



# Impacts of acute cold-stress in Pacific white shrimp *Litopenaeus vannamei*: investigating the tissue-specific stress resistance response

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## Abstract

Temperature is one of the key environmental factors in *Litopenaeus vannamei* (Boone, 1931) culture, affecting the growth and survival of shrimp. Our previous study found that stress resistance related pathways (endoplasmic reticulum stress, immune, and apoptosis) were significantly increased under low temperature stress, but studies about their regulation are still limited. Therefore, the histological changes and the genes expression related to the above pathways in *L. vannamei* were examined under conditions of cooling from 28 to 13 °C with a cooling rate of 2.5 °C /2 h and then maintained at 13 °C for 12 h. The results showed that the gill and intestine of *L. vannamei* were obviously damaged after low temperatures stress, while the muscle tissue of shrimp did not change significantly. Most of the relative expression of unfolded protein response (UPR) pathway (*GRP78*, *ATF6*, *IRE1*, and *PERK*), immune (*proPO*), as well as apoptosis (*CASP3*) gene in each tissue were significant changed after cooling. The results suggested that muscle may be more structurally stable than other tissues in shrimp. The stress resistance mechanisms could be activated under low temperatures, and 18 °C may be the critical point for *L. vannamei* to adjust its anti-stress system to maintain organism homeostasis. Moreover, the results also reflect that the stress resistance system is tissue-specific under low temperature stress in *L. vannamei*. This study can enrich our understanding of the responses at the histological and genetic levels in shrimp under low temperature stress, helping to promote further development of shrimp culture industry.

**Keywords** *Litopenaeus vannamei* · Temperature · UPR pathway · Immunity · Apoptosis

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## Introduction

*Litopenaeus vannamei* (Boone, 1931), also known as Pacific white shrimp or white-leg shrimp, is the most economically valuable cultured shrimp in the world (FAO 2018). Temperature, one of the key environmental factors in shrimp culture, may affect the growth and survival of shrimp by affecting their tissue structure and the expression of genes (Wang et al. 2020d), proteins (Wang et al. 2020c), and metabolites (Duan et al. 2021). However, many extreme climate events, which were associated with drastic temperature fluctuation, have occurred often since global climate change and caused negative effects to animals' health (He et al. 2018; Zhang et al. 2019). Studies have shown that *L. vannamei* begins to exhibit the phenomenon of feeding cease when the water temperature drops to 18 °C, and the survival of *L. vannamei* is challenged when the water temperature drops to 13 °C (Fan et al. 2013; Huang et al. 2017). Therefore, it is important to study the response of *L. vannamei* under low temperature stress to promote the healthy culture of *L. vannamei*.

Environmental stresses such as pH (Tao et al. 2016), ammonia nitrogen (Liang et al. 2016), nitrite (Li et al. 2019), and sulfide (Duan et al. 2018b) could cause tissue structural changes or damage in shrimp. However, most of the relevant studies have focused histological analysis on the hepatopancreas of shrimp. Our previous studies also showed that low temperature stress caused the necrosis of hepatic tubules and the rupture of basement membranes in shrimp hepatopancreas (Wang et al. 2019b). The gill and intestine, which undertake many important physiological functions, are also important organs of shrimp, but their histological changes under low temperature remain to be investigated. Besides, few studies have involved the muscle histological changes of shrimp under environmental stress.

Endoplasmic reticulum stress (ER stress) is triggered by cells experiencing endogenous or exogenous stress. Unfolded protein response (UPR) is a self-protective mechanism that can promote the survival of cells in response to ER stress (Chen and He 2019). Glucose-regulated protein 78 kDa (GRP78), also known as immunoglobulin-binding protein (BIP), is the central regulator of the UPR (Nakka et al. 2010). Among the pathways activated by ER stress, the activating transcription factor 6 (ATF6) pathway, the inositol-requiring enzyme-1 (IRE1) pathway, and the protein kinase RNA (PKR)-like ER kinase (PERK) pathway are the three most studied classical pathways (Mori 2009), while the studies were mainly focused on vertebrates (Moncan et al. 2021; Ren et al. 2021b), the ER stress in invertebrates need to be further studied.

What's more, animals' immune systems can be triggered in reaction to environmental stress. The prophenoloxidase (proPO) system is an important component of the immune defense system of invertebrates, which can resist pathogen infection, kill microorganisms, and promote wound healing (Cerenius et al. 2008; Cerenius et al. 2010; Piti et al. 2013). Apoptosis is also an effective defense system against harmful biological stimuli in invertebrates. Cysteine containing aspartate specific proteases (Caspases) are a family of proteases that carry out apoptosis in animals (Shalini et al. 2015). The activation of caspase 3 (CASP3) indicates that apoptosis has entered an irreversible stage (Morishima et al. 2002).

