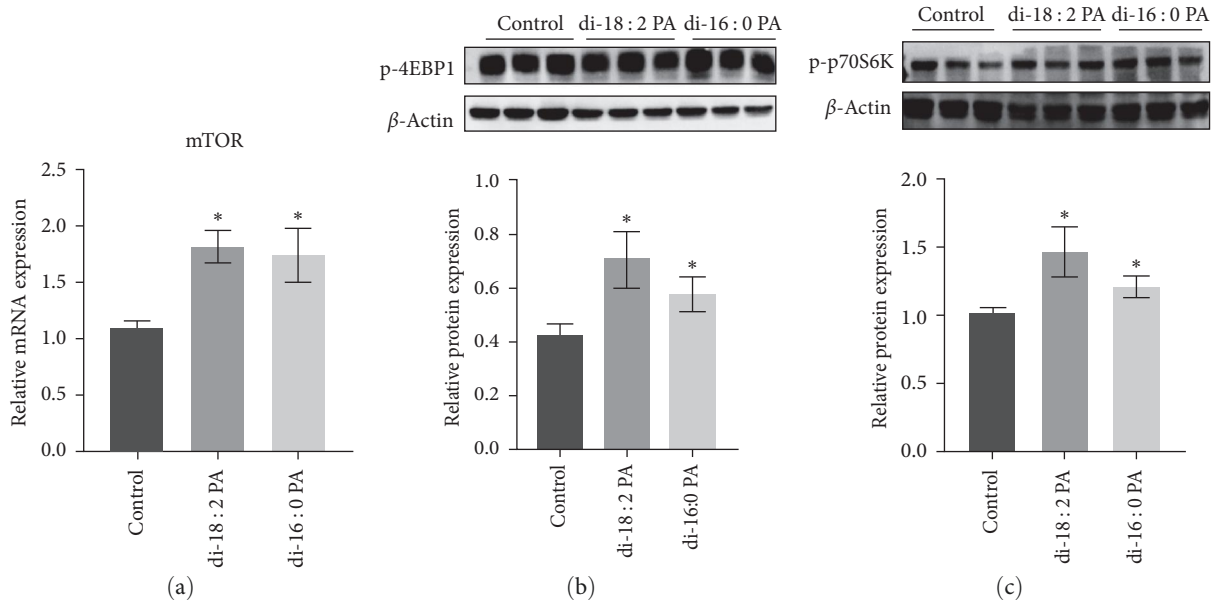


FIGURE 3: Effect of phosphatidic acid on the mTOR signaling pathway in the muscle of *Sinonovacula constricta*. Densitometry was quantified by ImageJ software. Values are means \pm SEMs ($n = 3$). Difference between control and PA treatment group was analyzed by *t*-tests (* $P < 0.05$). mTOR, mammalian target of rapamycin; 4EBP1, eIF4E binding protein 1; p70S6K, p70S6 kinase 1.

