Journal of Oceanology and Limnology
Vol. 39 No. 4, P. 1523-1537, 2021
**https://doi.org/10.1007/s00343-020-0181-8**

# Molecular characterization and expression of the *SiUCP2* gene in sea urchin *Strongylocentrotus intermedius**

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Received May 8, 2020; accepted in principle Jul. 3, 2020; accepted for publication Sep. 1, 2020


**Abstract** Uncoupling protein 2 (UCP2) is a proton transporter located in the inner mitochondrial membrane, and inhibits the formation of adenosine triphosphate and reactive oxygen species by uncoupling oxidative phosphorylation. To provide a theoretical basis for the role of *SiUCP2* in lipid metabolism, a 2 341-bp full-length cDNA of *SiUCP2* from sea urchin *Strongylocentrotus intermedius*, which encodes 323 amino acids (predicted MW 36.11 kDa) was obtained, and the structure and function of the *SiUCP2* gene and its expression at the mRNA and protein level were studied. *SiUCP2* had high homology with *UCP2* of other species. Expression of *SiUCP2* was detected in the order of tube feet > gonads > coelomocytes > intestines. The expression level was the highest in prismatic larvae and lowest in the two-cell stage. Moreover, using in-situ hybridization, we found that SiUCP2 protein was expressed in the gonads and intestine. This study provided a theoretical basis for subsequent studies on the role of *SiUCP2* and its regulatory mechanism in lipid metabolism, and for the improvement of gonad quality to obtain a higher economic value from sea urchins.

**Keyword**: *Strongylocentrotus intermedius*; *SiUCP2*; gene cloning; lipid metabolism; in-situ hybridization; western blot

## 1 INTRODUCTION

Adenosine triphosphate (ATP) represents one of the final compounds produced by animal organisms to store chemical energy converted from food. The generation of ATP is related to the energy requirements of the organism and is closely related to the metabolic rate. The metabolic rate quantifies the energy requirements of the organism, which is related to the size of the animal; therefore, the larger the body size is, the higher the metabolic rate is (McCGraham, 1967). The metabolic rate is always in a state of fluctuation, which varies with the amount of energy used by the animal. When the animal is in a stage of reproduction or stress, the metabolic rate would change accordingly. Most organisms maintain a basal metabolic rate (BMR) before they consume additional energy for life activities. Therefore, organisms have evolved a feedback system called “energy balance” that responds to energy requirements, so that the metabolic rate can change with energy requirements (Campbell and Reece, 2005).

When starvation occurs, there lacks sufficient energy substances to meet the needs of the juvenile perch (*Perca fluviatilis*), thus the organism first uses glycogen stored in the body, and then uses fat as energy substance, which is oxidized and decomposed to maintain energy metabolism, and finally the organism uses protein (Mehner and Wieser, 1994). However, some aquatic animals use energy substances in a different order during starvation. Fish *Sciaenops ocellatus* (Jiang et al., 2002) and juvenile *Eriocheir sinensis* (Wen et al., 2002) prioritize the use of fats and carbohydrates, while a few species of fish, such

* Supported by the National Key Research and Development Program of China (No. 2018YFD0901601) and Chinese Outstanding Talents in Agricultural Sciences (for Yaqing CHANG)
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as *Pagrus major* (Zhang et al., 2000) mainly use protein as an energy source during starvation. Fatty acids (FAs) are an important source of energy for cells and are the primary raw material for triglyceride synthesis. Polyunsaturated fatty acids (PUFAs) play important functional roles in a variety of tissues, such as promoting nervous system development, mediating immunity, and promoting coagulation processes. Fatty acid derivatives and metabolites are involved in mediating multiple gene expression and enzymatic reactions, ion channel and membrane receptor activities, etc. FA metabolism in animals is a complex process that is regulated by several factors such as genetics, hormones, nutrition, and immunity. Factors such as inheritance influences fat deposition (Geri et al., 1990). Fat deposition depends on the effects of several enzymes during fat synthesis and catabolism, as well as the regulation of hormones involved in fat metabolism (Malavazos et al., 2007; Jiang et al., 2008; Kang et al., 2017). Researchers have undertaken several studies on fat deposition and its regulatory mechanism in livestock for many years, and found that candidate genes such as *UCP1*, *UCP2*, *UCP3*, *LPL*, *PPARs*, *Leptin*, and *SREBP* were closely associated with fat metabolism and meat quality (Douaire et al., 1992; Taouis et al., 1998; Huang and Xie, 2004; Li et al., 2010).

Uncoupling proteins 2 (UCP2) belongs to UCPs family which is a class of proton transporters located in the inner mitochondrial membrane and are members of the mitochondrial anion carrier superfamily (MACF) (Fleury and Sanchis, 1999; Luévano-Martínez, 2012). Studies have shown that *UCP2* is involved in multiple metabolic processes such as food intake (Coppola et al., 2007; Andrews et al., 2008), FA metabolism (Klingenberg and Echtay, 2001; Brookes et al., 2008), insulin secretion (Wang et al., 2010), and the immune response (Tagen et al., 2009; Emre and Nübel, 2010). Currently, the *UCP2* gene has been cloned from rat (Matsuda et al., 1997), pig (Werner et al., 1999), carp and zebrafish (Stuart et al., 1999), and silver carp (Liao et al., 2006), but the cloning of *UCP2* from Echinoderms has not yet been reported.

*Strongylocentrotus intermedius* was introduced to China from Japan by Dalian Ocean University in 1989 and is currently distributed in Liaoning and Shandong Peninsula. It is one of the main species of sea urchins cultured in China (Chang et al., 2004). The gonads of sea urchins are the only edible part of the organism. These organs contain essential nutrients such as lipids and PUFAs, which not only determine the nutritional value of sea urchins, but also ensure the normal growth and reproduction of the organisms during cultivation processes. Uneven feeding or excessive feeding often happens in the aquaculture of sea urchins. Moderate deposits of fat help the sea urchins maintain normal metabolic activities, but excessive deposits will not only affect the health of the sea urchins but also reduce the quality of the gonads (Zhou et al., 2008). Artificially cultured sea urchins are often exposed to starvation stress due to environmental and seasonal changes. During starvation, sea urchins will consume stored glycogen and fat to maintain their basal metabolism (Lares and Pomory, 1998). *UCP2* regulates lipid metabolism directly or indirectly, but the structure and expression of *UCP2* and its role in lipid metabolism in *S. intermedius* have not been reported.