In our previous study, we sequenced the transcriptome of *L. vannamei* under low temperature stress and found that ER stress, immune, and apoptosis-related pathways were significantly increased (Wang et al. 2020a; Wang et al. 2020b), but the specific regulation of these pathways under low temperature stress remains to be investigated deeply. Therefore, in this study, the histological changes of gill, intestine, and muscle in *L. vannamei* were observed under low temperature stress, and the expressions of genes related to the above

pathways were examined. These results could enrich our understanding of the responses at the histological and genetic levels in shrimp under low temperature stress.

## Materials and methods

### Experimental shrimp and culture conditions

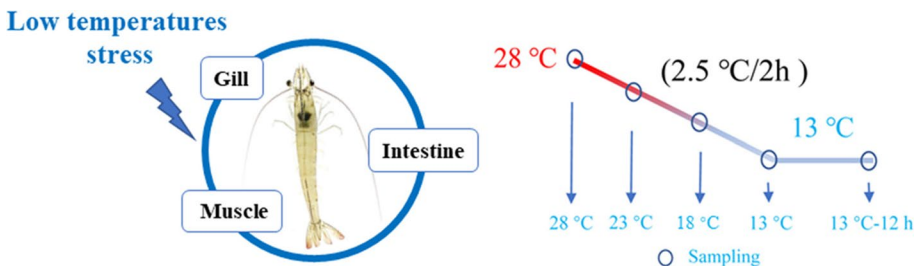
*L. vannamei* were purchased from a commercial farm in Guangzhou (Guangdong, China). The shrimp were placed in a 500 L circulating culture system in our laboratory for 1 week before the experiment. During the acclimation period, the salinity of water in the culture system is 5 ‰, the pH value is  $8.3 \pm 0.1$ , the temperature is  $28 \pm 1$  °C, and the dissolved oxygen is  $7.5 \pm 1.0$  mg/L. Shrimp were fed twice daily with a commercial shrimp feed (Haida Feed, China) at a rate of 5% of the shrimp's body weight. The above culture conditions were consistent with the water and feeding conditions of the commercial farm.

### Low temperature stress

Forty-five healthy and inter-moult *L. vannamei* ( $5.28 \pm 0.50$  g) were randomly divided into three tanks (30 L), and then placed in an artificial climate incubator (Laifu, China). The temperature setting of the incubator was based on our previous study (Wang et al. 2019a), cooling from 28 to 13 °C at a change rate of 2.5 °C/2 h, and then maintained at 13 °C for 12 h.

### Sample collection

There were five sampling points in this experiment, which were 28 °C, 23 °C, 18 °C, 13 °C, and 13 °C for 12 h (Fig. 1). Six shrimp were randomly selected from each sampling point. Three of them were used for histological assay: the gill, intestine, and muscle were collected from the shrimp, with the dendrobranchiate selected from the gills, midgut from the intestine, and second segment from the muscle. The collected tissues were placed in centrifuge tubes with 4% paraformaldehyde and stored at 4 °C for subsequent histological observations. The other three were used for the gene expression assay: 40–50 mg of gill, intestine, and muscle tissues were also collected from shrimp and immediately frozen in liquid nitrogen, then stored at –80 °C for subsequent gene expression analysis.



**Fig. 1** Overview of treatment and sample collection in this study

## Index detection

### Histological analysis

After fixed in paraformaldehyde for 24 h, gill, intestine, and muscle tissues were embedded in paraffin. The middle of each tissue was selected for section. Sections were then stained with hematoxylin and eosin. Histological sections were observed with a microscope (Nikon), and were photographed and analyzed with the Mshot image analysis system.

### Gene relative expression analysis

RNAiso Plus (Takara) was used to extract RNA from each tissue. Then RNA integrity was tested by 1% agarose gel electrophoresis, and its concentration was detected by spectrophotometer (VWR, mySPEC). The first-strand cDNA was synthesized by ReverTra Ace qPCR RT Master Mix (TOYOBO, FSQ-301) according to the manufacturer's instructions. RT-qPCRs were performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, QPS-201) and then assayed on a CFX Connect real-time System (Bio rad, Singapore) instrument. Primer 5.0 (<http://www.premierbiosoft.com/>) was used for primer designing. The primers were presented in Table 1, and  $\beta$ -actin was used as the reference gene.

### Statistical analyses

Relative gene expression was calculated by the  $2^{-\Delta\Delta C_t}$  method. The data were analyzed by one-way ANOVA with SPSS Statistics 19.0 followed by Tukey test. The results were expressed by mean  $\pm$  standard deviation (S.D.), and  $P < 0.05$  indicated the significance of the difference. The Pearson's correlation analysis, principal component analysis (PCA), and all figures in this study were processed with the software Origin 2021.