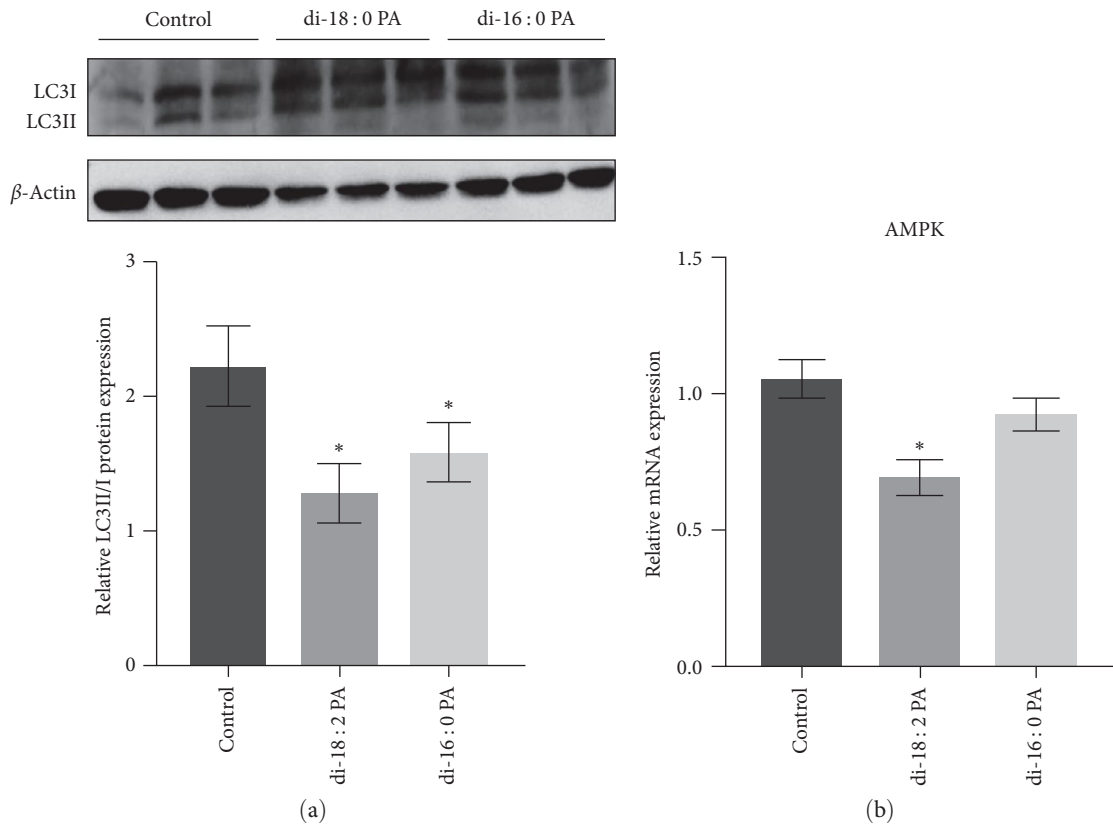


FIGURE 4: Effect of phosphatidic acid on the marker of autophagy in the muscle of *Sinonovacula constricta*. Densitometry was quantified by ImageJ software. Values are means \pm SEMs ($n = 3$). Difference between control and PA treatment group was analyzed by *t*-tests (* $P < 0.05$). LC3, microtubule-associated protein 1 light chain 3 and AMPK, AMP activated protein kinase.

