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Effects of RNA Interference with *Acetyl-CoA Carboxylase* Gene on Expression of Fatty Acid Metabolism-Related Genes in *Macrobrachium rosenbergii* under Cold Stress

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Abstract: *Macrobrachium rosenbergii* is a warm water species, and low temperature is a limiting factor for its growth and survival. In order to explore the role of the *acetyl-CoA-carboxylase* (*ACC*) gene in response to the cold stress of *M. rosenbergii*, we investigated the effects of RNA interference (RNAi) with the *ACC* gene on the expression of fatty acid metabolism-related genes and the mortality of *M. rosenbergii* under cold stress. The results showed that different siRNA sequences and different injection concentrations had different inhibiting effects on *ACC* gene expression, and siRNA-III with an injection concentration of 2.0 μg/g (siRNA/prawn body weight) had the best interference effect. With the optimal siRNA and the optimal concentration under cold stress, the expressions of three fatty acid metabolism-related genes, *FabD*, *echA*, and *ACOT*, were generally significantly down-regulated. Compared to negative (scrambled-siRNA) and blank (PBS) control groups, the expression of *FabD* in the interference group was extremely significantly down-regulated at 12 h in the hepatopancreas and at 18 h in the muscles and gills; *EchA* was highly significantly down-regulated at 6 and 12 h in the muscles and gills; and *ACOT* was extremely significantly down-regulated and kept declining in the gills. Within 6–18 h after injection under cold stress, the mortality rate of the siRNA interference group (75%) was much lower than that of the negative (95%) or blank control group (97.5%), and all prawns died after 24 h. In conclusion, RNA interference with the *ACC* gene inhibited the expression of some fatty acid metabolism-related genes, and could partly improve the tolerance of *M. rosenbergii* to cold stress, indicating that the *ACC* gene might play an important role in the response of *M. rosenbergii* to cold stress.

Keywords: *Macrobrachium rosenbergii*; *ACC*; RNA interference; cold stress

Key Contribution: ACC is a key enzyme in fatty acid metabolism, mainly regulating fatty acid synthesis and β-oxidation, and plays an important role in fat deposition. The present results confirmed that the expression of fatty acid metabolism-related genes in *M. rosenbergii* was affected by RNA interference with the *ACC* gene, which might play a crucial role during cold stress response.

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1. Introduction

As an important environmental factor, water temperature can affect the growth, development, survival, and distribution of aquatic organisms [1]. Poikilothermic aquatic animals are particularly sensitive to changes in water temperature. When the water temperature exceeds their tolerance range, stress reactions will occur, such as moving slowly, reduced feeding, loss of balance, and even death. Lipids are essential nutrients for aquatic animals [2] and serve as biofuels for energy. Therefore, the metabolic regulation of lipids

plays a crucial role in aquatic animals' response to cold stress [3]. As an important lipid in the animal body, fatty acids, including saturated and unsaturated fatty acids, are the main components of cell membranes. Some studies have shown that cold stress can affect the lipid metabolism of aquatic animals [4,5] and cause changes in relevant indexes of the lipid metabolism [6].

The giant freshwater prawn *Macrobrachium rosenbergii*, native to southeast Asia and belonging to the genus *Macrobrachium* of the family Palaemonidae, is well-known worldwide for its high nutritional and economic value [7]. As a poikilothermal aquatic animal, *M. rosenbergii* is sensitive to water temperature, with an optimum survival temperature of 18–34 °C [8,9]. Its ability to withstand low temperatures is poor, and its vitality decreases when the water temperature is lower than 17 °C [10,11]. In most regions of China, the water temperature in winter is lower than the survival threshold for *M. rosenbergii*, leading to strict limitations on the cultivation of this species [12]. Therefore, improving the cold tolerance of *M. rosenbergii* is crucial and urgent for the development of its industry.

Low temperatures can induce cold stress in animals, and the damage caused by low temperatures can be mitigated by regulating the expression of the genes associated with low temperature tolerance [13–15]. Fatty acid anabolic metabolism is catalyzed by acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), and fatty acid synthase II (Fab). As the rate-limiting enzyme in the first step of fatty acid synthesis [16], ACC has multiple functional domains [17]. It can regulate fatty acid synthesis and β-oxidation, playing an essential role in lipid metabolism and fat deposition [18]. Acyl-CoA Thioesterase (ACOT) can catalyze the hydrolysis of acyl-CoA in fatty acid anabolism, thereby releasing fatty acids from acyl-CoA [19]. In studies of cancer cells, it was found that the *ACOT* gene is significantly down-regulated in response to genotoxic stress treatments such as ionizing radiation (IR) and Doxorubicin (Doxo) [20]. β-Ketoacyl-Acyl Carrier Protein Synthase III (FabD), involved in the bacterial fatty acid synthesis process, can catalyze the chemical reaction between β-ketoacyl-acyl and carrier protein, facilitating the synthesis of new fatty acids [21]. Epoxide hydrolase (echA), the key enzyme in fatty acid β-oxidation, is involved in epoxide metabolism and plays an important role in the biodegradation of environmental pollutants and drug metabolism.

Previous studies on the *ACC* gene in medicine have found that cancer cells can reprogram the lipid metabolism during malignant progression, and ACC plays a key role in regulating the growth and differentiation of leukemic initiation cells. In addition, adenosine monophosphate-activated protein kinase (AMPK) can inhibit ACC, reduce the consumption of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) in fatty acid synthesis, and generate homeostasis [22–24]. At present, there are few studies on the changes in *ACC* gene expression in aquatic animals under cold stress. Some studies have been reported on fishes. For example, Mininni et al. (2014) applied transcriptome sequencing to *Sparus aurata* under cold stress and found that a large number of lipid-metabolism-related genes in the liver were involved in the response to cold stress [4]. *ACC* gene expression decreased under cold stress in *Takifugu fasciatus* [3] and *Scophthalmus maximus* [25]. Additionally, some scholars found that *ACC* gene expression also decreased in *Cherax quadricarinatus* under cold stress [26]. Therefore, cold stress has a significant impact on the expression of lipid-metabolism-related genes, indicating that regulating lipid metabolism may be a crucial mechanism for aquatic animals to adapt to cold stress.