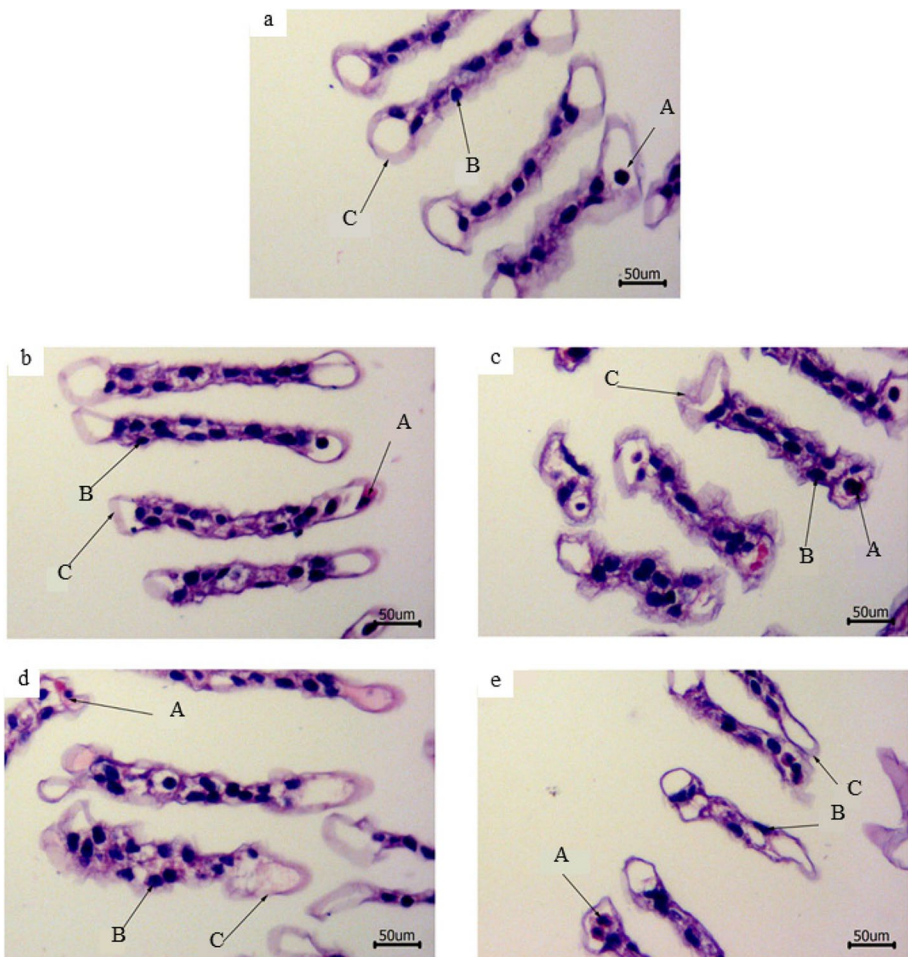
**Table 1** Nucleotide sequences used for the real-time PCR primers in this study

Gene	Nucleotide sequences (5'–3')	GenBank accession number
<i>proPO</i>	F:GTGATTCTTCGCCTCACGCA R:AGTCACCTCCTTCGGCTCTC	AY723296
<i>GRP78</i>	F:TCATTGCCAACGACCAGGGT R:TCCGATGAGACGCTTGGCAT	XM_027371138.1
<i>CASP3</i>	F:ACATTTCTGGGCGGAACACC R:GTGACACCCGTGCTTGTACA	KC660103.1
<i>ATF6</i>	F:CTGTTGGGACAAGGACCATAAGC R:GAATTGTAGGTGTGGCAGCTGTTA	AYM00394.1
<i>PERK</i>	F:TCCTGACATCATCATTATCATCTCC R:TGAAGCTCATGCTCTCTGCCAATCC	XP_027239142.1
<i>IRE1</i>	F:TGGTGAGAAGCAGCTTGTGTGG R:ACTGTTGATGAAGAGCCACTGTAGC	AFQ62792.1
<i><math>\beta</math>-actin</i>	F:GACTACCTGATGAAGATCC R:TCGTTGCCGATGGTGATCA	AF300705