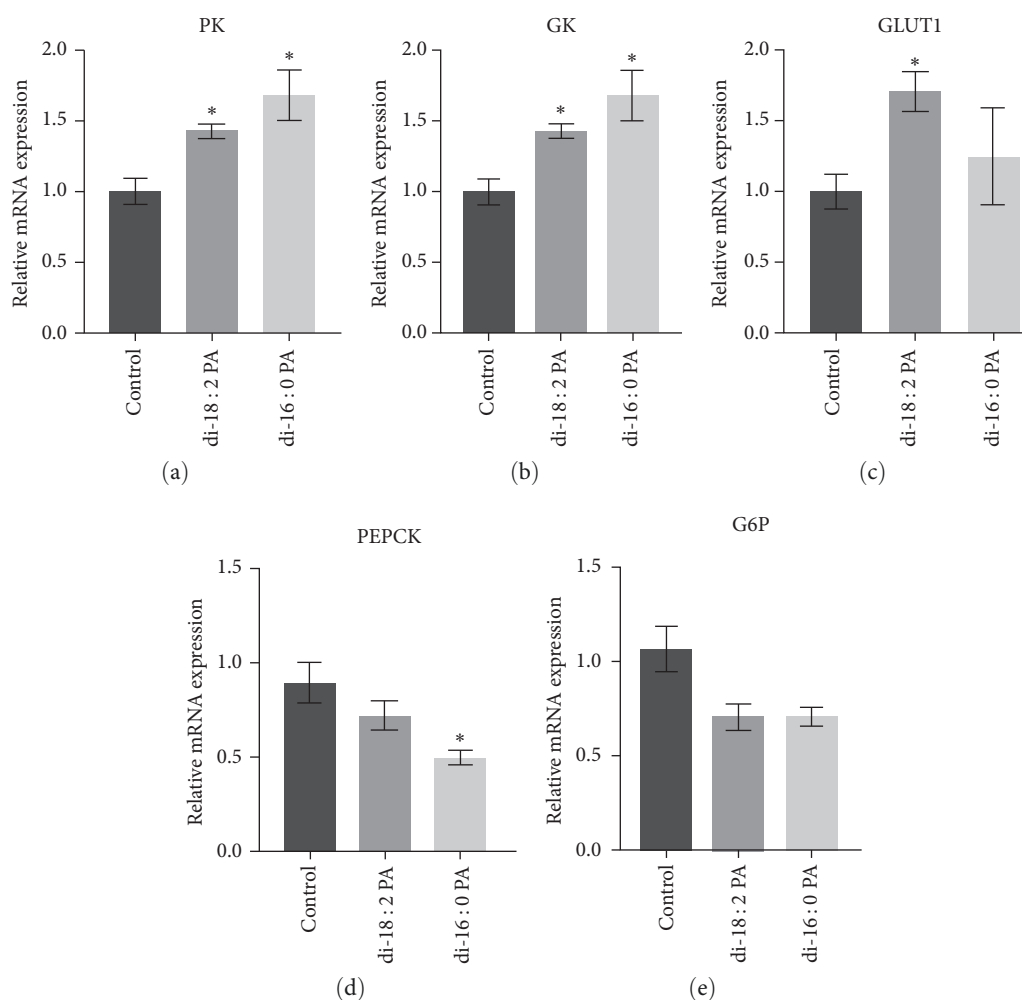


FIGURE 5: Effect of phosphatidic acid on glucose metabolism in the muscle of *Sinonovacula constricta*. Values are means \pm SEMs ($n=6$). Difference between control and PA treatment group was analyzed by t-tests ($*P<0.05$). PK, pyruvate kinase; GK, glucokinase; GLUT1, glucose transporter 1; PEPCK, phosphoenolpyruvate carboxykinase; G6P, glucose-6-phosphatase.

increased by di-18:2 PA, however di-16:0 PA did not influence the mRNA level of SCD (Figure 6(d)).

PA treatment significantly increased the mRNA levels of citrate synthase (CS) and NADP-isocitrate dehydrogenase (NADP) compared with control (Figures 7(a) and 7(b)).

4. Discussion

As a master regulator of growth and metabolism, mTOR integrates nutrient signal, especially amino acids, to mediate the metabolic pathways that control growth [18]. In this study, the downstream targets of mTOR (the phosphorylation levels of 4EBP1 and p70S6K) were significantly increased by an intermediate of lipid metabolism, PA. These results indicate that PA induces the activation of mTOR signaling pathway and protein synthesis in the muscle of razor clam. This is similar to previous results observed in mammalian [12, 13] and fish [16]. Mechanistically, the stability and activity of mTOR complex require the interaction of PA with the FRB domain of mTOR [11]. In addition, the subcellular localization of mTOR is also modulated by PA [18]. Endogenous

suppressing PA production prevents the activation of mTOR signaling pathway in mammal cell lines [11]. However, several studies have shown that exogenous PA activates mTOR signaling through multiple indirect mechanism, rather than through direct binding to mTOR. For example, results from Winter et al. [19] suggested that exogenous PA is metabolized to lysophosphatidic acid (LPA), which subsequently activates the LPA receptor, then leading to activation of mTOR signaling via ERK pathway. Pioneering works also indicated that PA with some degree of unsaturation is required for a functional interaction with mTOR [20, 21]. In this study, both PA species containing 16:0–16:0 and 18:2–18:2 stimulated mTOR activity, but di-16:0 PA did show less potential for mTOR activation than di-18:2 PA. Whether there is a mechanism for mTOR to distinguish between PA species with some degree of unsaturation remains to be determined in razor clam.

Though the role of mTOR signaling in suppressing catabolism, such as autophagy has been well elucidated, few studies have focused on the effects of exogenous PA on autophagy. The results from Dall'Armi et al. [22] suggested that PA is required for autophagy, and pharmacological and