In this study, the *SiUCP2* gene was cloned from *S. intermedius* using the rapid-amplification of cDNA ends (RACE) technique, and the expressions of this gene in different tissues and at different development stages and in different starvation periods of *S. intermedius* were studied using quantitative real-time PCR (qRT-PCR). Gene expression in intestinal and gonadal tissues was also examined by in-situ hybridization. Finally, the expression of *SiUCP2* recombinant protein in the intestine and gonads were examined using western blotting. This study aimed to investigate the structure and function of the *SiUCP2* gene from *S. intermedius* and its expression at the mRNA and protein level to provide a theoretical basis for subsequent studies on the role of *SiUCP2* and its regulatory mechanism the fatty acids synthesis of *S. intermedius*. In the future, we will continue to pay attention to *SiUCP2*, study which fatty acids are produced by *SiUCP2*, clarify the mechanism of *SiUCP2* in the synthesis of fatty acids, improve the content of fatty acids in sea urchins, and further improve the gonadal quality of sea urchins to obtain sea urchins with a higher economic value.

## 2 MATERIAL AND METHOD

Sea urchins and rabbits used in this study were farmed, and all experiments were conducted in accordance with the ethics committee of Dalian Ocean University and national guidelines. No endangered or protected species were involved in this test. The location of the training experiment does not require special permission.

**Table 1 The sequences of PCR primers used in this study**

| Primer name | Sequence (5′→3′) | Purpose |
|---|---|---|
| 3′F1 | GTCAGTATCGGGGAGCCACGGAAT | 3′RACE |
| 3′F2 | AGCTTTCTATAAAGGATTCACGCCACAGTTT | 3′RACE |
| 5′R1 | CACTGGCGTTGACGACA | 5′RACE |
| 5′R2 | GGCATAGTTCCTTTCCAGA | 5′RACE |
| 18s rRNA-F | TGAGCCGCAACAGTAATC | qRT-PCR |
| 18s rRNA-R | AAGGGAAAAGGAAGTGAAAG | qRT-PCR |
| *SiUCP2*-F | CGACTGGTAAAGAACTACGGGC | qRT-PCR |
| *SiUCP2*-R | GCCATTTAGAACCGCATTACCC | qRT-PCR |
| Dig probe | CAAGAUAUUACAAUCUGUUAAAGAAAGGCAACCUGUCUUCAAUG | In-situ hybridization |

### 2.1 Animals, rearing conditions, and sample collection

One-year-old *S. intermedius* adults used in this study were bred at Dalian Ocean University. Healthy sea urchins were homogeneous in size with test heights of 21.80±1.20 mm, shell lengths of 37.62±1.53 mm, and weights of 24.27±1.46 g.

The tube feet, coelomocytes, Aristotle's lantern, stomachs, and gonads, intestines, were dissected from three sea urchins. To collect samples from different developmental stages, we selected three females and three males and injected with 1-mL KCL (40 μL/g of body mass) to induce spawning. Unfertilized eggs were transferred to a beaker containing 1.5-L fresh sea water (salinity: 31) in density of 20–30 ind./mL in temperature of 19±0.5 °C. They were then dripped into semen (sperm-egg ratio was 1 000∶1). Water was stirred to ensure sperm and eggs in good contact, and stayed for 15 min. After artificial fertilization, eggs were washed three times every 30 min to remove extra sperm and impurities. Morphological changes in larvae were observed under a microscope (Kelly et al., 2000). The fertilized eggs develop in three stages, early embryonic development, planktonic larva, and competent larvae, and four major developmental stages, the blastula, prismatic larval, long-arm larval, and juvenile sea urchin. All samples were frozen in liquid nitrogen immediately and stored at -80 °C.

A starvation experiment commenced in May 2019, in which 25 sea urchins were cultured in a 500-L tank in water temperature of 19 °C. Feces were observed every day since feeding was stopped. Once fecal excretion was significantly reduced (Li et al., 2004), five samples were taken as the control group, and the hunger experiment officially commenced. The intestines and gonads of five sea urchins were taken on Days 0, 7, 14, and 21 of starvation and rapidly frozen in liquid nitrogen, and stored at -80 °C.

### 2.2 Total RNA extraction and reverse transcription

Total RNA was extracted from different tissues, at different development stages and different starvation periods of *S. intermedius*, using an RNAprep Pure Kit for tissue (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. Standard RNAs were used for cDNA preparation. The cDNA for qRT-PCR was prepared using a Prime Script™ RT Reagent Kit (TaKaRa, Japan). The reaction was performed in a total volume of 10 μL containing 2-μL 5×Primer script Buffer, 0.5-μL Primer script Enzyme MixI, 0.5-μL Oligo dT Primer, 0.5-μL Random Primer, 500-ng Total RNA and $ddH_2O$. The PCR reaction conditions were 37 °C for 15 min and 85 °C for 5 s. All cDNA samples were diluted five times and stored at -20 °C before subsequent experiments.

### 2.3 Cloning the full-length cDNA of *SiUCP2* using rapid amplification of cDNA ends (RACE) PCR

The partial cDNA sequence of *SiUCP2* was obtained from our transcriptome assembly data (http://gsa.big.ac.cn/, accession number: PRJCA000276), and the gene-specific primers were designed by Primer 5.0 and obtained from Sangon Biotech, Shanghai as shown in Table 1. The cDNA for RACE was prepared using the M-MuLV First Strand cDNA Synthesis Kit (Sangon, Shanghai, China). The PCR reactions for 5′-RACE and 3′-RACE were conducted following the manufacturer's instructions. Products from RACE were extracted and purified using a Quick Gel Extraction kit (Transgen Biotech, China). For specific experimental content, refer to kits instructions and Han et al. (2019). After the full-length cDNA of *SiUCP2* was amplified, it was ligated into the PEASY-1 vector and sequenced.

### 2.4 Bioinformatics analysis

The open reading frame (ORF) of *SiUCP2* was

analyzed using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and the deduced amino acid (AA) sequence was determined using the Expert Protein Analysis System (http://www.expasy.org/). The physical and chemical parameters of the deduced protein were computed by the ProtParam tool (http://web.expasy.org/protparam/). The functional domains of the SiUCP2 protein were predicted by InterPro (http://www.ebi.ac.uk/interpro/). Multiple sequence alignment of the deduced *SiUCP2* AA sequence was performed using DNAMAN 6.0. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0.