RNA interference (RNAi) is a form of molecular biotechnology that specifically inhibits gene expression by degrading specific mRNA through double-stranded RNA (dsRNA) mediation [27,28]. Presently, RNAi technology has been widely used to verify gene function in crustaceans [29]. For example, Tan et al. [30] utilized RNAi technology to knock out the *M. rosenbergii Insulin-like Androgenic Gland* (*MrIAG*) gene, which regulates male sex differentiation and gonadal development in *M. rosenbergii*. The results indicated that knocking out the *MrIAG* gene could impact the sex differentiation of *M. rosenbergii*, leading to the transformation of males into neo-females. A similar study was also reported in *Litopenaeus vannamei* [31].

In our previous research, the *Aacetyl CoA carboxylase* (*ACC*) gene, a key gene in the lipid and energy metabolism pathway, was enriched based on the transcriptome data of *M. rosenbergii* under cold stress [32]. In order to investigate the role of the *ACC* gene in the cold stress response of *M. rosenbergii*, RNAi technology was applied in this study to interfere with the *ACC* gene. Real-time quantitative PCR (RT-qPCR) was used to detect the expression changes of the *ACC* gene, meanwhile, we investigated the effects of interference with the *ACC* gene on the survival of *M. rosenbergii* and the expression of other genes related to fatty acid metabolism, including the *ACOT*, *FabD*, and *echA* genes in *M. rosenbergii* under cold stress. Since the hepatopancreas and muscles are vital organs for energy conversion and metabolism, and gills are directly in contact with water and are sensitive to changes in water temperature, we chose these three tissues to test the gene expression under cold exposure. The present study can provide a reference for further analyses of the molecular mechanism of lipid metabolism in *M. rosenbergii*.

2. Materials and Methods

2.1. Prawns Used for Experiments

Around 600 healthy and vigorous prawns with a body weight of about 8 g were randomly selected from Jiangsu Shufeng Prawn Breeding Co., Ltd. (Gaoyou, China), and all selected prawns were temporarily raised in the recirculating water system for about two weeks. The RNAi experiment was performed after all prawns were adapted to the environment and no prawn death occurred. Tissues including the hepatopancreas, muscles, and gills were taken and temporarily stored in liquid nitrogen for 24 h, and then they were transferred to the refrigerator at −80 °C for reserve during experiments.

2.2. Experimental Methods

2.2.1. Screening the Optimal siRNA

The partial *ACC* gene sequence was obtained from the transcriptome data of our previous study, and sequencing data were stored in the NCBI SRA database, with the SRA entry number PRJNA596398 [32,33]. Three different siRNA sequences (siRNA-I, siRNA-II, and siRNA-IIII, Table 1) were designed using an online website (https://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designO, accessed on 16 June 2023) and synthesized by Wuhan Tianyi Huiyuan Co., Ltd. (Wuhan, China) (Table 1). Four groups were established, including one blank control group and three interference groups designed based on three different siRNAs. Thirty prawns were involved in each group, all of which were cultured under the same conditions, keeping the water temperature stable at 25 °C. Prawns in the blank control group were injected with 100 μL of PBS, while individuals in the three interference groups were injected with 100 μL of siRNA at a concentration of 1.2 μg/g (siRNA/prawn body weight). The injection was administered into the third abdominal segment using a 1 mL disposable sterile syringe. Hepatopancreas and muscle tissues from three prawns in each group were collected at 2 h, 6 h, 12 h, and 24 h after injection, respectively. The total RNA was extracted and reverse-transcribed into cDNA for the expression analysis of the *ACC* gene, and the optimal siRNA was selected based on this analysis result for the next experiment.

Table 1. siRNA sequences and RT-qPCR primers used in the present study.

Primer Name	siRNA/Primer Sequences (5′–3′)	SiRNA or Primer Length/bp	TM/°C
siRNA-I	CAGCACCUGGCAAGCUUCUUAAUUA	25	/
siRNA-II	GGUCGAGAUGUGAUAUGCAUUUGUA	25	/
siRNA-III	CCCAGUUAGGUGGUGUACAAAUUAU	25	/
scrambled-siRNA	CGUACAUGCGUAGUAUAGAUGUACU	25	/
ACC-F	TGGCAGCATTGGAGGTGTA	19	60.8
ACC-R	GATGAGATGATGGCAGCAGAA	21	60.8

Table 1. *Cont.*

Primer Name	siRNA/Primer Sequences (5′–3′)	SiRNA or Primer Length/bp	TM/°C
ACOT-F	TCCACTGTCCTGTLTTCAT	19	56.8
ACOT-R	CGTCAACCTCACCATTCC	19	56.8
FabD-F	GCATTGGTGTAGCAGGTT	19	63.0
FabD-R	GTLTTGAATLTGGTCCGTAT	20	63.0
echA-F	GGCTLTCAATGCTLTATGT	19	59.3
echA-R	CCTGCTGTGCTGTAATCA	18	59.3
18S-F	TATACGCTAGTGGAGCTGGAA	21	/
18S-R	GGGGAGGTAGTGACGAAAAAT	21	/

2.2.2. Screening the Optimal siRNA Injection Concentration

After confirming the optimal siRNA, the optimal injection concentration was determined. We established one blank control group and three interference groups corresponding to three injection concentrations (1.2 μg/g, 2.0 μg/g, and 2.8 μg/g) of the optimal siRNA. Thirty prawns were involved in each group. The experimental procedure and sampling setting were the same as those in Section 2.2.1. Based on the *ACC* gene expression analysis, the optimal siRNA injection concentration of C μg/g (herein, C means the optimal siRNA concentration value which will be determined by this concentration gradient test) was selected for the following experiment.

2.2.3. RNAi with *ACC* Gene in *M. rosenbergii* under Cold Stress

Three groups were established, including an interference group, negative control group, and blank control group, with 60 prawns (among which, 20 were used for sampling and 40 for statistics of mortality) allocated to each group. The water temperature decreased from 25 °C to 18 °C at a rate of 4 °C/h by adding ice, and then gradually dropped to 16 °C at a rate of 2 °C/h, where it was maintained. Injections were performed when the water temperature stabilized at 16 °C. These injections were conducted on the third abdominal segment of the prawns in the three groups with 100 μL of the optimal siRNA (interference group), scrambled-siRNA (negative control group), and PBS (blank control group). The optimal concentration of C μg/g was selected as the injection concentration of the siRNA and scrambled-siRNA. At each time point of 2 h, 12 h, and 18 h after injection, tissue samples including the hepatopancreas, gills, and muscles of 3 individuals from each group were taken for subsequent analyses of gene expression, and three technical replicates were performed for RT-qPCR analyses of each sample. Meanwhile, the survival of the prawns was observed during the experiment.