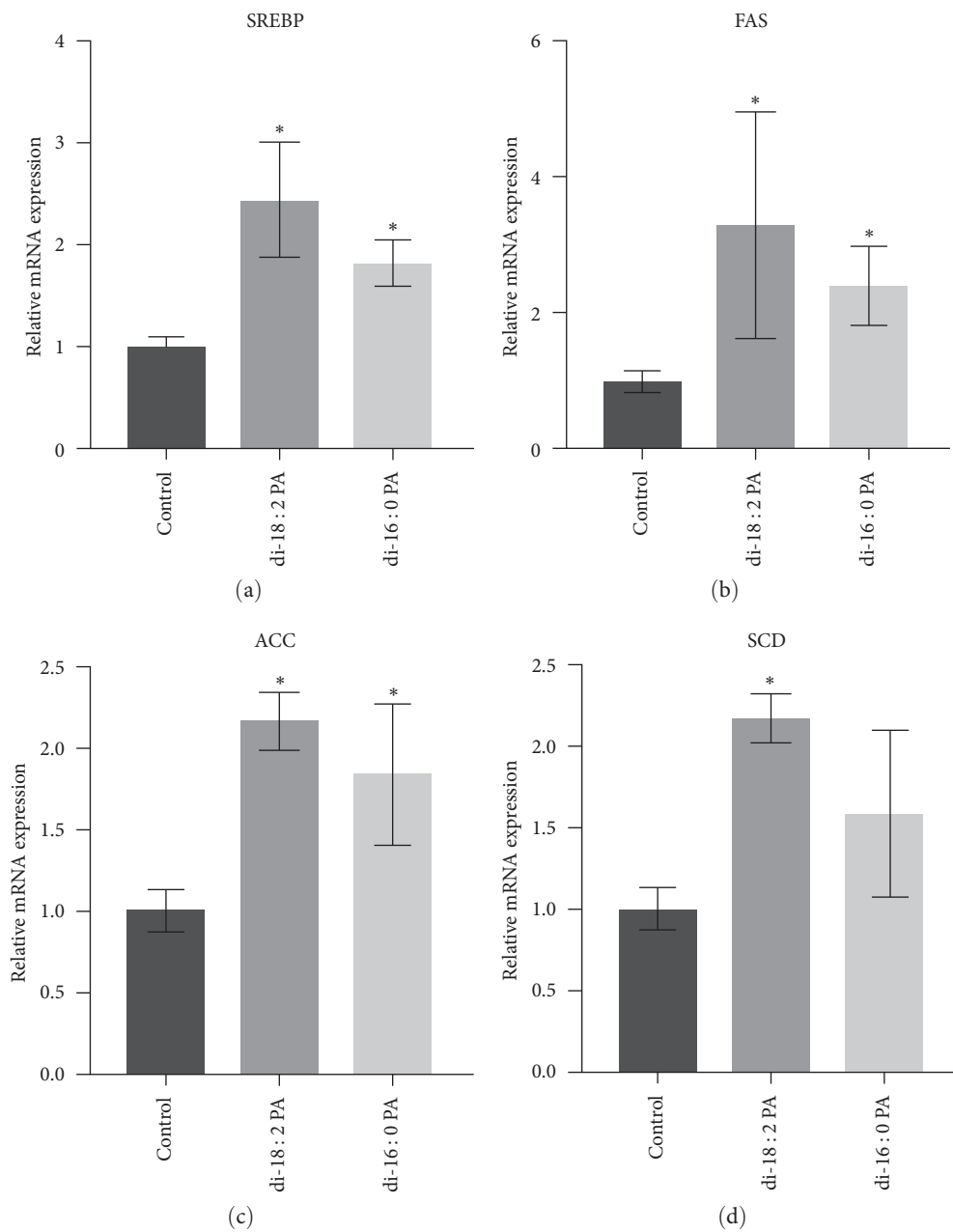


FIGURE 6: Effect of phosphatidic acid on lipid synthesis in the muscle of *Sinonovacula constricta*. Values are means \pm SEMs ($n = 6$). Difference between control and PA treatment group was analyzed by t -tests ($*P < 0.05$). SREBP, sterol responsive element binding protein; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; SCD, stearoyl-CoA desaturase.

genetic inhibition of PA production decrease the starvation-induced autophagy in mouse cells. In this study, the protein level of LC3-II/I, a marker for autophagy, was significantly downregulated by PA, suggesting exogenous PA treatment decreased autophagy and catabolism in the muscle of razor clam, potentially due to induction of mTOR signaling. In support of this hypothesis, the mRNA level of another autophagy activator, AMPK, was significantly decreased by di-18:2 PA in the muscle of razor clam. Meanwhile the reduced mRNA level of AMPK after di-18:2 PA treatment probably indicates a higher energy status in the muscle of razor clam.