### 2.5 Real-time PCR analyses of *SiUCP2* expression

The relative expression levels of *SiUCP2* mRNA transcripts in different tissues (coelomocytes, tube feet, Aristotle's lantern, gonads, intestines and stomachs), were assessed at developmental stages (eggs (1–2 min after fertilization), two cells (40 min after fertilization), eight cells (2 h after fertilization), blastula (5–6 h after fertilization), gastrula (17–20 h after fertilization), prism larvae (24 h after fertilization), two-arm larvae (30–40 h after fertilization), eight-arm larvae (7–13 d after fertilization), juvenile sea urchins (30 d after fertilization)) and intestines and gonads of sea urchins that were fasted for 0, 7, 14, and 21 d were analyzed with quantitative real-time PCR (qRT-PCR) using the Applied Biosystems 7500 Real-time System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq (SYBR PrimeScript™ RT-PCR kit II, TaKaRa, Japan) following the manufacturer's instructions. The 18s rRNA gene was used as an internal reference gene. The amplification was performed in a total volume of 20 μL containing 2 μL of 1∶5 diluted original cDNA, 10 μL of 2×SYBR Green Master mix (TaKaRa, Japan), 0.4 μL of ROX Reference Dye, 6 μL of PCR grade water and 0.8 μL (10 mmol/L) of each primer. The reaction conditions were followed by 40 cycles of 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The final extension step was at 72 °C for 5 min. Three independent biological replicates and three technical repetitions of each group were carried out. The relative expression levels of the target gene were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### 2.6 Statistical analysis

All data were expressed as the mean±SD ($n$=5). $P$ value was adjusted for multiple tests using a false discovery rate (Benjamini-Hochberg). Significant differences ($P$<0.05) for each variable were firstly detected using the one-way ANOVA test, followed by Tukey's test and Tamhane's T2 method. Statistical analysis was performed using SPSS software version 19.0 (IBM, Armonk, NY, USA).

### 2.7 In-situ hybridization

In-situ hybridization was performed using a DIG High Prime DNA Labeling and Detection Starter Kit I (Sangon). Probe primers were based on the full-length cDNA sequence of *SiUCP2*. The intestines and gonads of *S. intermedius* were collected at the three stage of reproductive cycle the gonads and soaked in a 4% paraformaldehyde solution overnight. After the tissues were fixed, dehydrated with gradient alcohol, waxed, and embedded, they were cut into slices and baked in oven at 62 °C for 2 h. Paraffin sections were dewaxed by placing in water and sequentially in xylene I for 15 min, xylene II for 15 min, anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, 85% alcohol for 5 min, 75% alcohol for 5 min, and washed in diethylpyrocarbonate (DEPC). Slices were placed in the repair solution, boiled for 10–15 min, and cooled naturally. Proteinase K (20 μg/mL) was added over 30 min at 37 °C and washed with pure water, the pre-hybrid solution was added and incubated at 37 °C for 1 h and then decanted. The hybridization solution containing the probe was added at a concentration of 5 ng/μL, and the mixture was mixed overnight at 37 °C in an incubator. To wash away the hybridization solution, the sections were washed by 2×saline sodium citrate (SSC) at 37 °C for 10 min, washed by 1×SSC at 37 °C for 5 min, and finally washed by 0.5×SSC at room temperature for 10 min. Serum bovine serum albumin (BSA) was added and incubated at room temperature for 30 min. After pouring off the sealing solution, anti-digoxin-labeled alkaline phosphatase was added and incubated at 37 °C for 40 min. Then washed for 5 min with phosphate buffer saline (PBS) for four times. After the sections were slightly dried, the freshly prepared nitro-blue tetrazolium (NBT) color rendering solution was added, and the color rendering time was controlled under the microscope. The sections were washed with pure water to terminate the color rendering, and then nuclear solid red dye was added to stain the nucleus, which was washed with pure water after an appropriate degree of staining. Finally, neutral gum was used to seal the film and an optical microscope was used to observe the results.

### 2.8 Construction of prokaryotic expression plasmid and preparation of antibody

2.8.1 Construction of prokaryotic expression plasmid

The encoding region of *SiUCP2* was amplified by a gene-specific primer with the enzyme cleavage site Nde I/Xho I. After sequencing, it was inserted into the expression vector pET22b (Sangon) to construct the recombinant plasmid pET22b-*SiUCP2*, which was expressed in *Escherichia coli*.

2.8.2 Animal immunity

Four-month-old healthy female New Zealand white rabbits weighing 2.1 kg were used. For the first immunization, the protein antigen was emulsified with an equal volume of Freund's complete adjuvant and injected (100 μL) into the lymph nodes of the hind legs. A second immunization was performed after 21 d. The protein antigen was emulsified with an equal volume of Freund's incomplete adjuvant and injected (100 μL) into the lymph nodes of the hind legs. The third immunization was performed after 35 d. After 42 d, 1 mL of blood was collected from the ear vein and the antiserum titer was detected by enzyme-linked immunosorbent assay (ELISA).

2.8.3 Indirect ELISA test

The antigen was coated with 0.05-mol/L carbonate (pH=9.6) at 100 μL/well, incubated at 4 °C overnight, removed and washed for 3 min with 0.05% Tween-20 (PBST) for three times. Blocking solution (150 μL of 5% skimmed milk) was added to each well and blocked at 37 °C for 60 min, and then washed for 3 min three times with 0.05% Tween-20 (PBST). The rabbit serum was diluted 1∶1 000 and then incubated at 37 °C for 1 h, and washed three times with 0.05% Tween-20 (PBST) for 3 min per time. Horseradish-labeled enzyme goat anti-rabbit IgG (H+L) (Sangon) was diluted 1∶8 000 and incubated at 37 °C for 45 min. The plate was washed five times with 0.05% Tween-20 (PBST) and 100 μL/well of substrate solution (TMB) was added, the reaction was carried out for 15 min. Finally, 100 μL of 2-mol/L sulfuric acid was added to terminate the reaction. Optical density (OD) value was measured at a wavelength of 450 nm using a microplate reader (KeHua ST-360).

### 2.9 Extraction and expression of the SiUCP2 protein

About 20-mg gonads were taken and 200-L RIPA lysate was added (with 1-mmol/L PMSF, broad-spectrum protease inhibitor, and phosphatase inhibitor). Low temperature and high speed homogenate for 3 times, 10 s each time, 30 s pause, then ice lysis for 2 h, and take out ice bath ultrasound once in the middle. At 4 °C, 12 000×*g* high speed centrifugation was performed, the supernatant was absorbed and centrifuged again, and the intermediate clarified sample was taken again until the obtained protein solution was clarified and transparent, which was temporarily stored at 4 °C. The protein concentration was determined. In this study, western blotting was used to detect SiUCP2 in the gonads of *S. intermedius*, with an antibody that was diluted 1∶2 000 and β-actin acted as a house-keeping gene. The resultant protein was isolated on a reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% separating gel and 5% stacking gel, 40-μg protein extract was loaded into lanes for electrophoresis) and visualized after staining with Coomassie Brilliant Blue R-250.