2.2.4. RNA Extraction and Expression Analyses of the Target Genes

The total RNA was extracted from the hepatopancreas, muscles and gills of each individual using RNAiso Plus (Takara, Dalian, China). The extracted RNA concentrations were determined by the Qubit® RNA Assay Kit (Life Technologies, San Jose, CA, USA), and the purity and integrity were examined by a NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA) and Nano 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA). At the same time, agarose gel electrophoresis was used to assess the extracted RNA. The PrimeScriptTMRT reagent Kit with gDNA Eraser (Takara, Dalian, China) was used for reverse transcription with 1.5 μg of total RNA according to its instructions.

The sequences of the target genes *ACC*, *ACOT*, *FabD*, and *echA* were searched from the existing transcriptome data [32], and 18S rRNA was used as the reference gene. Primer 6.0 software was used to design the primers (Table 1) of each gene for RT-qPCR. The total volume of the reaction system was 25 μL, including 12.5 μL of TB Green® Premix Ex TaqTM II (2×), 8.5 μL of ddH_2O, 1 μL of forward primer, 1 μL of reverse primer, and 2 μL of cDNA (10 pg~100 ng cDNA). The reaction procedure included pre-denaturation at 95 °C for 3 min, and then 40 cycles of denaturation at 95 °C for 5 s and annealing at 59.3–63 °C (depending

on different primers, see Table 1 for details) for 30 s. $2^{-\Delta\Delta CT}$ methods were used to calculate the relative expression levels of the target genes.

2.2.5. Statistical Analysis

All statistical analyses were performed by one-way analysis of variance in SPSS 25.0. The significance level was set at $p < 0.05$, and the extremely significant level was $p < 0.01$. GraphPad Prism 9 software was applied for plotting.

3. Results

3.1. Optimal siRNA for Interference with ACC Gene

The results of the optimal siRNA screening showed that the interference effects of different siRNAs on the *ACC* gene were different (Figure 1). In the hepatopancreas (Figure 1A), siRNA-I inhibited the expression of the *ACC* gene only at 12 h after the siRNA injection, while siRNA-II interfered with the *ACC* gene both at 12 h and 24 h, and siRNA-III had an interference effect at 2 h, 6 h, 12 h, and 24 h, but there were no significant differences ($p > 0.05$) compared with PBS and the other two siRNAs. In general, it seemed that siRNA-III had the best interference effect among the three siRNAs, and it could maintain the inhibiting effect for 24 h, followed by siRNA-II, with siRNA-I having the weakest effect.

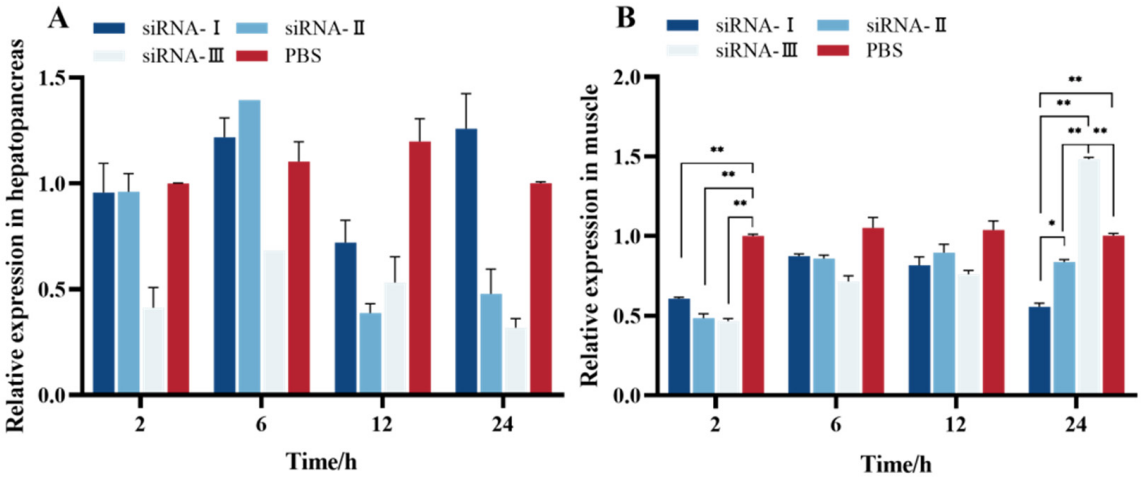

Figure 1. Relative expression level of *ACC* gene in *M. rosenbergii* after RNAi with *ACC* gene by three different siRNAs. (**A**): in hepatopancreas and (**B**): in muscles. * indicates a significant difference ($p < 0.05$) and ** indicates an extremely significant difference ($p < 0.01$).

In the muscles (Figure 1B), all three siRNAs had a down-regulated *ACC* gene expression with extremely significant differences compared to the PBS group at 2 h after the siRNA injection ($p < 0.01$). At 6 h and 12 h after the injection, the three siRNAs interfered with the *ACC* gene, but with no significant differences compared to the PBS group ($p > 0.05$). At 24 h, the siRNA-III group had a significantly higher expression of the *ACC* gene than other groups ($p < 0.01$), with no interference effect, while both siRNA-I and siRNA-II significantly inhibited the *ACC* gene expression. On the whole, the interference effects of both siRNA-I and siRNA-II were maintained for 24 h, while the effect of siRNA-III was only maintained for 12 h. However, the interference efficiency of siRNA-III on the *ACC* gene was the best within the first 12 h. Table A1 lists the changes in the gene expression of the *ACC* gene, showing the interference effects of different siRNAs compared with the control group.

Considering its interference effect in the hepatopancreas and muscles of *M. rosenbergii*, siRNA-III was selected for the next experiment, screening the optimal concentration of siRNA.

