Characterization of Free Fatty Acid Receptor 4 and Its Involvement in Nutritional Control and Immune Response in Pacific Oysters (Crassostrea gigas)

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ABSTRACT: Free fatty acid receptor 4 (FFAR4) has various physiological functions, including energy regulation and immunological homeostasis. We examined the only FFAR4 homologue in the Pacific oyster Crassostrea gigas (CgFFAR4), which functions as a sensor of long-chain fatty acids. CgFFAR4 is 1098 bp long and contains a seven-transmembrane G protein-coupled receptor domain. CgFFAR4 expression was high in the hepatopancreas, but it was downregulated after fasting, indicating that it plays an essential role in food digestion. Lipopolysaccharide stimulation downregulated CgFFAR4 level, probably as an immune response of the animal. Reduced glycogen level alongside decreased insulin receptor, insulin receptor substrate, and C. gigas glycogen synthase transcription levels after CgFFAR4 knockdown revealed that CgFFAR4 was involved in the regulation of fatty acid and glycogen levels via the insulin pathway. Accordingly, this is the first study on an invertebrate FFAR and provides new insights into the role of this receptor in immune response and nutritional control.

1. INTRODUCTION

Fatty acids (FAs) are carboxylic acids with long saturated or unsaturated aliphatic chains.1 FAs are essential dietary nutrients for animals and important cellular components.2 Circulating plasma FAs are called free fatty acids (FFAs) (aka, nonesterified FAs). FFAs bind to glycerol and form triglycerides, a major energy source in animals.3 FAs provide one of the main flavors in food. For example, certain short-chain FFAs have been used as indicators of cheese flavor to assess the quality of Swiss cheese.4 Additionally, FFAs also function as essential physiological molecules that participate in various FHA-receptor-mediated signaling pathways.5,6 FHA receptors, which are G protein-coupled receptors (GPCRs), contain the typical seven-transmembrane domain and function in maintaining the energy and immune homeostasis.

To date, four FFARs have received considerable attention in mammals. Of the identified FFARs, FFAR2 (GPR43) and FFAR3 (GPR40) are activated by short-chain FAs (SCFAs),6,7 whereas FFAR1 (GPR40) and FFAR4 (GPR120) function as the sensors of medium- FFAR1 (GPR40) and long-chain FAs (FFAR1 and FFAR4).8 FFAR4 is widely expressed in various tissues, especially high in digestive or immune-related organs.9 Consistent with this expression pattern, FFAR4 has multiple physiological functions, such as energy regulation,9 immunological homeostasis,10 and neuronal functions.11

FFAR4 is highly expressed in mammalian proinflammatory macrophages and mature adipocytes.12 Recently, a marked decrease in FFAR4 levels has been shown to be induced by interleukin (IL)-1β and tumor necrosis factor α (TNF-α), two major proinflammatory cytokines in human adipocytes13 and macrophages.8 Additionally, lipopolysaccharide (LPS) inhibits macrophage activation and FFAR4 expression in macrophages, thereby enhancing the proinflammatory response.6,14 However, ω-3 FAs or GW9508 (GPR120 agonist) treatment broadly represses the LPS-stimulated inflammatory response through GPR120.8 These results indicate that FFAR4 plays an important role in the immune response.

To date, no FFAR has been identified in invertebrates, unlike in mammals. Herein, we identified and cloned the only FFAR homolog in Crassostrea gigas (CgFFAR4) to investigate the role of this receptor in the immune response and nutritional control. In this study, we investigated CgFFAR4 mRNA levels in various tissues during homeostasis and after LPS stimulation or during fasting. To further analyze the function of CgFFAR4, we knocked down CgFFAR4 and characterized the resulting phenotypes. This is the first study about an FFAR in an invertebrate and significantly improves our mechanistic understanding of FFARs and their roles in immune response and nutritional control.
Table 1. Primer Used in the Study

| sequence ID | forward | sequences (5′-3′)                  | reverse | application |
|-------------|---------|-----------------------------------|---------|-------------|
| CgFFAR4     | forward | ATGCCAATGTTGAAAAGGTCTCC          | reverse | CDS amplification |
| qCgFFAR4    | forward | TCGGAACCGGCTCAAAAG               | reverse | q-RT-PCR*    |
| qCgGN       | forward | CAGAAGACGAGAATGACGGCA            | reverse |             |
| qCgG5       | forward | GTACGCAAGGACCAAACT               | reverse |             |
| qCgEF       | forward | AGTCACCAAGGGTCACAGAAT            | reverse |             |
| qCgCIR      | forward | TCCGACGTATTCTTCTTTGCGAT          | reverse |             |
| qCgRS       | forward | GGAGTTGAGGGTGAC                  | reverse |             |
| qCgGP       | forward | GATAAGTGGGTTCTGACGA              | reverse |             |