## 3 RESULT

### 3.1 Analysis of *SiUCP2* sequence

The *SiUCP2* gene was obtained from *S. intermedius* by RACE technology and the sequence was deposited in the GenBank database, under the accession number MN065154. *SiUCP2* was 2 341 bp in length and contained a 969-bp ORF encoding 323 AAs, a 232-bp 5′UTR, and a 1 140-bp 3′UTR containing a polyadenylation signal (AATAAAA). The molecular weight of *SiUCP2* was 36.11 kDa, and the theoretical pI was 9.68. Analysis of the AA sequence predicted that *SiUCP2* had no signal peptide and three characteristic features of carrier proteins in the inner mitochondrial membrane. Analysis of the AA hydrophilicity/hudrophobicity indicated that *SiUCP2* had an aliphatic index of 87.24 and a grand average of hydropathicity (GRAVY) of 0.014, thus *SiUCP2* was a hydrophobic protein (Fig.1).

### 3.2 Multiple alignment and evolution analysis of SiUCP2 amino acid sequence

The AA sequence of SiUCP2 was aligned with UCP2 AA sequences from other species using DNAMAN 6.0 software. The results showed that *SiUCP2* shared a high homology with all *UCP2* from vertebrates and invertebrates as shown in Table 2.

To reveal the phylogeny of the *SiUCP2* cloned in this study, a phylogenetic tree was constructed using the neighbor-joining method. The result in Fig.2 shows that *SiUCP2* from *S. intermedius* and *UCP2*


**Fig.1 Nucleic acid sequence of *SiUCP2* in *S. intermedius* and its deduced amino acid sequence**

The starting codon ATG is indicated in bold, the termination codon TAA is indicated by bold italics and the box contains the characteristic domain of the carrier protein in the mitochondrial inner membrane. The shaded region represents the characteristic sequence of uncoupled proteins, and the underlined region represents the polyA tail signal (AATAAAA).

**Table 2 Homologous comparisons of *SiUCP2* with *UCP2* in vertebrates and invertebrates**

| Species | Similarity with *S. intermedius* (%) |
|---|---|
| *Homo sapiens* (CAA11402.1) | 45.93 |
| *Mus musculus* (NP_035801.3) | 45.45 |
| *Sus scrofa* (AAU94639.1) | 52.58 |
| *Xenopus laevis* (NP_001080223.1) | 46.17 |
| *Danio rerio* (CAB46268.1) | 44.98 |
| *Cyprinus carpio* (CAB46248.1) | 46.17 |
| *Salmo salar* (XP_014064971.1) | 47.13 |
| *Strongylocentrotus purpuratus* partial *UCP2* (XP_782840.3) | 47.37 |
| *Mizuhopecten yessoensis* (OWF50193.1) | 46.61 |
| *Crassostrea giga* (XP_011412835.1) | 40.11 |
| *Pocillopora damicornis* (XP_027048867.1) | 41.46 |
| *Stylophora pistillata* (PFX28316.1) | 41.46 |

**Table 3 One-way ANOVA of different tissues**

| Item | Quadratic sum | df | Mean square | *F* | Significance |
|---|---|---|---|---|---|
| Between the groups (merger) | 443.952 | 5 | 88.790 | 92.757 | 0.000 |
| Linear term comparison | 126.594 | 1 | 126.594 | 132.249 | 0.000 |
| Deviation | 317.358 | 4 | 79.340 | 82.884 | 0.000 |
| In group | 45.947 | 48 | 0.957 | | |
| Total | 489.899 | 53 | | | |

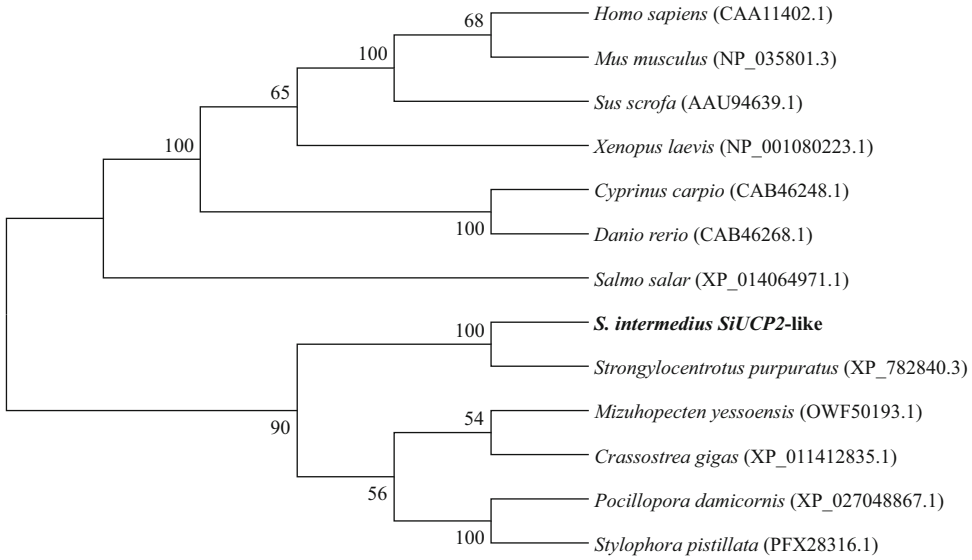


**Fig.2 Consensus neighbor-joining tree based on the amino acid sequences of *SiUCP2* genes from other species**

The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees where the associated taxa cluster together in the bootstrap test (1 000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA 7.0.