In this study, the glycogen concentration in the muscle of razor clam was significantly increased by PA. Glycogen is the primary metabolic reserve in bivalves, and its concentration is positively related to the growth, gonad development, and gametogenesis [23, 24]. In fact, glycogen concentration is a physiological marker of health in bivalves [25]. Moreover, the glycogen concentration is closely associated with the flavor and the quality of the bivalve [26]. PA supplement may therefore represent a nutritional strategy to promote the growth while increasing its glycogen content in bivalves, such as razor clam. In addition to replenishing muscle

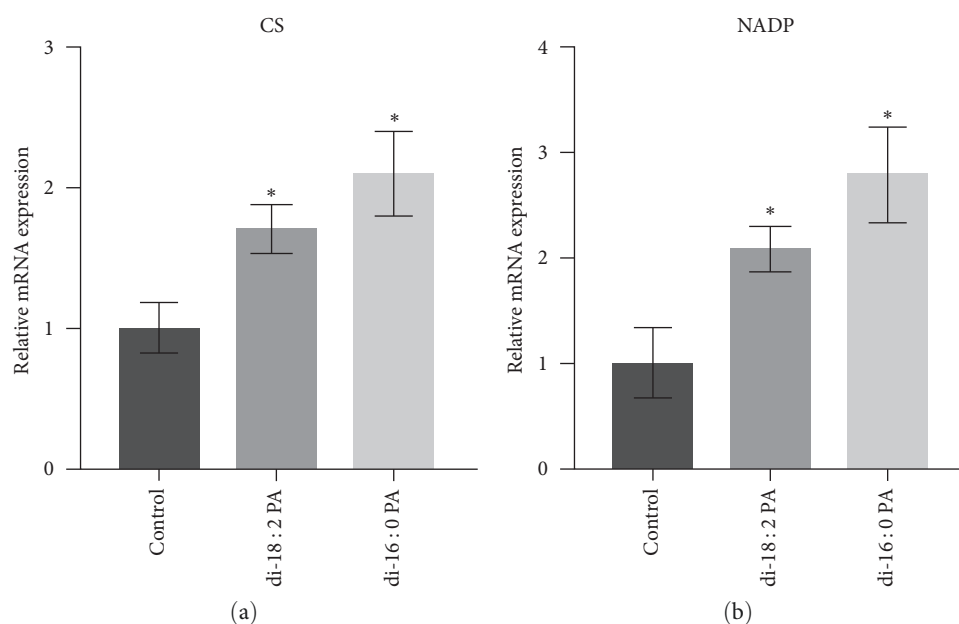


FIGURE 7: Effect of phosphatidic acid on the expression of genes related to tricarboxylic acid cycle in the muscle of *Sinonovacula constricta*. Values are means \pm SEMs ($n=6$). Difference between control and PA treatment group was analyzed by t -tests (* $P<0.05$). CS, citrate synthase and NADP, NADP-isocitrate dehydrogenase.

glycogen, PA supplement can promote the uptake of circulating glucose and stimulate glycolysis. Previous study in mammals showed that PA supplementation increases the mRNA level of glucose transporter, potentially increasing glucose sensitivity in an mTOR-dependent manner [13]. In primary muscle cells of turbot, the mRNA expressions of two key enzymes of glycolysis (GK and PK) are also upregulated by PA, potentially providing intermediates for anabolism and ATP production [16]. In this study, we did observe an increased in the mRNA level of GLUT1 (di-18:2 PA), GK and PK in the muscle of razor clam after PA treatment. Furthermore, the mRNA level of the rate enzymes (PEPCK) in gluconeogenesis was also reduced by di-16:0 PA. Thus, the results in the current study suggest that PA can increase the uptake of excess circulating glucose, stimulate glycolysis, while inhibit gluconeogenesis in the muscle of razor clam. This synchronized glucose metabolism may be crucial for providing intermediates and ATP for anabolism after PA treatment.

Our findings that PA increased the mRNA levels of SREBP, FAS, ACC, and SCD support that PA promotes lipogenesis in an mTOR-dependent manner. Previous study in primary muscle cells of turbot also showed that the mRNA levels of genes involved in lipogenesis (SREBP1 and FAS) are upregulated by PA [16]. For more than a decade, it has been well-documented that mTOR can regulate the transcription of lipogenic genes such as FAS, ACC, and SCD through SREBP [27]. On the one hand, mTOR regulates the expression of SREBP at the level of transcription and translation, subsequently controlling lipogenesis [28]. On the other hand, mTOR mediates SREBP activity by modulation nuclear localization of lipin 1, which is a PA phosphatase that converts PA to diacylglycerol and serves as a repressor of SREBP