*qPCR, quantitative polymerase chain reaction.

2. MATERIALS AND METHODS

2.1. Animals. Pacific oysters (C. gigas) in this study were collected from the aquaculture basement of Jiaonan, Qingdao, Shandong, China. No specific permissions were required for this study, and all the experiments were conducted with approval from the Experimental Animal Ethics Committee, Institute of Oceanology, Chinese Academy of Sciences, China. Eight types of oyster tissue, including the adductor muscle, intestine, gonads, stomach, mantle, gill, hepatopancreas, and labial palps, were dissected from 18 live oysters in July 2017. Samples were immediately placed into liquid nitrogen and then preserved at −80 °C.

2.2. Characterization of the Full-Length CgFFAR4 and Phylogenetic Analysis. The CgFFAR4 coding sequence was downloaded from the National Center for Biotechnology Information (NCBI) and confirmed by PCR amplification using the specific primers. The open reading frame (ORF) was predicted using ORFfinder in the NCBI database (https://www.ncbi.nlm.nih.gov/orffinder/). To construct the phylogenetic tree, FFAR4 sequences from various species were downloaded from the NCBI, and multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/). A phylogenetic tree of FFAR4 was constructed in MEGAX using the neighbor-joining algorithm. The reliability of the estimated tree was evaluated with 1000 bootstrap replicates by using the Poisson model. The molecular weight and theoretical isoelectric point of the predicted CgFFAR4 were computed using ProtParam (http://web.expasy.org/protparam/).

2.3. RNA Extraction and qRT-PCR. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to investigate the tissue expression pattern of CgFFAR4. Total RNA from embryos or larvae in 10 typical developmental stages (zygote, two-cell, early morula, morula, posttrophectoderm, gastrula, Nauplius, and larval) was isolated using the RNAprep Pure Tissue Kit (Tiangen, Beijing, China), following the protocol of the manufacturer. The RNA integrity and concentration were assessed with agarose gel electrophoresis and UV spectrophotometer, respectively. For cDNA construction, 1 μg of total RNA was reverse-transcribed using the PrimeScript RT Reagent kit with a gDNA Eraser (Takara Bio Inc., Shiga, Japan), following the instructions of the manufacturer. Afterward, qRT-PCR was performed in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The 20 μL reaction volume contained 10 μL of 2× SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 5.8 μL RNase-free water, 0.4 μL of each 10 mM gene-specific primer (Table 1), 0.4 μL of 50× ROX reference dye, and 3 μL oyster cDNA template at 1:20 dilution. The thermocycling program was set at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. C. gigas elongation factor (CgEF) was used as an internal control, and the primers used were the same as in our previous study (15) (Table 1). A melting curve analysis was performed at the end of the reaction to confirm amplification specificity. Gene transcript levels were normalized to the expression of the internal control, and the comparative 2^{-ΔΔCt} method was used to analyze sample gene expression using Prism 8.0 software (GraphPad).