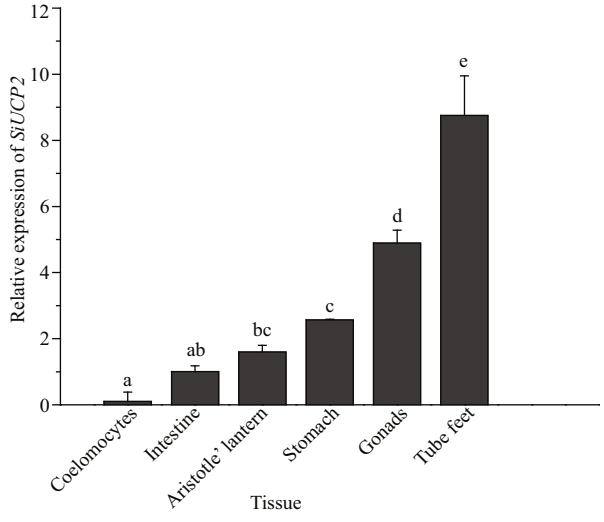


**Fig.3 Relative expression levels of *SiUCP2* in different tissues**

Each vertical bar represents the mean±SD ($n$=5), 18s rRNA was used as a reference gene. Letters above the bars indicate significant differences at $P$<0.05, in different tissues.

from *S. purpuratus* were clustered into one branch, indicating that these two species were closely related.

### 3.3 Relative expression of *SiUCP2* in different tissues

The tissue distribution of *SiUCP2* mRNA in *S. intermedius* was investigated by qRT-PCR. The results in Fig.3 show that the *SiUCP2* gene was expressed in all tissues examined. The data analysis results are shown in Table 3. Descriptive statistical analysis is shown in Supplementary Table S1. The relative expression level was the highest in the tube feet, followed by the gonads, and the relative expression level was the lowest in the coelomocytes.

### 3.4 Relative expression of *SiUCP2* at different development stages

The expression of *SiUCP2* in *S. intermedius* at different development stages, including fertilized eggs, two cells, eight cells, blastulae, gastrula, prism larvae, two-arm larvae, eight-arm larvae, and juvenile sea urchins, was determined to understand the role of the *SiUCP2* gene in different development stages. As shown in Fig.4, The data analysis results are shown in Table 4. Descriptive statistical analysis is shown in

**Table 4 One-way ANOVA of different development stages**

| Item | Quadratic sum | df | Mean square | *F* | Significance |
|---|---|---|---|---|---|
| Between the groups (merger) | 321.265 | 8 | 40.158 | 46.305 | 0.000 |
| Linear term comparison | 160.579 | 1 | 160.579 | 185.157 | 0.000 |
| Deviation | 160.686 | 7 | 22.955 | 26.469 | 0.000 |
| In group | 15.611 | 18 | 0.867 | | |
| Total | 336.876 | 26 | | | |

**Table 5 One-way ANOVA of gonads at the different starvation periods**

| Item | Quadratic sum | df | Mean square | *F* | Significance |
|---|---|---|---|---|---|
| Between the groups (merger) | 8.979 | 3 | 2.993 | 62.523 | 8.979 |
| Linear term comparison | 0.955 | 1 | 0.955 | 19.951 | 0.955 |
| Deviation | 8.024 | 2 | 4.012 | 83.809 | 8.024 |
| In group | 1.532 | 32 | 0.048 | | 1.532 |
| Total | 10.511 | 35 | | | 10.511 |

**Table 6 One-way ANOVA of intestines at the different starvation periods**

| Item | Quadratic sum | df | Mean square | *F* | Significance |
|---|---|---|---|---|---|
| Between the groups (merger) | 6.390 | 3 | 2.130 | 40.816 | 0.000 |
| Linear term comparison | 3.456 | 1 | 3.456 | 66.230 | 0.000 |
| Deviation | 2.934 | 2 | 1.467 | 28.108 | 0.000 |
| In group | 1.670 | 32 | 0.052 | | |
| Total | 8.060 | 35 | | | |

Supplementary Table S2. *SiUCP2* was expressed at all nine development stages and the relative expression at the prism larval stage was the highest, while the expression at the 2-cell stage was significantly lower than at other development stages. During the whole development process, the overall expression of *SiUCP2* showed a pattern of first increasing and then decreasing.

### 3.5 Expression of *SiUCP2* during starvation

The relative expression of *SiUCP2* in the intestines and gonads of *S. intermedius* at different starvation periods was determined using qRT-PCR to understand the expression and role of *SiUCP2* during starvation. The data analysis results are shown in Tables 5–6. Descriptive statistical analysis is shown in Supplementary Tables S3–S4. As shown in Fig.5, the trends of different periods of starvation on *SiUCP2* expression in the intestines and gonads of *S. intermedius* were similar. The relative expression levels of *SiUCP2* in the intestines and gonads during starvation showed a pattern of first decreasing, then increasing and then decreasing again.

### 3.6 In-situ hybridization of *SiUCP2*

In-situ hybridization of *SiUCP2* in the intestines and gonads of *S. intermedius* was carried out. The results are shown in Fig.6. Scattered blue-violet positive signals in the gonads were observed under a microscope. The signals were present in nutritive

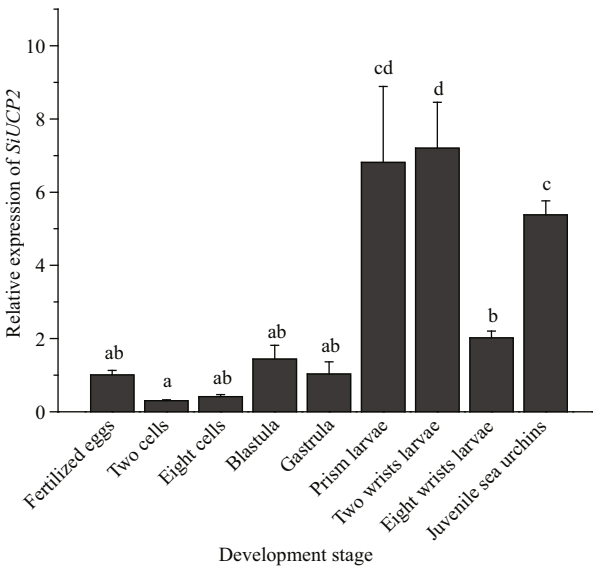


**Fig.4 Relative expressions of *SiUCP2* during the development of *S. intermedius***

18s rRNA was used as a reference gene. Each vertical bar represents the mean±SD ($n$=3). Letters above the bars indicate significant differences at $P$<0.05, in different development stages.

**Table 7 ELISA assay data**

| Group | Negative | Blank | 1K | 2K | 4K | 8K | 16K | 32K | 64K | 128K | 256K | 512K |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0.077 | 0.072 | 1.235 | 1.163 | 1.113 | 1.036 | 0.893 | 0.743 | 0.629 | 0.517 | **0.296** | 0.182 |
| B | 0.076 | 0.083 | 1.166 | 1.106 | 1.153 | 1.094 | 0.974 | 0.892 | 0.776 | 0.658 | 0.511 | **0.366** |

The antibody efficacy value ≥2.5×negative value (marked in bold).

phagocytes, while no positive signals were observed in the intestines, indicating that *SiUCP2* was expressed in small amounts in the gonads and intestines.