3.2. Optimal Concentration of siRNA for Interference with ACC Gene

Figure 2 shows the *ACC* gene expression after being inhibited by three different concentrations of siRNA-III. In the hepatopancreas (Figure 2A), all three concentrations had interference effects at 2 h, 6 h, 12 h, and 24 h after the injection. At 2 h after the injection, the *ACC* gene expression had significant differences between the 1.2 μg/g of siRNA and PBS groups and between the 2.8 μg/g of siRNA and PBS groups, with 2.8 μg/g of siRNA having the lowest expression; at 6 h and 12 h, the 1.2 μg/g group had the best interference effect with extremely significantly (at 6 h, $p < 0.01$) and significantly (at 12 h, $p < 0.05$) lower expressions than the PBS group. However, at 24 h, the *ACC* gene expression in the 1.2 μg/g group increased rapidly, with no significant difference from the PBS group ($p > 0.05$), and both the 2.0 μg/g and 2.8 μg/g groups evidently had a significant interference effect ($p < 0.01$). These results indicate that the interference effects of all three concentrations on the *ACC* gene could be maintained for 24 h, with 2.0 μg/g and 2.8 μg/g being better.

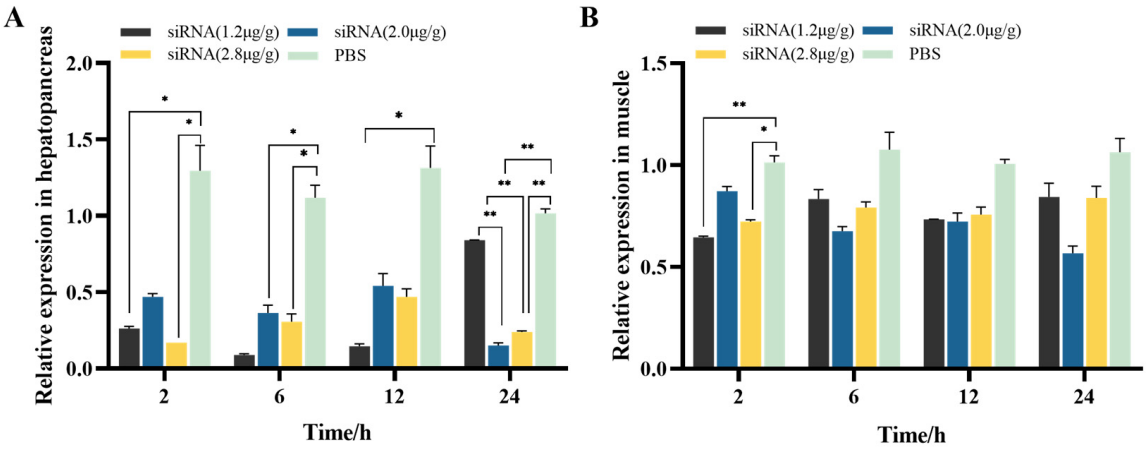

Figure 2. Relative expression level of *ACC* gene in *M. rosenbergii* after RNAi with *ACC* gene by three different siRNA-III concentrations. (**A**): in hepatopancreas and (**B**): in muscles. * indicates a significant difference ($p < 0.05$) and ** indicates an extremely significant difference ($p < 0.01$).

In the muscles (Figure 2B), the three concentrations inhibited the *ACC* gene at all four time points, but only the 1.2 μg/g group had an extremely significant difference ($p < 0.01$) and the 2.8 μg/g group had a significant difference ($p < 0.05$) from the PBS group at 2 h. For the 1.2 μg/g group, the *ACC* gene expression showed an upregulated trend on the whole with the extension of the interference time, but was lower than the PBS group, while for the 2.0 μg/g group, the trend of *ACC* gene expression was down-regulated. For the 2.8 μg/g group, there was no obvious expression change at four time points, but all were lower than the PBS group. Table A2 lists the changes in the *ACC* gene expression, showing the interference effects of different siRNA concentrations compared with the control group.

Considering the interference effect of the three concentrations of siRNA-III in the hepatopancreas and muscles, 2.0 μg/g was selected as the optimal injection concentration for the following experiment.

3.3. Effects of RNAi with ACC Gene on Mortality of M. rosenbergii under Cold Stress

After being injected with 100 μL of 2.0 μg/g of siRNA-III (interference group), scrambled-siRNA (negative control group), and PBS (blank control group) under cold stress, the mortality of the prawns in each group was observed, and the results are shown in Table 2 and Figure 3. The results showed that no mortality occurred in the first 6 h after injection. Within 12 h, the cumulative mortality of the negative control group was the highest, reaching 52.5%, while the mortality of the blank control group and interference group were similar, both lower than 30%. After 12 h, the mortality sharply increased, and at 18 h, the cumulative mortality of the negative control group and blank control group reached 95%

and 97.5%, respectively, but only 75% in the interference group. At 24 h after the injection, all prawns died.

Table 2. *Mortality* of *M. rosenbergii* after RNAi with *ACC* gene under cold stress.

Time (after Injection)	Interference Group (siRNA-III)		Negative Control Group (Scrambled-siRNA)		Blank Control Group (PBS)	
	Cumulative Deaths	Cumulative Mortality	Cumulative Deaths	Cumulative Mortality	Cumulative Deaths	Cumulative Mortality
2 h	0	0	0	0	0	0
6 h	0	0	0	0	0	0
12 h	9	22.50%	21	52.50%	11	27.50%
18 h	30	75.00%	38	95.00%	39	97.50%
24 h	40	100.00%	40	100.00%	40	100.00%

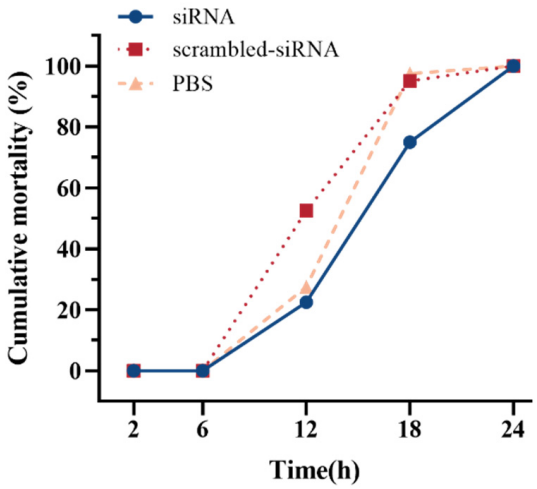

Figure 3. Cumulative mortality of *M. rosenbergii* after RNAi with *ACC* gene under cold stress.