2.4. CgFFAR4 Knockdown (KD) in the Oyster and Glycogen Content Assay. To investigate the role of FFAR4 in the glycogen metabolism of Pacific oyster, we knocked down CgFFAR4 expression via CgFFAR4-specific siRNAs (GenePharma Inc., Shanghai). We designed three siRNAs against CgFFAR4 and a negative control siRNA, which had no homology with CgFFAR4. Each siRNA was verified to knockdown CgFFAR4. To achieve the maximum KD efficiency, the three siRNAs were combined at equal amounts. The siRNA sequences are shown in Table 1. The siRNAs were dissolved in RNase-free water to reach 100 μg/mL. We obtained 30 oysters from the aquaculture basement of Jiaonan, Qingdao, Shandong, China, and acclimatized them in the same environment for seven days. Thirty oysters were randomly divided into three groups, namely, experimental (injected with the siRNA mixture), blank control (injected with only water), and negative control. The oysters were anesthetized by mixed seawater (MgCl2: seawater, 500: 500 mL) following the protocol of Suquet. The three groups of oysters were injected on the adductor muscle. Seventy-two hours later, five oysters from each group were sampled into liquid nitrogen with a piece of gill used for RNA extraction and the other tissues for glycogen content. The mRNA levels of FFAR4 and the genes involved in the insulin pathway or glycogen metabolism were evaluated using qRT-PCR. The glycogen contents were
Table 2. Sequences of siCgFFAR4s

| sequence ID | sequences (5′−3′) | sequences (5′−3′) |
|-------------|------------------|------------------|
| siCgFFAR4-963 | CCGAGUCAAACCGCAGAAUUTT | AAUUCCGAGGUUUGACUCGGTT |
| siCgFFAR4-209 | GCACAGUCACCAACUACUUTT | AAGUAGUUGGUCAUCUGCTT |
| siCgFFAR4-102 | GCUCGGGUUACCUUACAUUTT | AAUGUAGGUAACCCGAGCTT |

Figure 1. Multiple sequence alignment of CgFFAR4 sequence with the FFAR4 sequences from Homo sapiens (>NP_859529.2 for long and >NP_001182684.1 for short), Rattus norvegicus (NP_001040553.1), and Mus musculus (NP_861413.1). The completely conserved residues across all the aligned species are shaded in black, and the residues labeled in gray indicate ≥75% identity. Dots refer to gaps.

Figure 2. Neighbor-joining phylogenetic tree of FFAR4. The phylogenetic tree was constructed using MEGA X software, based on FFAR4 sequences from C. gigas and other species, including Mus musculus FFAR4 (NP_861413.1), Rattus norvegicus FFAR4 (NP_001040553.1), Homo sapiens GPR120-L (NP_859529.2), Homo sapiens GPR120-S (NP_001182684.1), Anas platyrhynchos FFAR4 (NP_001297760.1), Astyanax mexicanus FFAR4 (XP_022537849.1), Lingula anatina FFAR4 (XP_0133904.1), Pipra flicauda FFAR4 (XP_027598952.1), Xenopus tropicalis FFAR4 (XP_02032042.0), Cimex lectularius FFAR4-like (XP_012440460.1), Crassostrea virginica FFAR4-like (XP_022332461.1), Malanaphis sacchari FFAR4-like (XP_022504151.1), Mizuhopecten yessoensis FFAR4-like (XP_021365995.1), Octopus vulgaris FFAR4-like (XP_029654917.1), Onthophagus taurus FFAR4-like (XP_022910859.1), and Zootermopsis nevadensis FFAR4-like (XP_021919308.1).
analyzed using a kit for liver and muscle glycogen content detection from Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China. The samples were freeze-dried and ground into a powder before the analysis. The procedure was as follows: 0.5 μg of the sample was added to alkaline liquor and incubated for 20 min at 100 °C and diluted 16-fold with water. Subsequently, color reagent was added to the hydrolysate and incubated at 100 °C for 5 min. Finally, the optical density of the samples was measured at 620 nm with a path length of 1 cm and normalized using the blank control (Table 2).

2.5. Oyster Starvation and LPS Treatment. To evaluate the expression of FFAR4 under starvation, 50 oysters were cultured at 18 °C with filtered seawater daily changed without food for 10 days and then refed with marine algae (Arthrosira platensis) daily for 14 days. The hepatopancreas and visceral ganglia of five oysters were sampled at days 0, 5, and 10 of starvation, as well as 7 and 14 days after starting refeeding.

To activate the immune response, LPS was injected into the oyster adductor muscle. One hundred and twenty oysters were anesthetized using mixed seawater, and then 100 μL of LPS (0.5 mg mL⁻¹) and phosphate-buffered saline (control pH = 7.4) were injected into the adductor muscle. The gills of 15 oysters were randomly sampled at 0, 6, 12, 24, 48, and 72 h postinjection.