### 3.7 Indirect ELISA results

The prepared antibody was subjected to indirect ELISA. The OD values were measured using a microplate reader at 450-nm wavelength. The results are presented in Table 7.

### 3.8 Expression of the SiUCP2 protein in the gonads of *S. intermedius*

To investigate the function of the SiUCP2 protein, total protein was extracted from the gonads of three sea urchins. The SiUCP2 protein was detected by western blotting using β-actin as an internal reference. Through the pre-experiment, we determined the destination band in the gonads, as shown in Fig.7. The results of SDS-PAGE in Fig.8 show that a clear band of about 36 kDa appeared in the gonads.

## 4 DISCUSSION

Sea urchins are considered delicacies in many countries. The gonads of sea urchins contain essential nutrients such as lipids and PUFAs, which not only determine the nutritional value of the sea urchins but also ensure the proton transporters, are located in the inner mitochondrial membrane. Free FAs provide necessary free carboxyl groups for UCPs, making proton transport possible or facilitating proton transport. Currently, the mechanisms of proton leaks caused by UCPs are still controversial (Garlid et al., 2000). UCPs that are present in the inner mitochondrial membrane are capable of disrupting the coupling between oxidation in the electron transport chain and adenosine triphosphate (ATP) synthesis, thus dissipating energy in the form of heat and possibly affecting metabolic efficiency (Thompson and Kim, 2004). The full-length cDNA of a gene is the basis for studying gene structure, function, and protein expression. The *SiUCP2* gene of *S. intermedius* was characterized in this study. DNAMAN sequence alignment showed that *SiUCP2* shared high homology

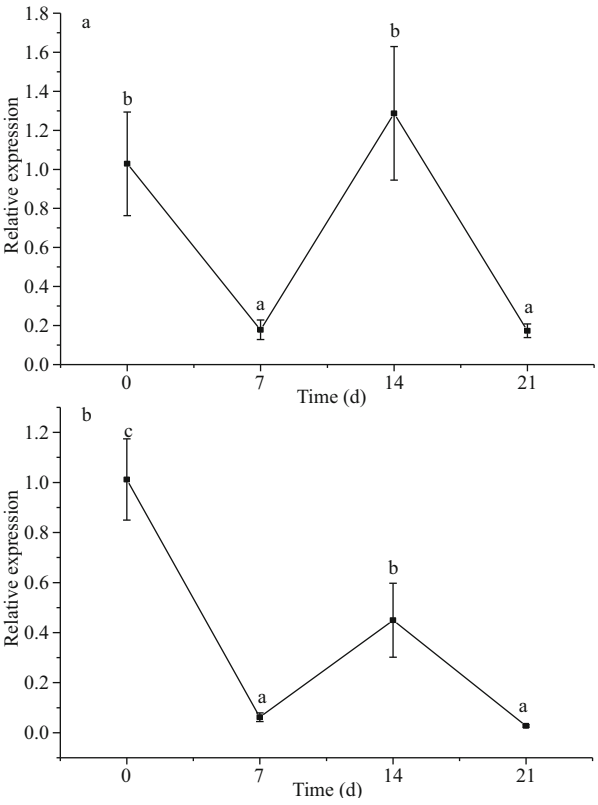


**Fig.5 Effects of starvation on the relative expression of *SiUCP2* gene in gonads (a) and intestines (b)**

18s rRNA was used as a reference gene. Each vertical bar represents the mean±SD ($n$=5). Different letters above the bars indicate significant differences at $P$<0.05, at different times.

with *UCP2* from other species and had similar domains; therefore *SiUCP2* may also have similar biological functions to other homologs from different species. Phylogenetic analysis showed that *SiUCP2* from *S. intermedius* and *UCP2* from *S. purpuratus* were clustered into one branch, indicating that these two species were closely related and distantly related to other vertebrates. Sequence similarity, conserved domains, and phylogenetic analysis provided evidence that the cloned gene was *SiUCP2* cDNA.

*UCP2* was highly expressed in the liver of *Pagrus major*, but almost undetectable in visceral mesenteric adipose tissue (Liang et al., 2003). The relative expression levels of *UCP2* were high in the tissues of liver, stomach, and eye of *Siniperca chuatsi*, but low in the spleen, intestine, and brain (Wen et al., 2002).