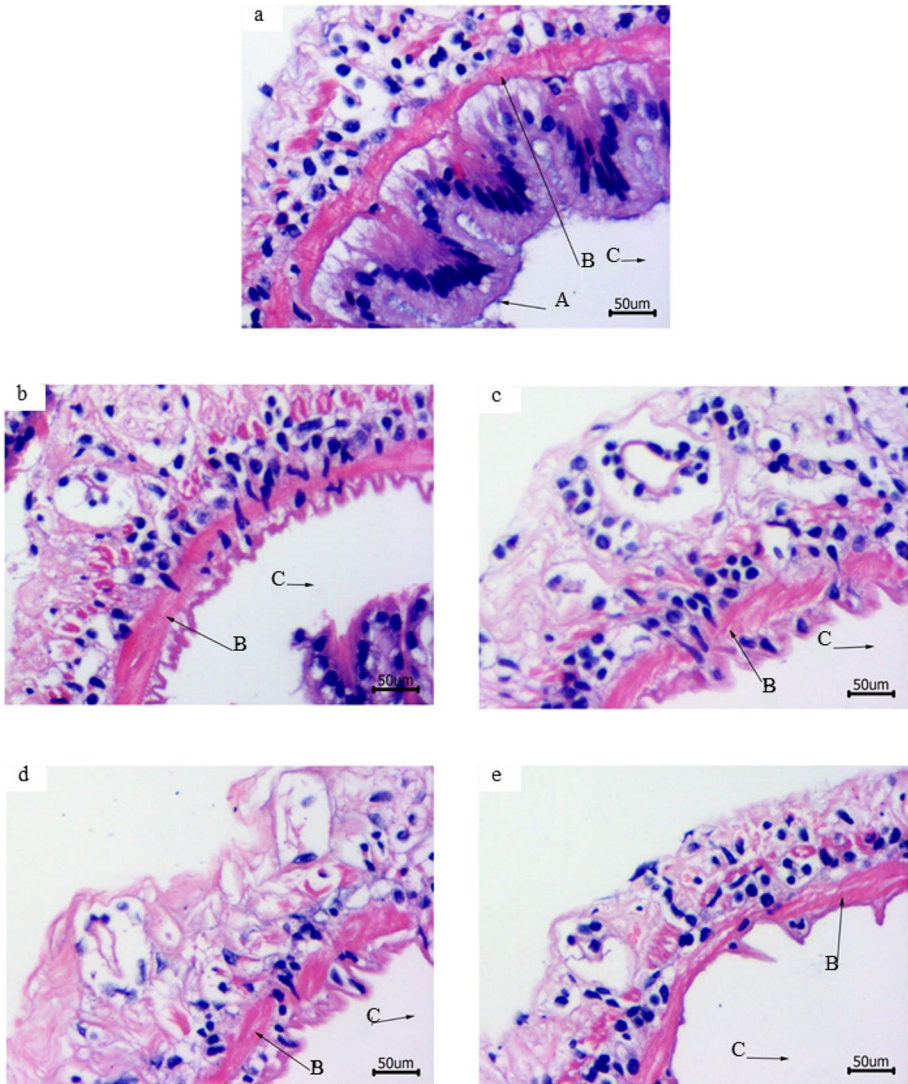
## Results

### Histological analysis

According to HE staining, the gill and intestine of *L. vannamei* were obviously damaged after low temperatures stress. The gill structure was clear, and the cuticle membrane was relatively smooth at 28 °C and 23 °C. However, when the temperature drops to 18 °C, the gill structure is obviously damaged, distorted, and the cuticle membrane is damaged or even shed (Fig. 2). For the intestine, the intestine villi have been severely exfoliated at 23 °C. Most of the cells were necrotic, vacuoles were formed, and the cell spaces were blurred when the temperature was below 23 °C (Fig. 3). However, the muscle tissue of shrimp did not change significantly during the cooling process (Fig. 4).



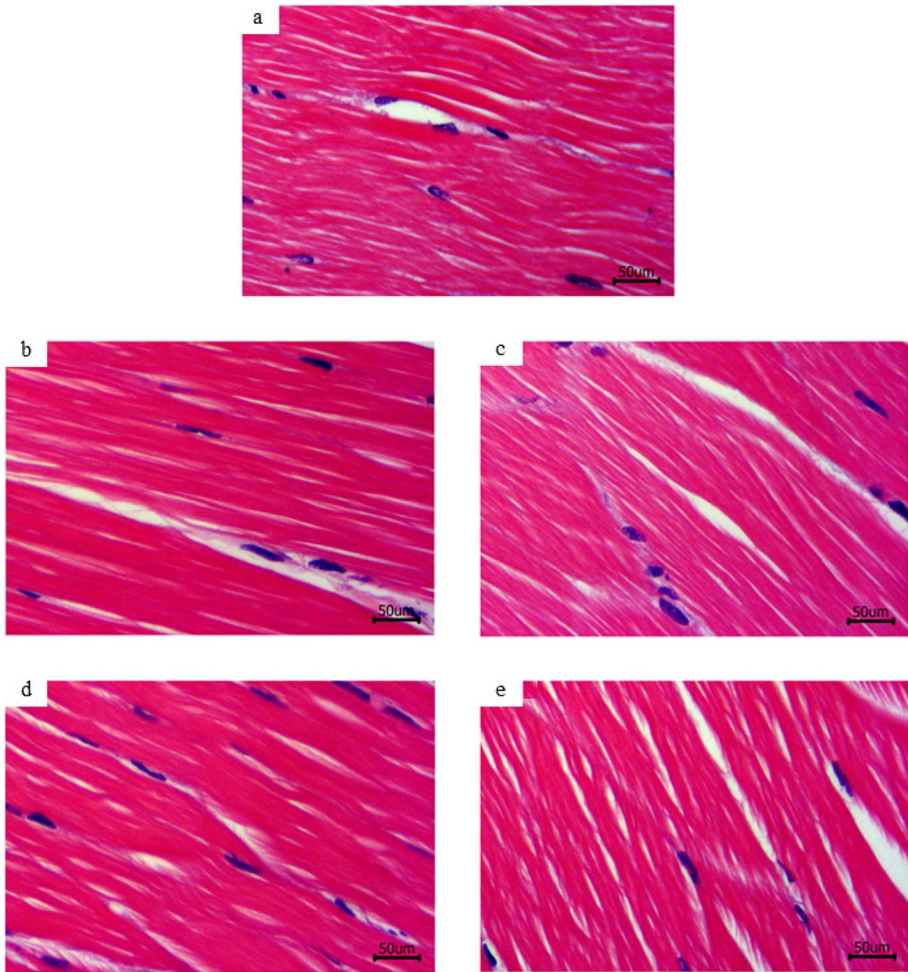
**Fig. 2** Gill tissue structure of *L. vannamei* after low temperature stress ( $\times 400$ ). The gill tissue structure at 28 °C (the control group), 23 °C, 18 °C, 13 °C, and 13 °C for 12 h are represented by **a**, **b**, **c**, **d**, and **e**, respectively. The letters in the figure indicated that: A, hemocyte; B, gill cells; and C, cuticle membrane