2.6. Statistical Analysis. Statistical analyses were performed using Prism 8.0 software (GraphPad). Comparisons between two groups were performed using the unpaired, two-tailed t-test. Multiple comparisons among more than two groups were performed using one-way ANOVA, followed by Bonferroni’s posthoc test or two-way ANOVA. P-values were two-tailed, and values <0.05 were considered statistically significant. *P < 0.05, **P < 0.01, and ***P < 0.001 are designated in all the figures with *, **, and ***, respectively.

3. RESULTS

3.1. FFAR4 Characterization in C. gigas. The full-length cDNA of the FFAR4 in C. gigas was cloned. CgFFAR4 ORF was 1098 bp, encoding 365 amino acids. The predicted molecular weight was 4.1 kDa, and the theoretical pI was 9.71. CgFFAR4 was found to include a seven-transmembrane GPCR domain from position 41–320, as revealed by SMART (Simple Modular Architecture Research Tool) analysis (http://smart.embl-heidelberg.de/smart), which indicated that CgFFAR4 belonged to the GPCR family (Figure 1). CgFFAR4 shares 62.96% similarity with human GPR120 (FFAR4).

To analyze the relationship of CgFFAR4 with the homologs in other species, a phylogenetic tree was constructed with FFAR4 sequences from C. gigas and 16 other species downloaded from the NCBI. Invertebrate and vertebrate FFAR4s were found to be separated into two distinct clusters (Figure 2).

3.2. CgFFAR4 mRNA Levels in Various Tissues and Different Developmental Stages. Among the embryonic and larval developmental stages, CgFFAR4 mRNA level increased at the D-shape larval stage (Figure 3A). Among the eight tissues, CgFFAR4 was found to be relatively highly transcribed in the hepatopancreas, stomach, and intestine, which constitutes the oyster digestive tract (Figure 3B).

3.3. CgFFAR4 Expression after Fasting or LPS Treatment. Figure 4A indicates that the relative mRNA level of CgFFAR4 to that of CgEF in the hepatopancreas and visceral ganglia significantly declined after 5–10 days of fasting and gradually recovered in 7–14 days after refeeding. After LPS injection, CgFFAR4 mRNA level significantly decreased relative to that under phosphate-buffered saline injection (Figure 4B).

3.4. Glycogen Content of Oysters after CgFFAR4 KD. To further elucidate the involvement of CgFFAR4 in glycogen content in oysters, we knocked down FFAR4 in the oysters. Seventy-two hours after siRNA injection, a piece of gill was collected to inspect KD efficiency, and the other tissues were harvested for glycogen content assessment. Figure 5A indicates that 72 h after siRNA injection, CgFFAR4 was significantly downregulated compared with the level in the negative control. In the five knocked-down oysters, the glycogen contents were also significantly decreased (Figure 5B).

3.5. Expression Levels of Insulin-Signaling- or Glycogen-Synthesis-Related Genes after CgFFAR4 KD. FFAR4 has been reported to participate in insulin resistance. To further investigate the relationship between FFAR4 and the insulin pathway, we analyzed the expression levels of insulin-signaling- or glycogen-synthesis-related genes after FFAR4 KD in the oysters. Figure 6A indicates that insulin receptor (CIR) and insulin receptor substrate (IRS) decreased upon FFAR4 KD in C. gigas. CIR and IRS sequences were obtained from the oyster genome. Among the three key regulators of the glycogen content, glycogen synthase level decreased upon CgFFAR4 KD (Figure 6B). Glycogenin (CgGN) participates in glycogen synthesis, and CgFFAR4 KD did not significantly alter the level of glycogen phosphorylase (CgGP), the main protein involved in glycogenolysis (Figure 6B).
4. DISCUSSION