activity [29]. Interestingly, di-16:0 PA significantly decreased the triglyceride content, though the lipogenesis was upregulated by di-16:0 PA in the muscle of razor clam. Researches conducted by Joy et al. [12] and Escalante et al. [30] have demonstrated that there is a trend for PA to decrease body fat in human. Indirectly, it is widely reported that mTOR inhibition induces triglyceride accumulation in yeast and fruit flies [31, 32] and in nutrient starved mammalian cells [33, 34]. The recent research indicates that cells might channel *de novo* synthesized lipids toward new membrane biogenesis when mTOR is active, while mTOR inhibition leads to a shift of lipids from membrane phospholipid to triglyceride [35]. Thus, this lipid-lowering effect of PA might also be mTOR-dependent in razor clam.

In this study, the mRNA levels of key enzymes involved in tricarboxylic acid (TCA) cycle (CS and NADP) were upregulated by PA, which supports the findings in primary muscle cells of turbot that PA promotes TCA cycle or mitochondrial metabolism [16]. In fact, TCA cycle intermediates and ATP are required for macromolecular biosynthesis and cell growth [36], and mTOR inhibition impairs mitochondrial activity and results in a decrease in the amounts of TCA cycle intermediates [37]. Besides pyruvate from the above-mentioned glycolysis, amino acids are also important source of TCA cycle intermediates. In this study, the concentration of several amino acids, including Asp, Glu, Ala, Cys, Val, Met, Ile, Leu, Phe, Arg, and Pro was reduced by di-18:2 PA or/and di-16:0 PA. In Jurkat cells, Val, Met, Leu, Phe, Arg, and Pro levels are upregulated by rapamycin, a well-known mTOR inhibitor [38]. In proliferating mammalian cells, mTOR promotes glutaminolysis which converts Glu into TCA cycle intermediate, α -ketoglutarate [39, 40]. Taken together, our results raise the possibility that as with glycolysis, PA

enhances the utilization of amino acids as TCA cycle intermediates for macromolecular biosynthesis and energy production through mTOR.

In conclusion, PA activated mTOR signaling pathway, and resulted in the increase of anabolism and the inhibition of catabolism in *S. constricta*. Our results indicate that PA is a potential growth promoting supplement that might be incorporated in artificial diet of *S. constricta*.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kai Liao contributed in validation, data curation, and writing—original draft and review. Qian Zhang, Yangyang Qiu, Yuxiang Zhu, Yang Liu, and Hailong Huang contributed in investigation. Deshui Chen, Bin Ma, Lin Zhang, and Zhaoshou Ran contributed in writing—review and editing. Kai Liao, Xiaojun Yan, and Jilin Xu contributed in project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Supplementary Materials

Figure S1: the dosage and timing effects of phosphatidic acid on the expression of p-4EBP1 in the muscle of *S. constricta*. (*Supplementary Materials*)