**Fig. 3** Intestine tissue structure of *L. vannamei* after low temperature stress ( $\times 400$ ). The intestine tissue structure at 28 °C (the control group), 23 °C, 18 °C, 13 °C, and 13 °C for 12 h are represented by a, b, c, d, and e, respectively. The letters in the figure indicated that: A, intestinal epithelial cell; B, circular muscle; and C, lumen

### Relative expression analysis of UPR pathway related genes

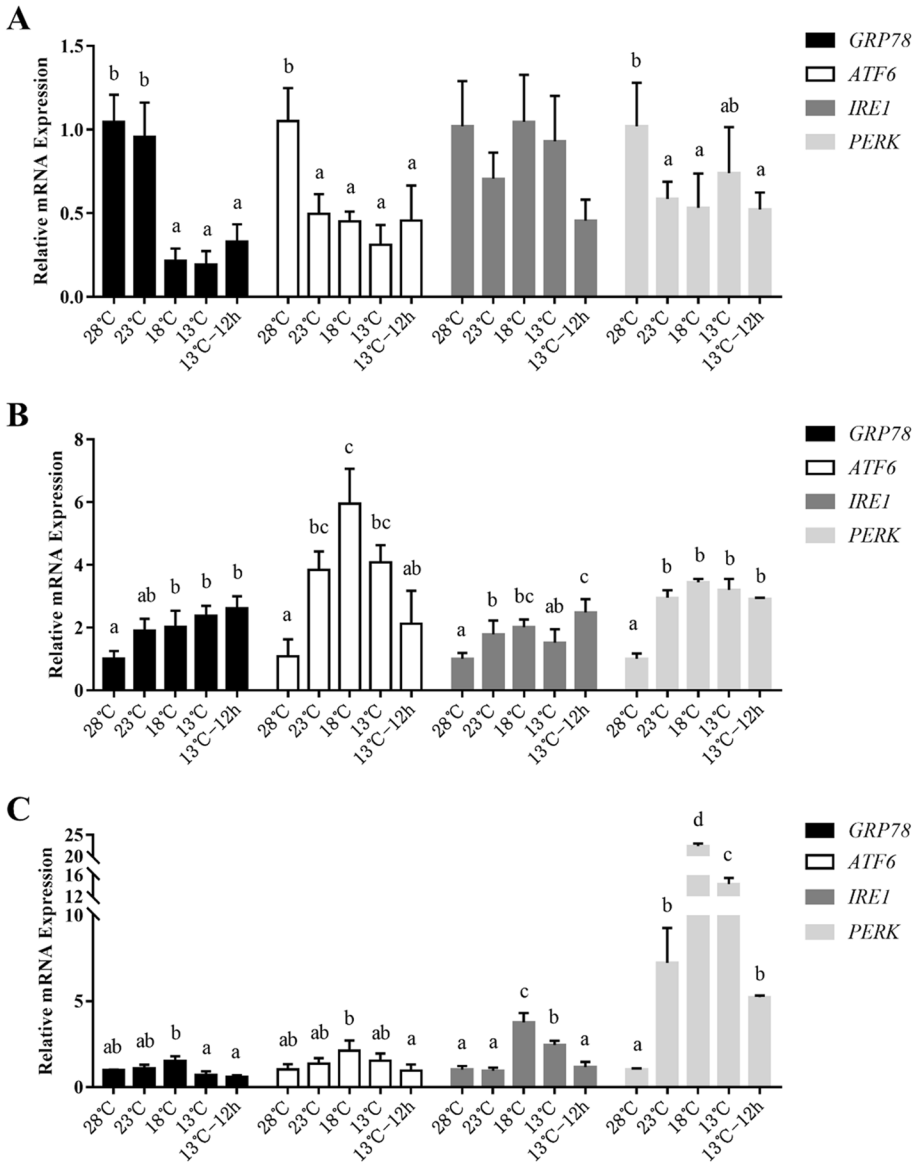
In this study, the changes of UPR pathway in *L. vannamei* under low temperature stress were investigated by detecting the relative expressions of *GRP78*, *ATF6*, *IRE1*, and *PERK* in each tissue. In the gill (Fig. 5A), after the temperature was lowered to 18 °C, the expression of *GRP78* was significantly decrease than that at 28 °C and 23 °C, and it remained relatively stable thereafter. The expressions of *ATF6* and *PERK* were significantly lower



**Fig. 4** Muscle tissue structure of *L. vannamei* after low temperature stress ( $\times 400$ ). The muscle tissue structure at 28 °C (the control group), 23 °C, 18 °C, 13 °C, and 13 °C for 12 h are represented by a, b, c, d, and e, respectively

after 23 °C than that at 28 °C, and remained relatively stable after 23 °C. The expression of *IRE1* fluctuated to some extent under low temperature stress, while there was no significant difference throughout the whole process.