3.4. Effects of RNAi with ACC Gene on Expression of the Fatty Acid Metabolism-Related Genes in M. rosenbergii under Cold Stress

As shown in Figure 4, the *ACC* gene expression after interference showed differences in the three tissues, and all showed a certain inhibiting effect. In the hepatopancreas, the *ACC* gene expression decreased rapidly with the duration of cold stress. At 18 h, the average relative expression in the interference group was only 12.3% of that in the PBS group, and showed significant differences from the negative control group at 12 h and from the PBS group at 18 h ($p < 0.05$). In the muscles, the *ACC* gene expression in the interference group was lower and more stable than in the negative control group, with significant differences compared to both control groups at 12 h and 18 h ($p < 0.05$). In the gills, the expression in the interference group at 2 h was extremely significantly lower than both control groups ($p < 0.01$), and then increased but was still extremely significantly lower than the PBS group at 12 h ($p < 0.01$). The *ACC* gene expression changes at different times after interference with the *ACC* gene under cold stress are shown in Table A3.

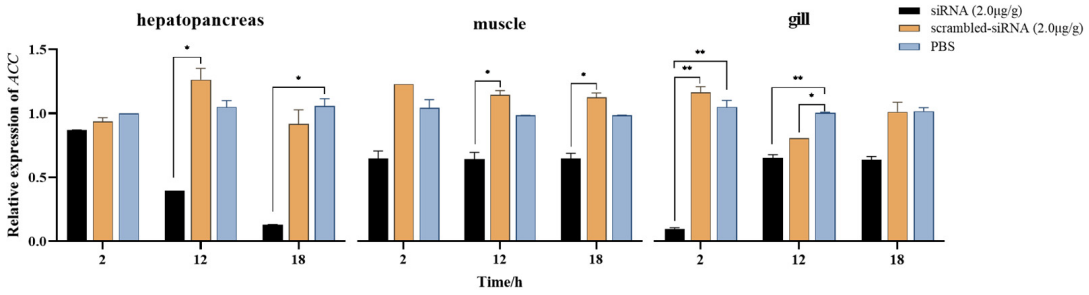

Figure 4. *ACC* gene expression at different time points and in different tissues after RNAi with *ACC* gene in *M. rosenbergii* under cold stress. * indicates a significant difference ($p < 0.05$) and ** indicates extremely significant difference ($p < 0.01$).

Figure 5 demonstrates the *ACOT* gene expression in three tissues after RNAi with the *ACC* gene. In the hepatopancreas, the expression in the interference group was extremely significantly lower than in both control groups at 2 h and 12 h ($p < 0.01$), and then increased and recovered to a normal level at 18 h. In the muscles, the expression was the lowest at 2 h, being evidently significantly different from both control groups ($p < 0.05$), and then increased at 12 h, with no significant difference compared to both control groups ($p > 0.05$). In the gills, the expression was significantly lower than in both control groups at all times ($p < 0.05$), showing a trend of gradually declining with the duration of cold stress, with the lowest at 18 h. The *ACOT* gene expression changes at different times after the *ACC* gene interference under cold stress are shown in Table A4.

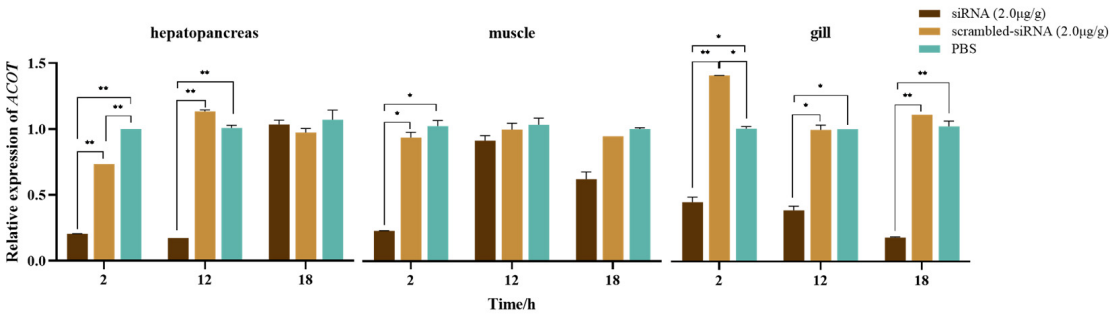

Figure 5. *ACOT* gene expression at different time points and in different tissues after RNAi with *ACC* in *M. rosenbergii* under cold stress. * indicates a significant difference ($p < 0.05$) and ** indicates extremely significant difference ($p < 0.01$).

The expression of the *echA* gene is shown in Figure 6. It shows that the expression in the interference group was lower than in both control groups in all three tissues at all three time points. In the hepatopancreas, the expression in the interference group reached the lowest at 18, with a significant difference compared to both control groups ($p < 0.05$). In the muscles, the expression in the interference group was extremely significantly lower than in both control groups at 2 h and 12 h ($p < 0.01$). At 18 h, the expression slightly increased, but was still lower than in both control groups. In the gills, the expression in the interference group showed a trend of first increasing and then decreasing, reaching the lowest at 18 h, being extremely significantly lower (at 2 h, $p < 0.01$) or significantly lower (at 12 h and 18 h, $p < 0.05$) than both control groups. The *EchA* gene expression changes at different times after *ACC* gene interference under cold stress are shown in Table A5.

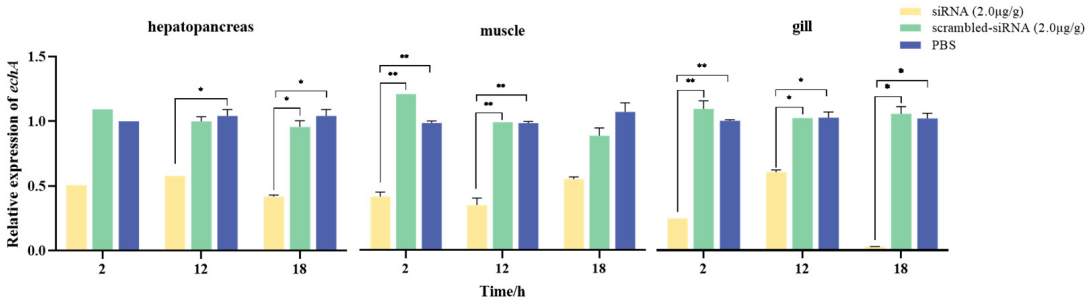

Figure 6. *EchA* gene expression at different time points and in different tissues after RNAi with *ACC* in *M. rosenbergii* under cold stress. * indicates a significant difference ($p < 0.05$) and ** indicates extremely significant difference ($p < 0.01$).