In oysters, glycogen and FFAs have been reported to be involved in the response pathways to stresses, such as temperature and toxicity stresses. Our previous study has identified that glycogen content is closely related to the FA content. However, it has remained unknown how FAs and glycogen interact with each other in oysters. In this study, we demonstrated that FFAR4 is a GPCR [also known as seven-(pass)-transmembrane domain receptor], and, together with the insulin signaling pathway, it is involved in the interaction between FAs and glycogen content in Pacific oysters. As an n-3 unsaturated FFA sensor, FFAR4 also contributes to the anti-inflammatory response. The inhibition of FFAR4 expression induced by LPS stimulation has been reported to enhance the proinflammatory reaction. In Pacific oysters, we found that LPS downregulated CgFFAR4, and this effect may contribute to the immune response of the oysters.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** FFAR4 expression relative to the EF expression in the oysters after fasting or LPS treatment. (A) CgFFAR4 mRNA level relative to the CgEF mRNA level in the oyster hepatopancreas and visceral ganglia after fasting for 5 or 10 days and then refeeding for 7 or 14 days. F5d: fasting for 5 days, F10d: fasting for 10 days, R7d: re-feeding for 7 days, and R14d: re-feeding for 14 days. Data are displayed as mean ± SE from five individuals. The mRNA levels were calculated using the comparative $2^{-\Delta\Delta Cq}$ method with the control samples as the reference samples in the two groups. (B) CgFFAR4 mRNA level relative to CgEF mRNA level in the oysters injected with phosphate-buffered saline (PBS) or LPS, 0, 6, 12, 24, 48, or 72 h postinjection. The mRNA levels were calculated using the comparative $2^{-\Delta\Delta Cq}$ method with the 0-h samples as the reference samples in the two groups. *P < 0.05; **P < 0.01, and ***P < 0.001 (two-way ANOVA).

![Figure 5](https://example.com/fig5.png)

**Figure 5.** CgFFAR4 knockdown efficiency and glycogen content after the knockdown. Data are displayed as mean ± SE from five individuals. ***P < 0.001 (one-way ANOVA). (A) CgFFAR4 mRNA level relative to CgEF mRNA level 72 h after CgFFAR4 siRNA injection. The mRNA level in the negative control was considered as the reference. (B) Whole-tissue glycogen content after CgFFAR4 knockdown.

![Figure 6](https://example.com/fig6.png)

**Figure 6.** The mRNA levels of insulin-signaling- or glycogen-synthesis-related genes after CgFFAR4 knockdown. (A) The relative mRNA levels of the insulin receptor and insulin receptor substrate in the H2O, negative control or siCgFFAR4 knockdown group. (B) The relative mRNA levels of glycogen synthase, glycogenin, and glycogen phosphorylase in the three groups. Data are displayed as mean ± SE from five individuals. *P < 0.05; and **P < 0.01 (two-way ANOVA). The mRNA levels were calculated using the comparative $2^{-\Delta\Delta Cq}$ method with the negative control sample used as the reference.
FFAs are essential dietary components and act as signaling molecules in multiple physiological processes. Multiple FFARs act as sensors for FFAs containing specific carbon chain lengths. These receptors have been reported to mediate physiological functions, such as anti-inflammatory effects, taste preference, and energy regulation. In mammals, the FFAR family comprises FFAR 1–4, formerly known as GPR40, 43, 41, and 120, which are now classified into a new GPCR family. Long-chain saturated and unsaturated FAs activate FFAR1 and FFAR4, whereas SCFAs activate FFAR2 and FFAR3. In the genome of a Pacific oyster, FFAR4 is the only gene annotated as FFAR and has the seven-transmembrane receptor structure similar to mammalian FFARs. The reason why there is only one FFAR homolog in Pacific oysters may be because the specialized FFAR functions observed in mammals can be executed by a single FFAR in evolutionarily low animals such as the oysters because of simpler physiology.

FFAs as nutrients are not only involved in nutrition control but also play an essential role in anti-inflammatory activity. For example, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can function through the cell surface receptor GPR120 to control the inflammatory signaling and related gene expression patterns. Incubation with IL-1β or TNF-α results in a dose-dependent downregulation of the GPR120 mRNA level in human adipocytes. LPS is a major component of the outer membrane of Gram-negative bacteria and can induce a strong immune response in mammalian cells and invertebrates. It is a common immune response stimulator in Pacific oysters. LPS has been reported to be able to activate the p-JNK signaling pathways and to be related to the multiple immune response processes, such as inflammation and apoptosis. In this study, the CgFFAR4 level significantly decreased after the LPS injection versus the phosphate-buffered saline injection, indicating that CgFFAR4 is involved in the anti-inflammatory response of Pacific oysters. In human or mouse cells, FFAR4 expression markedly decreases upon LPS challenge and inhibits LPS-stimulated cytokine expression. These observations indicate that the immune function of FFAR4 is conserved in invertebrates and mammals.