In the intestine (Fig. 5B), the expression of *GRP78* tended to increase throughout the cooling process, with significantly higher expression levels after 18 °C than that at 28 °C. The expression of *ATF6* tended to increase and then decrease, with a peak at 18 °C, and its relative expression was approximately six times higher than that at 28 °C. The expression of *IRE1* fluctuated after cooling, and its expression at 23 °C, 18 °C, and 13 °C-12 h was significantly higher than that at 28 °C, but its expression at 13 °C was not significantly different from that at 28 °C. The relative expression of *PERK* was significantly higher after cooling than that at 28 °C, and its expression during 23 °C to 13 °C-12 h was 2.92–3.45 times higher than that at 28 °C.



**Fig. 5** Relative gene expression of UPR pathway of *L. vannamei* after low temperature stress. A, B, and C represent the gene expression in gill, intestine, and muscle of *L. vannamei* under low temperature stress with change rate of 2.5 °C/2 h, respectively. The bars represent the mean ± S.D. (*n* = 3). Bars with different letters indicated statistical differences (*P* < 0.05)

In muscle (Fig. 5C), the expression of all ER stress-related genes in this study tended to increase and then decrease, and their highest expression, which is significantly different from that at 28 °C, appeared at 18 °C, being 1.15, 2.11, 3.76, and 22.42 times higher than at 28 °C, respectively.



### Relative expression analysis of immune- and apoptosis-related genes

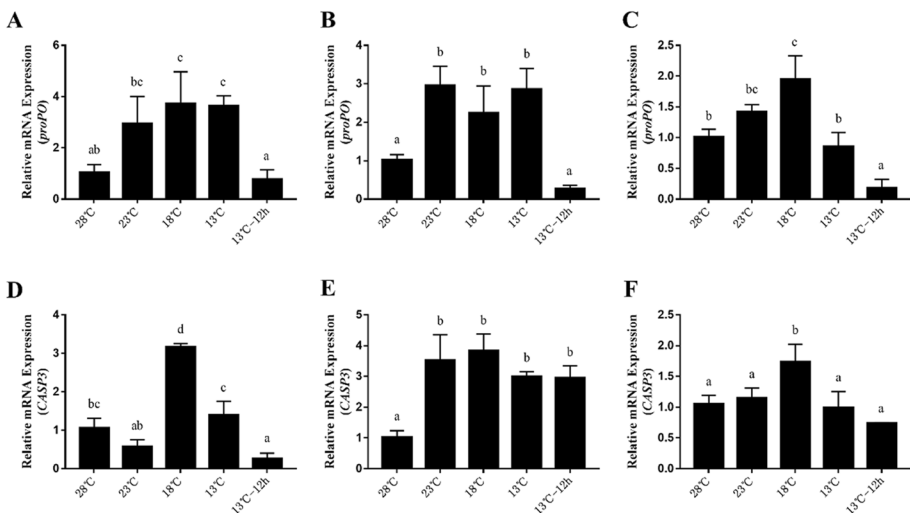
In this study, the changes of immune defense ability and apoptosis in *L. vannamei* under low temperatures stress were investigated by detecting the relative expression of *proPO* and *CASP3* in each tissue. In the gill (Fig. 6 A and D), with the decrease of temperature, the overall change trend of *proPO* and *CASP3* expressions were similar, showing a trend of first increasing and then decreasing. The highest expression occurs at 18 °C, and the lowest expression occurs at 13 °C-12 h. The difference between the expression at 18 °C and 13 °C-12 h was significant.

In the intestine (Fig. 6 B and E), *proPO* expression increased significantly with decreasing temperature and remained at higher levels at 23 °C, 18 °C, and 13 °C, being 2.96, 2.25, and 2.86 times higher than that at 28 °C, respectively. While then, it decreased significantly at 13 °C-12 h. The changes in *CASP3* expression followed the same trend as those in *PERK* in the intestine.