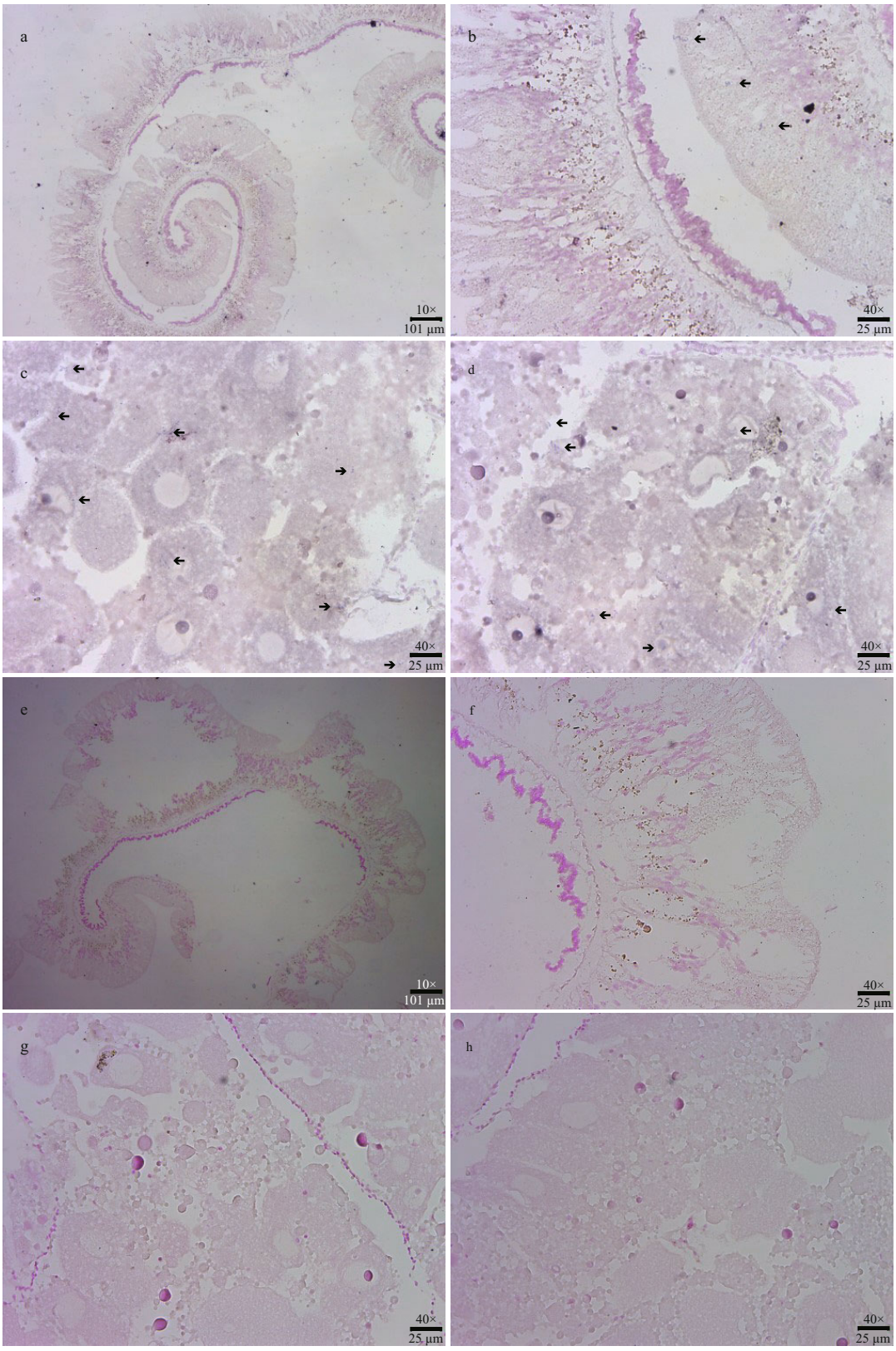


**Fig.6 In-situ hybridization of the *SiUCP2* gene in intestines and gonads**

a & b. hybridization of the digoxigenin-labeled RNA probe with the sea urchin intestines tissue; c & d. the digoxigenin-labeled RNA probe hybridizes with the sea urchin gonad; e & f. hybridization of the sense probe with the sea urchin intestines; g & h. hybridization of the sense probe with the sea urchin gonads. The black arrow indicates a positive signal. Scales are shown in the figure.

The qRT-PCR analysis showed that the *SiUCP2* gene was expressed in all examined tissues of *S. intermedius*, which was consistent with the study results in other species (Wen et al., 2002; Coulibaly et al., 2006; Liao et al., 2006). Sea urchin tube feet, which facilitate movement and adhesion, contain a large amount of inorganic residues (45.5%), protein (6.4%), lipid (2.5%), and neutral sugar (1.2%) (Santos et al., 2009). The highest relative expression level of *SiUCP2* in tube feet could be related to the energy consumption during movement and adhesion, which will be investigated in a follow-up study.

The high relative expression level of *SiUCP2* in gonads may be due to fat metabolism in the gonads. The gonads of sea urchins store fat and are the main sites for fat metabolism. The decomposition of triglycerides produces a large amount of free FAs, resulting in a high expression level of *SiUCP2* in the gonads, which was consistent with previous studies (Medvedev et al., 2002; Li et al., 2010). Sea urchin gonads contain nutritive phagocytes (NPs), which are versatile somatic cells that provide structural and nutritional microenvironments for germinal cells throughout sea urchin gametogenesis. In addition to mobilizing stored nutrients, NPs also continue to accumulate additional nutrients. NPs may also devour remaining eggs or sperm, to simply recover nutrients (Walker et al., 2013). *SiUCP2* may be expressed in NPs and participate in nutrient metabolism. Sea urchins feed mainly on large algae such as kelps, Wakame, and sea lettuces. Some algae contain toxins, and reactive oxygen species (ROS) are inevitably produced during the oxidation of toxins in sea urchins. Echtay et al. (2002) found that peroxides promoted *UCP2* gene expression, while a high expression of *UCP2* effectively inhibited overproduction. The pharynx of the sea urchin is contained in Aristotles' lantern. The pharynx, stomach, and intestine constitute the digestive tract of the sea urchin. It is an important place for digestion and absorption in the body. The digestive tract has a certain fat content and immune functions, which prevent pathogenic and poison invasion. Algae food with certain toxins enter the gastrointestinal tract through the mouth to be digested, and the coelomic fluid of sea urchins is not only involved in transport, secretion, and buffering but, more importantly, is involved in immune defense. Some studies have confirmed that amebocyte in sea urchin coelomic fluid play a role in phagocytosis to kill non-self-substances and mediate cytotoxic activity via the production of ROS (Ito et al., 1992). Therefore, low levels of *SiUCP2* expression in the coelomocytes, Aristotles' lantern and stomach may be related to ROS.

Lipids, including fat, phospholipids, and sterols, play a crucial role in individual life activities. The accumulation of total lipids, unsaturated FAs, and phospholipids in marine animals is essential for gonadal and early development (Palacios et al., 2007; Farhoudi et al., 2011). Previous studies have shown that the use of FAs in embryos varied with embryonic development stages (Yao and Zhao, 2006). During the early development of stingray embryos for example, high PUFA levels occur in the blastula stage, the gastrula stage and organogenesis, indicating that more PUFAs are needed during these periods to support the energy required for embryonic development (Yao et al., 2009). To understand the expression of the *SiUCP2* gene and its role in the development of embryos in *S. intermedius*, the relative expression levels of *SiUCP2* gene at different development stages of *S. intermedius* were determined using qRT-PCR. The results show that *SiUCP2* was expressed at all development stages in sea urchins. Decreased *SiUCP2* expression in early embryonic development may be related to fat utilization as an energy source, as developing embryos use their lipid reserves to meet their energy needs (Coulibaly et al., 2006). During the gastrula stage in sea urchins, the intestine is differentiated and they begin to take in external nutrients and FAs are synthesized. Meanwhile, lipid metabolism is relatively enhanced, so *SiUCP2* gene expression was slightly increased (Zuo et al., 2016). When sea urchins develop into prism larvae, they begin to float. When they develop into two-arm larvae, the range of the sea urchin movement is expanded, and the digestive tract is formed in the larvae, so their food intake is increased, thus lipid and energy metabolism are enhanced (Chang et al., 2004). Therefore, the expression level of *SiUCP2* gene was increased. When sea urchins undergo metamorphosis to the juvenile period, they became benthic, and their food changes from planktonic single-celled algae to benthic diatoms. Their food intake tends to be stable, so does the lipid metabolism and balanced. Therefore, the expression level of the *SiUCP2* gene was relatively decreased.