To further investigate the function of CgFFAR4, the expression patterns of CgFFAR4 in the tissues and developmental stages of C. gigas were evaluated by qRT-PCR. C. gigas undergoes the typical molluscan developmental stages with mosaic development, including trochophore and veliger larvae and metamorphosis. The oysters were sampled during each of the 10 developmental stages (from eggs to umbro larvae) for 14 days. CgFFAR4 mRNA level increased at the D-shape larval stage, which is the essential period for the development of the velum responsible for larval ingestion and swimming. Additionally, CgFFAR4 was variably expressed in all the major tissues. The highest expression was observed in the hepatopancreas, which is the digestive tract of mollusks. This organ combines the hepatic functions and pancreatic digestion observed in vertebrates and follows with the intestine and stomach. These data indicated that CgFFAR4 was closely related to the digestive function of the oysters, consistent with the data about the mammalian homologs. GPR120 is closely related to the digestive function, and it is present at the mRNA level in rodent pancreas. It is also expressed throughout the digestive tract of the goats. Decreased activities of digestive enzymes (such as amylase, lipase, and pepsin) have previously been observed in mollusks upon starvation treatment. Since then, this treatment has been applied to the study of digestive function in marine mollusks. Accordingly, to further investigate the role that CgFFAR4 plays in digestion, we subjected the oysters to starvation. The high CgFFAR4 levels in the hepatopancreas and stomach and CgFFAR4 downregulation upon starvation (Figure 4A) indicate that this protein plays an essential role in food digestion in C. gigas. Decreased CgFFAR4 transcription after starvation may be because of the low level of ingestion and digestion or a stress response.

In addition to being a digestion-related organ, the hepatopancreas also serves as the energy reserve and a metabolic organ in marine bivalves. Our previous data have indicated that FFAs are also involved in the regulation of the glycogen content. Thus, FFAR4 may be a mediator between FFAs and the glycogen metabolism. To further investigate how FFAR4 regulates FFAs and glycogen content, we performed CgFFAR4 knockdown with siCgFFAR4. The CgFFAR4 transcriptional level significantly decreased with qRT-PCR, following with reduction in the glycogen content.

It has been reported that FFAR4 is related to the insulin pathway. Insulin content decreases upon knocking out FFAR4 in mouse islets. The insulin signaling plays a critical role in the glycogen regulation in bivalve mollusks. In this study, CgCIR (C. gigas insulin receptor) and CgIRS (C. gigas insulin receptor substance) were downregulated alongside CgGS (C. gigas glycogen synthase) in FFAR4-KD oysters, whereas the expression levels of CgGN (C. gigas glycogenin) and CgGP (C. gigas glycogen phosphorylase) did not significantly differ between the control and KD groups (Figure 6). In Pacific oysters, glycogen synthase is closely linked to glycogen regulation and acts as a molecular marker of the glycogen metabolism. We hypothesize that FFAR4 KD in C. gigas induces the downregulation of glycogen content by reducing glycogen synthesis because of the involvement of the insulin signaling.

In conclusion, the present study characterized the only FFAR in C. gigas (CgFFAR4). FFAR4 in C. gigas has conserved structural motifs, such as seven-transmembrane helices. It plays an essential role in the immune response and is involved in the regulation of glycogen and FFA contents via the insulin signaling. CgFFAR4 may be the key regulator of the mechanism by which FFA content is associated with glycogen content and contributes to the immune response. Further research is required to confirm whether there is only one FFAR homolog in Pacific oysters and whether it can replace the functions of the FFARs in mammals. This study serves as the first step in the characterization of FFAs and provides an insight into their roles in the immune response and nutritional control in invertebrates.

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Funding

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (grant no. XDA24030105); Major Scientific and Technological Innovation Project of Shandong Province (2019JZZY010813); National Natural Science Foundation of China (31530079), and the Earmarked Fund for China Agriculture Research System (CARS-49).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to F.X. of the Institute of Oceanology Chinese Academy of Science for providing the fasting samples of Pacific oysters. The authors thank all the members of the laboratory for valuable discussions.

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