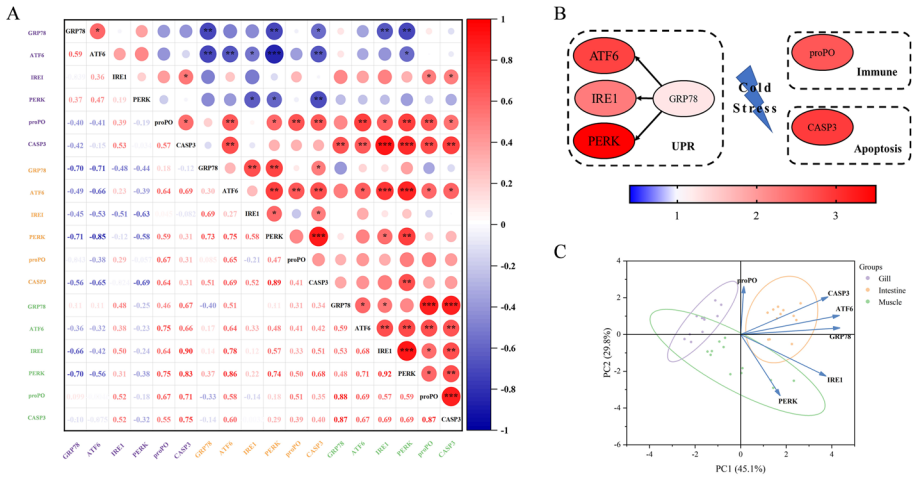
In the muscle (Fig. 6 C and F), the expression of *proPO* and *CASP3* tended to rise first and then fall, similar to their trends in the gill. That is, their expressions were highest at 18 °C and lowest at 13 °C-12 h. And their expressions at 18 °C were significantly higher than those at 28 °C.

### Multi-parameter integration analysis

To understand the relationship between ER stress, immunity and apoptosis parameters, Pearson’s correlation analysis was applied to find correlations between these variables (Fig. 7A).



**Fig. 6** Relative gene expression of *proPO* and *CASP3* of *L. vannamei* after low temperature stress. Gene expression of gill (A, D), intestine (B, E), and muscle (C, F) in *L. vannamei* after low temperature stress were detected with change rate of 2.5 °C/2 h, respectively. The bars represent the mean ± S.D. (n = 3). Bars with different letters indicated statistical differences (P < 0.05)



**Fig. 7** Multi-parameter integration analysis. **A** Pearson’s correlation analysis among UPR pathway, immune and apoptosis parameters. Asterisks (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ) indicate significant difference between the two groups; **B** Schematic diagram of stress resistance response in *L. vannamei* at 18 °C; **C** Principal component analysis (PCA) plots for stress resistance response parameters in different tissues

In the gill, the changes in the expression of UPR related genes (*GRP78*, *ATF6*, *IRE1*, and *PERK*) were mostly positively correlated with each other. A significant positive correlation was found between *GRP78* and *ATF6* ( $r = 0.59$ ;  $P < 0.05$ ). For immune and apoptosis related genes, they were significantly positively correlated with each other, but most of them were negatively correlated with ER stress-related genes in the gill, while the correlations were not significant.

In the intestine, most of the changes in the expression of UPR, immunity and apoptosis related genes were positively correlated. Among them, *GRP78* has a significant positive correlation with *IRE1*, *PERK*, and *CASP3*, and *ATF6* has a significant positive correlation with *PERK*, *proPO*, and *CASP3*. In addition, there was an extremely significant positive correlation between *PERK* and *CASP3* ( $r = 0.89$ ;  $P < 0.001$ ).

In the muscle, the correlations of the changes in the parameters were similar to those in the intestine, that is, the changes in expression between ER stress, immunity and apoptosis-related genes were all positively correlated, and significant positive correlations were found between all parameters except for the correlation between *GRP78* and *PERK*. Among them, the correlation between *IRE1* and *PERK* ( $r = 0.92$ ;  $P < 0.001$ ) was the strongest of all parameters in this study.

## Discussion

### Effects of low temperatures stress on tissue structure

In this study, tissue sections of the gill, intestine and muscle of *L. vannamei* were carried out under changes in water temperature to investigate the effects of low temperatures stress on its tissue structure. Though the histological analysis, we found that

during low temperature stress, tissue damage was observed in the gill and intestine of the shrimp, but there were no significant changes in the tissue structure of the muscle.

The gill is the main respiratory organ and the intestine is the main digestive organ of *L. vannamei*. Previous studies have shown that the histomorphology of gill and intestine could rapidly reflect alterations in the physiological state of organism under environmental stress (Duan et al. 2018b; Han et al. 2018a; Han et al. 2018b). The gill and intestine are responsible for more physiological functions than the muscles and we therefore speculate that their histology is more sensitive to changes in environmental stress than the muscles. Similar results were also observed in *Palaemon serratus* exposed to temperature stress (Madeira et al. 2015) and *Macrobrachium nipponense* exposed to hypoxic stress (Sun et al. 2015), which found that there was no significant change in the muscle tissue structure of animals under environmental stress. Based on the above, we concluded that the muscle in histological level were relatively stable under environmental stress compared to other organs such as gills, hepatopancreas, or intestine.