Figure 7 displays the expression of the *FabD* gene. The expression in the interference group was significantly or extremely significantly lower than in both control groups in three tissues, with the lowest in the muscles. In the hepatopancreas, the expression reached the lowest at 12 h after interference, and had a very significant difference compared to both control groups ($p < 0.01$). In the muscles, the expression in the interference group was very low for all three time points, with significant (at 2 h and 12 h, $p < 0.05$) or extremely significant (at 18 h, $p < 0.01$) differences compared to both control groups. In the gills, the expression in the interference group increased first and then decreased, and was extremely significantly (at 2 h and 18 h, $p < 0.01$) lower than both control groups. The *FabDA* gene expression changes at different times after the *ACC* gene interference under cold stress are shown in Table A6.

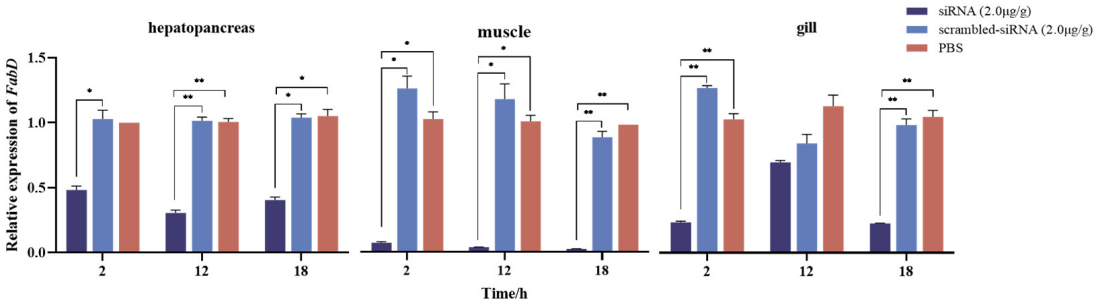

Figure 7. *FabD* gene expression at different time points and in different tissues after RNAi with *ACC* in *M. rosenbergii* under cold stress. * indicates a significant difference ($p < 0.05$) and ** indicates extremely significant difference ($p < 0.01$).

4. Discussion

4.1. Factors Affecting the Efficacy of siRNA Interference

The current findings from screening the optimal siRNA revealed that three distinct siRNAs designed for the same sequence of the *ACC* gene in *M. rosenbergii* exhibited varying interference effects on the expression of the *ACC* gene. The variations in the gene expression of *ACC* due to the interference effects of three different siRNAs were similar. Considering its expression in the hepatopancreas and muscles, siRNA-III had better interference effects than siRNA-I and siRNA-II. Ge et al. [34] designed three siRNAs, targeting the *insulin-like androgenic gland* (*IAG*) gene of *Procambarus clarkii*. They injected them with the same dose and found that only IAG-siRNA3 significantly inhibited the expression of the *IAG* gene, while the other two siRNAs had no inhibitory effect. Huang et al. [35] injected the same dose of three siRNAs to interfere with the *pyridoxal kinase* gene in *Bombyx mori* and found that the interference effects of the three siRNAs were significantly different. Some other studies also obtained similar results [36,37]. It was inferred that different siRNAs targeting the same gene might have various target sites, resulting in distinct interference effects on gene expression.

In addition, the interference effect of siRNA is also influenced by its injection dose. Tan et al. [30] designed four concentrations of siRNA, including 0.1 μg/g, 0.5 μg/g, 1.5 μg/g, and 3.0 μg/g, to interfere with the *MrIR* gene in *M. rosenbergii*. They found that the concentration of 0.5 μg/g had the most effective silencing effect on the *MrIR* gene. Wu et al. [36] suggested that a high dose of siRNA could potentially result in detargeting and cytotoxicity. Subsequently, a higher concentration of siRNA does not always result in a better interference effect. In this study, considering the interference effect of siRNA in the hepatopancreas and muscles, the concentration of 2.0 μg/g of siRNA-III was selected as the optimal injection concentration among three concentrations, including 1.2 μg/g, 2.0 μg/g, and 2.8 μg/g, which is consistent with the above conclusion. The interference of three concentrations of siRNA on *ACC* was compared with that of the PBS group, and the regulated state was down.

4.2. The Influence of Interfering with the ACC Gene on Fatty Acid Metabolism

ACC is a key enzyme in fatty acid metabolism, primarily regulating fatty acid synthesis and β-oxidation, and plays a crucial role in fat deposition [18]. Farkas et al. [38] confirmed that the fatty acid metabolism of *Cyprinus carpio* is highly sensitive to changes in environmental temperature. They found that the fish's body temperature decreased, leading to an increase in long-chain unsaturated fatty acids in phospholipids. Under cold stress, the β-oxidation of fatty acid was enhanced, while fatty acid synthesis was weakened, and the expression of the *ACC1* gene was significantly down-regulated in *Scaphesthes macrolepis* [39]. Our previous study, based on a transcriptome sequencing analysis, also showed that, under cold stress, differentially expressed genes were enriched in lipid and energy-related metabolic pathways. Pathways such as unsaturated fatty acid biosynthesis and fatty acid metabolism were identified, leading to the screening of some candidate genes related to low-temperature tolerance. These genes include *acetyl-CoA carboxylase* (*ACC*), *β-Ketoacyl-Acyl Carrier Protein Synthase III* (*FabD*), *fatty acid synthase* (*FAS*), and *long-chain acyl-CoA synthetase* (*ACSL*) [32].

Acyl-CoA thioesterase (ACOT) is a key regulator in fatty acid metabolism [40]. Its functions can be divided into three categories: firstly, it removes the metabolites generated by fatty acid β-oxidation and promotes the degradation of fatty acid; secondly, it hydrolyzes fatty acid CoA to form FFA and CoA, maintaining them at an appropriate level to ensure normal activities; and thirdly, as a transcription factor ligand, it participates in endocytosis, intracellular transport, signal transduction, and other activities [41]. Under cold exposure or a low ATP level, non-esterified fatty acids are activated to form fatty acyl-CoA in organisms. This, in turn, upregulates the activity of ACOT [42]. The results of an omics association analysis showed that the expression of unsaturated-fatty-acid-synthesis-related genes (*ACOT*) was upregulated under low temperature stress [32]. However, the present study showed that, after interference with the *ACC* gene, the overall *ACOT* gene expression exhibited a down-regulated trend under cold stress. This might be attributed to the down-regulation of *ACC* gene expression, leading to decreased CoA utilization and subsequent down-regulated expression of the *ACOT* gene due to negative feedback regulation.