References

- [1] J. Zhu and C. B. Thompson, "Metabolic regulation of cell growth and proliferation," *Nature Reviews Molecular Cell Biology*, vol. 20, pp. 436–450, 2019.
- [2] A. Szwed, E. Kim, and E. Jacinto, "Regulation and metabolic functions of mTORC1 and mTORC2," *Physiological Reviews*, vol. 101, no. 3, pp. 1371–1426, 2021.
- [3] M. Zachari and I. G. Ganley, "The mammalian ULK1 complex and autophagy initiation," *Essays in Biochemistry*, vol. 61, no. 6, pp. 585–596, 2017.
- [4] J. Kim, M. Kundu, B. Viollet, and K.-L. Guan, "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1," *Nature Cell Biology*, vol. 13, pp. 132–141, 2011.
- [5] E. Meyer and D. T. Manahan, "Gene expression profiling of genetically determined growth variation in bivalve larvae (*Crassostrea gigas*)," *Journal of Experimental Biology*, vol. 213, no. 5, pp. 749–758, 2010.
- [6] D. Hedgecock, J.-Z. Lin, S. DeCola et al., "Transcriptomic analysis of growth heterosis in larval Pacific oysters (*Crassostrea gigas*)," *Proceedings of the National Academy of Sciences*, vol. 104, no. 7, pp. 2313–2318, 2007.
- [7] S. Sforzini, M. N. Moore, C. Oliveri et al., "Role of mTOR in autophagic and lysosomal reactions to environmental stressors in molluscs," *Aquatic Toxicology*, vol. 195, pp. 114–128, 2018.
- [8] S. Sforzini, C. Oliveri, A. Barranger et al., "Effects of fullerene C₆₀ in blue mussels: role of mTOR in autophagy related cellular/tissue alterations," *Chemosphere*, vol. 246, Article ID 125707, 2020.
- [9] Q. Zhang, Y. Li, K. Liao et al., "mTOR plays a conserved role in regulation of nutritional metabolism in Bivalve *Simonovacula constricta*," *Journal of Marine Science and Engineering*, vol. 11, no. 5, Article ID 1040, 2023.
- [10] X. Wang, S. P. Devaiah, W. Zhang, and R. Welti, "Signaling functions of phosphatidic acid," *Progress in Lipid Research*, vol. 45, no. 3, pp. 250–278, 2006.
- [11] A. Toschi, E. Lee, L. Xu, A. Garcia, N. Gadir, and D. A. Foster, "Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin," *Molecular and Cellular Biology*, vol. 29, no. 6, pp. 1411–1420, 2009.
- [12] J. M. Joy, D. M. Gundermann, R. P. Lowery et al., "Phosphatidic acid enhances mTOR signaling and resistance exercise induced hypertrophy," *Nutrition & Metabolism*, vol. 11, Article ID 29, 2014.
- [13] C. B. Mobley, T. A. Hornberger, C. D. Fox et al., "Effects of oral phosphatidic acid feeding with or without whey protein on muscle protein synthesis and anabolic signaling in rodent skeletal muscle," *Journal of the International Society of Sports Nutrition*, vol. 12, no. 1, Article ID 32, 2015.
- [14] E. B. Sobotik, J. T. Lee, S. Hagerman, and G. S. Archer, "Evaluation of the use of phosphatidic acid in the diet on growth performance and breast meat yield in broilers," *Animals*, vol. 8, no. 6, Article ID 87, 2018.
- [15] H. Jiang, H. Zhou, C. Liu, X. Wang, K. Mai, and G. He, "Effects of phosphatidic acid on growth and antioxidant capacity in juvenile turbot, *Scophthalmus maximus* L., fed with high plant protein-based diets," *Journal of the World Aquaculture Society*, vol. 52, no. 4, pp. 947–960, 2021.
- [16] T. Wang, X. Wang, H. Zhou, H. Jiang, K. Mai, and G. He, "The mitotic and metabolic effects of phosphatidic acid in the primary muscle cells of turbot (*Scophthalmus maximus*)," *Frontiers in Endocrinology*, vol. 9, Article ID 221, 2018.
- [17] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.
- [18] M. A. Frias, S. Mukhopadhyay, E. Lehman et al., "Phosphatidic acid drives mTORC1 lysosomal translocation in the absence of amino acids," *Journal of Biological Chemistry*, vol. 295, no. 1, pp. 263–274, 2020.
- [19] J. N. Winter, T. E. Fox, M. Kester, L. S. Jefferson, and S. R. Kimball, "Phosphatidic acid mediates activation of mTORC1 through the ERK signaling pathway," *American Journal of Physiology-Cell Physiology*, vol. 299, no. 2, pp. C335–C344, 2010.
- [20] M.-S. Yoon, C. L. Rosenberger, C. Wu, N. Truong, J. V. Sweedler, and J. Chen, "Rapid mitogenic regulation of the mTORC1