The intestines of sea urchins may be the site for FA synthesis (Han et al., 2019) and the gonads of sea urchins are the place where lipids are stored (Zuo et al., 2016). To understand clearly the role of *SiUCP2* in lipid metabolism, the difference in expression of

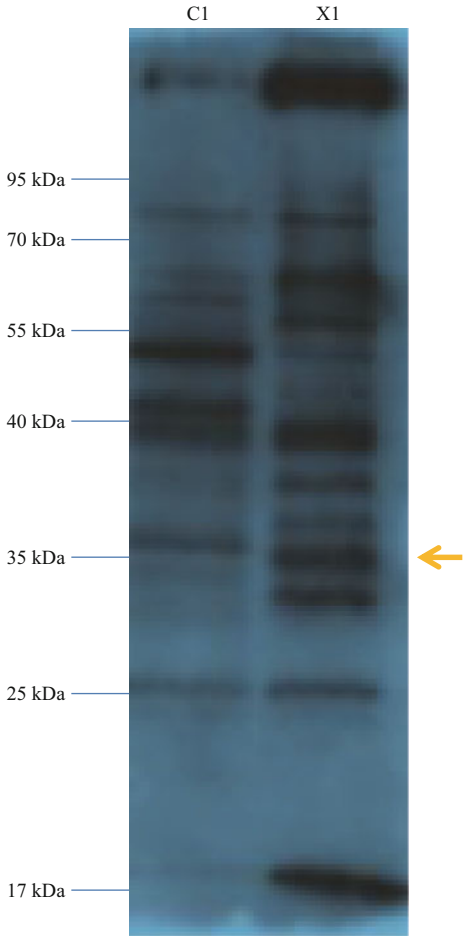


**Fig.7 Pre-experiment of expression of the SiUCP2 protein in the intestine and gonad of *S. intermedius***

C1: the intestine; X1: the gonads; arrow: the destination band.

*SiUCP2* in the intestines and gonads during starvation was analyzed using qRT-PCR. A previous study showed that *UCP2* expression in the lungs and stomach of starved or polysaccharide-treated mice were increased (Pecqueur et al., 2001). In this study, as the starvation period was prolonged, *SiUCP2* expression showed a pattern of first decreasing, then increasing, and decreasing again in the intestine and gonads. The sea urchins used food storage as energy after starvation of 0–7 d, and their metabolism was slowed down. Therefore, the expression level of *SiUCP2* was decreased after starvation for 0–7 d. When starvation was extended to 7–14 d, the expression levels of *SiUCP2* were gradually increased in the intestine and gonads, and the expression level in the gonads was greatly increased, suggesting that sea urchins may maintain their life activities by consuming lipids and proteins in the gonads and intestine after 7–14 d of starvation, which further indicated that *SiUCP2* played a role in lipid metabolism (Solanes et al., 2003; Tang et al., 2013),

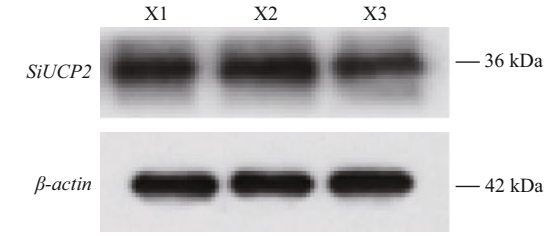


**Fig.8 Expression of the SiUCP2 protein in gonads of *S. intermedius* (*n*=3)**

X1, X2, and X3 represent the gonads of the three sea urchins.

and *UCP2* reduced ROS production that mediated oxidative damage in mitochondria (Echtay et al., 2002). After 14–21 d, the nutrients in sea urchins are exhausted therefore *SiUCP2* expression gradually decreases.

Song (2002) used in-situ hybridization to detect the expression of *UCP2* in mouse liver. The results show that *UCP2* was not expressed in mouse hepatocytes but expressed in macrophages under normal healthy conditions. When treated with lipopolysaccharides (LPS), *UCP2* was expressed in mouse hepatocytes. In this study, DIG-labeled specific *SiUCP2* probes were designed to perform fluorescence in-situ hybridization in the intestines and gonads of *S. intermedius*. The results show that *SiUCP2* was expressed in the intestine and gonads, which is consistent with our qRT-PCR results. Owing to the scattered positive signals in the gonads, it was presumed that *SiUCP2* was expressed in gonadal nutritive phagocytes, which is consistent with the findings of Larrouy et al. (1997). In the future, study of role of *SiUCP2* in the gonads will be conducted.

In this study, the antibodies for SiUCP2 of *S. intermedius* were prepared successfully. Subsequently, we extracted the UCP2 protein and used Western blot to detect its expression in the intestines and gonads of *S. intermedius*. The results of SDS-PAGE show that the SiUCP2 protein with a molecular weight of 36.11 kDa was successfully obtained. This size was very similar to the molecular weight of human UCP2 (Pecqueur et al., 1999) and Chinese perch (Wen et al., 2002). Furthermore, the SiUCP2 protein was expressed in the gonads. The above-mentioned results are consistent with our in-situ hybridization results, which further demonstrated that UCP2 was closely related to lipid and energy metabolism.

Based on the results of this study, we speculated that the *SiUCP2* gene might be related to the fatty acids synthesis of *S. intermedius*. In the future, we will continue to pay attention to *SiUCP2*, study which fatty acids are produced by *SiUCP2*, clarify the

mechanism of *SiUCP2* in the synthesis of fatty acids, improve the content of fatty acids in sea urchins, and further improve the gonadal quality of sea urchins. We hope to combine molecular technology with aquaculture in the future to select and breed more nutritious and economic value of *S. intermedius*.

## 5 DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

## 6 AUTHOR CONTRIBUTION

Jun DING, Bing HAN, and Lingshu HAN contributed to the conception of the study. Lingshu HAN and Zijiao QUAN performed the experiments. Lingshu HAN, Zijiao QUAN, Xiaofang HUANG, and Beichen DING performed the data analyses. Lingshu HAN drafted the manuscript. Lingshu HAN, Jun DING, Bing HAN, and Heng WANG revised the manuscript. Jun DING and Yaqing CHANG approved the final version.

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## Electronic supplementary material

Supplementary material (Supplementary Tables S1–S4) is available in the online version of this article at https://doi.org/10.1007/s00343-020-0181-8.