Fatty acid β-oxidation is the primary source of energy in organisms [43]. Epoxide hydrolase (echA) is a crucial enzyme in fatty acid β-oxidation, and its abnormal metabolism can lead to disorders in fatty acid metabolism [44]. In the current study, the expression of the *echA* gene was down-regulated after interfering with the *ACC* gene in *M. rosenbergii* under a cold challenge. Previous transcriptome results showed that the expression of the *echA* gene was upregulated under low temperatures [32]. This inconsistency may have resulted from the decrease in fatty acid synthesis after *ACC* gene knockdown, leading to a reduction in the fatty acid β-oxidation rate and the down-regulation of the *echA* gene expression.

As a key enzyme in the second pathway of fatty acid synthesis, β-Ketoacyl-Acyl Carrier Protein Synthase III (FabD) catalyzes the conversion of Malonyl-CoA and acyl carrier protein (ACP) into malonyl-monoacyl ACP, which serves as an extended substrate for the fatty acid synthesis process [45,46]. After the *ACC* gene was knocked down under cold

stress, the expression of the *FabD* gene was down-regulated. However, the transcriptome results showed that the *FabD* gene expression was upregulated under cold stress [32]. It was inferred that the inhibited expression of the *ACC* gene reduced malonyl-CoA, contributing to the down-regulated expression of the *FabD* gene.

In addition, this study found that the mortality rate of the siRNA interference group was lower than that of both the negative and blank control groups within 6 to 18 h after injection under cold stress. However, all prawns died after 24 h. It is speculated that interference with the *ACC* gene might improve the cold tolerance of *M. rosenbergii* to some extent. Still, this improvement was not significant at the late stage of interference. The reason for this might be that the reserved fatty acids in the organisms were used to provide energy for β-oxidation. Meanwhile, the synthesis rate of fatty acids decreased due to the down-regulated expression of the *ACC* and *FabD* genes after RNAi with the *ACC* gene. Therefore, the supply of fatty acids was far lower than the body's requirement to resist low temperatures.

5. Conclusions

Different siRNA sequences and different injection concentrations had different interference effects on genes. After interference with the *ACC* gene of *M. rosenbergii* during a cold challenge, the expression of fatty acid metabolism-related genes, including the *FabD*, *echA*, and *ACOT* genes, which were initially up-regulated under cold stress, was down-regulated. The mortality of *M. rosenbergii* in the interference group was relatively lower than that in the control groups, suggesting that interference with the *ACC* gene can improve the cold tolerance of *M. rosenbergii* to a certain extent, but this improvement can only last for a short period of time, which may be due to the supply of fatty acid being lower than that required in later stages of interference. In conclusion, the *ACC* gene might be the key gene for *M. rosenbergii* responding to low-temperature stress, but its detailed function and molecular mechanism need to be further investigated.

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Institutional Review Board Statement: This study was performed according to the Guidelines for the Care and Use of Laboratory Animals developed by the Ministry of Science and Technology (Beijing, China). All experiments were approved by Huzhou University (the ethical approval code: 20190625).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: Zhenglong Xia, Miaoying Cai and Guoliang Yang are employed at Jangsu Shufeng Prawn Breeding Company Limited. The authors declare this conflict of interest did not influence the content and results of the study. The other authors declare no conflict of interest.

Appendix A

Table A1. Changes in *ACC* gene expression showing interference effect of different siRNAs compared with control group (PBS).

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
hepatopancreas	2 h	siRNA-I vs. PBS	−0.043	0.929	down
		siRNA-II vs. PBS	−0.040	0.934	down
		siRNA-III vs. PBS	−0.587	0.246	down
	6 h	siRNA-I vs. PBS	0.115	0.734	up
		siRNA-II vs. PBS	0.291	0.402	up
		siRNA-III vs. PBS	−0.419	0.238	down
	12 h	siRNA-I vs. PBS	−0.478	0.365	down
		siRNA-II vs. PBS	−0.810	0.143	down
		siRNA-III vs. PBS	−0.667	0.218	down
	24 h	siRNA-I vs. PBS	0.258	0.632	up
		siRNA-II vs. PBS	−0.523	0.343	down
		siRNA-III vs. PBS	−0.682	0.225	down
muscles	2 h	siRNA-I vs. PBS	−0.394	<0.01	down
		siRNA-II vs. PBS	−0.517	<0.01	down
		siRNA-III vs. PBS	−0.535	<0.01	down
	6 h	siRNA-I vs. PBS	−0.178	0.394	down
		siRNA-II vs. PBS	−0.193	0.356	down
		siRNA-III vs. PBS	−0.338	0.125	down
	12 h	siRNA-I vs. PBS	−0.222	0.387	down
		siRNA-II vs. PBS	−0.142	0.573	down
		siRNA-III vs. PBS	−0.279	0.282	down
	24 h	siRNA-I vs. PBS	−0.446	<0.01	down
		siRNA-II vs. PBS	−0.166	0.079	down
		siRNA-III vs. PBS	0.480	<0.01	up

Table A2. Changes in *ACC* gene expression showing interference effect of different siRNA concentrations compared with control group.

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
hepatopancreas	2 h	1.2 vs. PBS	−1.033130301	<0.05	down
		2.0 vs. PBS	−0.826504288	0.085	down
		2.8 vs. PBS	−1.125366284	<0.05	down
	6 h	1.2 vs. PBS	−1.030205555	0.060	down
		2.0 vs. PBS	−0.755244227	<0.05	down
		2.8 vs. PBS	−0.812130448	<0.05	down
	12 h	1.2 vs. PBS	−1.166649643	<0.05	down
		2.0 vs. PBS	−0.770975262	0.114	down
		2.8 vs. PBS	−0.843998366	0.088	down
	24 h	1.2 vs. PBS	−0.1767506	0.076	down
		2.0 vs. PBS	−0.865795579	<0.01	down
		2.8 vs. PBS	−0.776727349	<0.01	down
muscles	2 h	1.2 vs. PBS	−0.36783873	<0.01	down
		2.0 vs. PBS	−0.141112979	0.212	down
		2.8 vs. PBS	−0.289875935	<0.05	down
	6 h	1.2 vs. PBS	−0.242709411	0.381	down
		2.0 vs. PBS	−0.400211499	0.165	down
		2.8 vs. PBS	−0.284508805	0.308	down
	12 h	1.2 vs. PBS	−0.273136694	0.109	down
		2.0 vs. PBS	−0.283068528	0.098	down
		2.8 vs. PBS	−0.250016135	0.137	down
	24 h	1.2 vs. PBS	−0.219449812	0.476	down
		2.0 vs. PBS	−0.495745874	0.130	down
		2.8 vs. PBS	−0.22408862	0.467	down

Table A3. Expression changes in *ACC* gene at different times after interference with *ACC* gene under cold stress.