- inhibitor, DEPTOR, by phosphatidic acid," *Molecular Cell*, vol. 58, no. 3, pp. 549–556, 2015.
- [21] C. Zhang, A. A. Wendel, M. R. Keogh, T. E. Harris, J. Chen, and R. A. Coleman, "Glycerolipid signals alter mTOR complex 2 (mTORC2) to diminish insulin signaling," *PNAS*, vol. 109, no. 5, pp. 1667–1672, 2012.
- [22] C. Dall'Armi, A. Hurtado-Lorenzo, H. Tian et al., "The phospholipase D1 pathway modulates macroautophagy," *Nature Communications*, vol. 1, Article ID 142, 2010.
- [23] T. Yurimoto, "Seasonal changes in glycogen contents in various tissues of the edible bivalves, pen shell *Atrina lischkeana*, ark shell *Scapharca kagoshimensis*, and Manila clam *Ruditapes philippinarum* in West Japan," *Journal of Marine Biology*, vol. 2015, Article ID 593032, 5 pages, 2015.
- [24] M. M. Hassan, J. G. Qin, and X. Li, "Gametogenesis, sex ratio and energy metabolism in *Ostrea angasi*: implications for the reproductive strategy of spermcasting marine bivalves," *Journal of Molluscan Studies*, vol. 84, no. 1, pp. 38–45, 2018.
- [25] B. Vodáková and K. Douda, "Variation in glycogen distribution among freshwater bivalve tissues: simplified protocol and implications," *Journal of Aquatic Animal Health*, vol. 31, no. 1, pp. 107–111, 2019.
- [26] B. Li, K. Song, J. Meng, L. Li, and G. Zhang, "Integrated application of transcriptomics and metabolomics provides insights into glycogen content regulation in the Pacific oyster *Crassostrea gigas*," *BMC Genomics*, vol. 18, Article ID 713, 2017.
- [27] N. F. Brown, M. Stefanovic-Racic, I. J. Sipula, and G. Perdomo, "The mammalian target of rapamycin regulates lipid metabolism in primary cultures of rat hepatocytes," *Metabolism-Clinical and Experimental*, vol. 56, no. 11, pp. 1500–1507, 2007.
- [28] Q. Dong, G. Majumdar, R. N. O'Meally, R. N. Cole, M. B. Elam, and R. Raghov, "Insulin-induced de novo lipid synthesis occurs mainly via mTOR-dependent regulation of proteostasis of SREBP-1c," *Molecular and Cellular Biochemistry*, vol. 463, pp. 13–31, 2020.
- [29] T. R. Peterson, S. S. Sengupta, T. E. Harris et al., "mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway," *Cell*, vol. 146, no. 3, pp. 408–420, 2011.
- [30] G. Escalante, M. Alencar, B. Haddock, and P. Harvey, "The effects of phosphatidic acid supplementation on strength, body composition, muscular endurance, power, agility, and vertical jump in resistance trained men," *Journal of the International Society of Sports Nutrition*, vol. 13, no. 1, Article ID 24, 2016.
- [31] I. Bjedov, J. M. Toivonen, F. Kerr et al., "Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*," *Cell Metabolism*, vol. 11, no. 1, pp. 35–46, 2010.
- [32] J. B. Madeira, C. A. Masuda, C. M. Maya-Monteiro, G. S. Matos, M. Montero-Lomelí, and B. L. Bozaquel-Morais, "TORC1 inhibition induces lipid droplet replenishment in yeast," *Molecular and Cellular Biology*, vol. 35, no. 4, pp. 737–746, 2015.
- [33] T. B. Nguyen, S. M. Louie, J. R. Daniele et al., "DGAT1-dependent lipid droplet biogenesis protects mitochondrial function during starvation-induced autophagy," *Developmental Cell*, vol. 42, no. 1, pp. 9–21.e5, 2017.
- [34] A. S. Rambold, S. Cohen, and J. Lippincott-Schwartz, "Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics," *Developmental Cell*, vol. 32, no. 6, pp. 678–692, 2015.
- [35] A. M. Hosios, M. E. Wilkinson, M. C. McNamara et al., "mTORC1 regulates a lysosome-dependent adaptive shift in intracellular lipid species," *Nature Metabolism*, vol. 4, pp. 1792–1811, 2022.
- [36] M. G. V. Heiden, L. C. Cantley, and C. B. Thompson, "Understanding the warburg effect: the metabolic requirements of cell proliferation," *Science*, vol. 324, no. 5930, pp. 1029–1033, 2009.
- [37] M. Morita, S.-P. Gravel, V. Chénard et al., "mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation," *Cell Metabolism*, vol. 18, no. 5, pp. 698–711, 2013.
- [38] A. Ramanathan and S. L. Schreiber, "Direct control of mitochondrial function by mTOR," *Proceedings of the National Academy of Sciences*, vol. 106, no. 52, pp. 22229–22232, 2009.
- [39] A. Csibi, G. Lee, S.-O. Yoon et al., "The mTORC1/S6K1 pathway regulates glutamine metabolism through the eIF4B-dependent control of c-Myc translation," *Current Biology*, vol. 24, no. 19, pp. 2274–2280, 2014.
- [40] A. Csibi, S.-M. Fendt, C. Li et al., "The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4," *Cell*, vol. 153, no. 4, pp. 840–854, 2013.