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
hepatopancreas	2 h	siRNA vs. scrambled-siRNA	−0.070	0.430	down
		siRNA vs. PBS	−0.132	0.159	down
	12 h	siRNA vs. scrambled-siRNA	−0.867	<0.05	down
		siRNA vs. PBS	−0.653	0.07	down
	18 h	siRNA vs. scrambled-siRNA	−0.788	0.07	down
		siRNA vs. PBS	−0.928	<0.05	down
muscles	2 h	siRNA vs. scrambled-siRNA	−0.591	0.061	down
		siRNA vs. PBS	−0.399	0.171	down
	12 h	siRNA vs. scrambled-siRNA	−0.508	<0.05	down
		siRNA vs. PBS	−0.345	0.106	down
	18 h	siRNA vs. scrambled-siRNA	−0.486	<0.05	down
		siRNA vs. PBS	−0.342	0.072	down
gills	2 h	siRNA vs. scrambled-siRNA	−1.062	<0.01	down
		siRNA vs. PBS	−0.949	<0.01	down
	12 h	siRNA vs. scrambled-siRNA	−0.152	0.076	down
		siRNA vs. PBS	−0.350	<0.01	down
	18 h	siRNA vs. scrambled-siRNA	−0.374	0.173	down
		siRNA vs. PBS	−0.380	0.168	down

Table A4. Expression changes of *ACOT* gene at different time after interference with *ACC* gene under cold stress.

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
hepatopancreas	2 h	siRNA vs. scrambled-siRNA	−0.530	<0.01	down
		siRNA vs. PBS	−0.795	<0.01	down
	12 h	siRNA vs. scrambled-siRNA	−0.961	<0.01	down
		siRNA vs. PBS	−0.835	<0.01	down
	18 h	siRNA vs. scrambled-siRNA	0.061	0.809	up
		siRNA vs. PBS	−0.036	0.887	down
muscles	2 h	siRNA vs. scrambled-siRNA	−0.706	<0.05	down
		siRNA vs. PBS	−0.793	<0.05	down
	12 h	siRNA vs. scrambled-siRNA	−0.084	0.725	down
		siRNA vs. PBS	−0.121	0.615	down
	18 h	siRNA vs. scrambled-siRNA	−0.326	0.093	down
		siRNA vs. PBS	−0.380	0.059	down

Table A4. *Cont.*

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
gills	2 h	siRNA vs. scrambled-siRNA	−0.959	<0.01	down
		siRNA vs. PBS	−0.557	<0.05	down
	12 h	siRNA vs. scrambled-siRNA	−0.609	<0.05	down
		siRNA vs. PBS	−0.613	<0.05	down
	18 h	siRNA vs. scrambled-siRNA	−0.929	<0.01	down
		siRNA vs. PBS	−0.841	<0.01	down

Table A5. Expression changes in *EchA* gene at different times after interference with *ACC* gene under cold stress.

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
hepatopancreas	2 h	siRNA vs. scrambled-siRNA	−0.013	0.964	down
		siRNA vs. PBS	0.080	0.774	up
	12 h	siRNA vs. scrambled-siRNA	−0.420	0.052	down
		siRNA vs. PBS	−0.464	<0.05	down
	18 h	siRNA vs. scrambled-siRNA	−0.539	<0.05	down
		siRNA vs. PBS	−0.624	<0.05	down
muscles	2 h	siRNA vs. scrambled-siRNA	−0.809	<0.01	down
		siRNA vs. PBS	−0.581	<0.01	down
	12 h	siRNA vs. scrambled-siRNA	−0.653	<0.01	down
		siRNA vs. PBS	−0.646	<0.01	down
	18 h	siRNA vs. scrambled-siRNA	−0.343	0.258	down
		siRNA vs. PBS	−0.531	0.102	down
gills	2 h	siRNA vs. scrambled-siRNA	−0.845	<0.01	down
		siRNA vs. PBS	−0.754	<0.01	down
	12 h	siRNA vs. scrambled-siRNA	−0.419	<0.05	down
		siRNA vs. PBS	−0.422	<0.05	down
	18 h	siRNA vs. scrambled-siRNA	−1.027	<0.05	down
		siRNA vs. PBS	−0.991	<0.05	down

Table A6. Expression changes in *FabD* gene at different times after interference with *ACC* gene under cold stress.

Tissue	Time	Comparison Group	Log2 Foldchange	*P*adj	Regulated
hepatopancreas	2 h	siRNA vs. scrambled-siRNA	−0.546	<0.05	down
		siRNA vs. PBS	−0.518	0.051	down
	12 h	siRNA vs. scrambled-siRNA	−0.709	<0.01	down
		siRNA vs. PBS	−0.701	<0.01	down
	18 h	siRNA vs. scrambled-siRNA	−0.635	<0.05	down
		siRNA vs. PBS	−0.646	<0.05	down

Table A6. *Cont.*

Tissue	Time	Comparison Group	Log2 Foldchange	*Padj*	Regulated
muscles	2 h	siRNA vs. scrambled-siRNA	−1.212	<0.05	down
		siRNA vs. PBS	−0.971	<0.05	down
	12 h	siRNA vs. scrambled-siRNA	−1.162	<0.05	down
		siRNA vs. PBS	−0.988	<0.05	down
	18 h	siRNA vs. scrambled-siRNA	−1.009	<0.01	down
		siRNA vs. PBS	−0.974	<0.01	down
gills	2 h	siRNA vs. scrambled-siRNA	−1.033	<0.01	down
		siRNA vs. PBS	−0.791	<0.01	down
	12 h	siRNA vs. scrambled-siRNA	−0.146	0.663	down
		siRNA vs. PBS	−0.433	0.223	down
	18 h	siRNA vs. scrambled-siRNA	−0.755	<0.01	down
		siRNA vs. PBS	−0.819	<0.01	down

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