### Effects of low temperatures stress on the stress resistance system

Environmental stress induces a variety of responses in the body, including ER stress (Yap et al. 2021). ER stress activates the UPR, which is able to eliminate misfolded proteins in the ER to maintain ER homeostasis and protect cells. Therefore, the UPR is considered to be one of the important stress resistance mechanisms in animals (Frakes and Dillin 2017). In addition, animals can respond to environmental stress by enhancing the body's immune response. As an invertebrate, *L. vannamei* relies exclusively on the innate immune system to recognize pathogens or environmental antigens (Iwanaga and Lee 2005). The proPO-activation pathway is considered to be the major pathway regulating the immune response in invertebrates. However, if the imbalance caused by environmental stress exceeds animal's regulatory capacity, apoptosis may be invoked. Apoptosis is also an important component of the organism's stress resistance system. Environmental stresses such as changes in temperature (Ren et al. 2021a) and salinity (Ma et al. 2020), as well as toxic substances in the water (Li et al. 2021) can induce apoptosis in aquatic organisms.

In this study, the relative gene expression of UPR pathway (*GRP78*, *ATF6*, *IRE1*, and *PERK*), immune (*proPO*), as well as apoptosis (*CASP3*) in each tissue were detected to explore the changes in the stress resistance system of shrimp under low temperature stress. The variation patterns of each detection index in various tissues were not completely consistent in our present results. Overall, most of these gene expressions at 18 °C were significantly different from those at 28 °C in all tissues (gill, intestine, muscle). Comparative analysis of the mean expression of each gene in all tissues at 18 °C with its expression at 28 °C revealed that the expression levels of genes involved in the UPR pathway, immune and apoptosis in this study were up-regulated, indicating that the stress resistance system of *L. vannamei* had been activated at this time (Fig. 7B). Moreover, obvious tissue damage occurred in the gill and intestine of shrimp at 18 °C in histological analysis, and further cooling led to severe damage in these tissues. Therefore, combined with histological and gene expression analysis, we deduced that 18 °C may be the critical point for *L. vannamei* to adjust its anti-stress system to maintain its own stability under low temperature stress, and further cooling imbalances the regulation of the stress resistance system and thus affects the survival of shrimp.

### Tissue specific responses under low temperatures stress

The correlations between the gill, intestine, and muscle were analyzed in this study. The correlation of expression changes of each gene was different in different tissues. What's more, principal component analysis (PCA) was used for comparative analysis among tissues, showing an overlap between gill and muscle, but a clear separation between gill and intestine (Fig. 7C). These results suggested that the response of anti-stress system to cold stress is inconsistent in gill, intestine, and muscle. Based on the above results, we can conclude that the regulation of UPR pathway, immune, as well as apoptosis in *L. vannamei* under low temperature stress is tissue-specific.

Previous studies have shown significant changes of the UPR-related genes or proteins in the hepatopancreas of shrimp after low temperature stress (Fan et al. 2016; Wang et al. 2019a). These results are consistent with the present study. In previous studies, the change trend of GRP78 expression in hepatopancreas was consistent with that of ATF6, but has some different from that of IRE1 and PERK. It is reported that ATF6 was the first sensor to respond to UPR, while for IRE1 and PERK, GRP78 is a regulating element rather than a switch (Pincus et al. 2010; Gardner et al. 2013). However, in this experiment, the correlations between GRP78 and the three classical UPR related pathways were different in different tissues. IRE1 and PERK were more responsive to follow the changes of GRP78 than ATF6 in the intestine, which is inconsistent with previous studies. Therefore, we speculate that UPR may have different regulation patterns in different tissues of organism. The specific mechanisms of UPR regulation in animals under environmental stress need to be further explored.

### Conclusions

In summary, low temperature stress causes tissue damage to the gill and intestine in *L. vannamei*, while has no significant effect on muscle tissue structure. The stress resistance mechanisms such as UPR, immunity and apoptosis would be activated under low temperatures. Eighteen degree Celsius is the critical point of adaptation to temperature stress in *L. vannamei*, and rapid cooling below 18 °C may exceed the ability of the shrimp to maintain its own homeostasis. Moreover, the stress resistance system under low temperature stress is tissue-specific in *L. vannamei*. However, the specific differential responses of various tissues to environmental stress require additionally attention in future studies (Fig. 8).

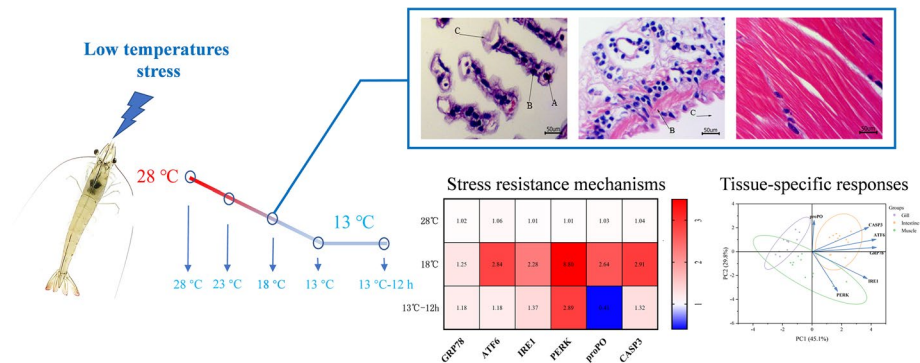


Fig. 8 Schematic diagram of impacts in *L. vannamei* under cold-stress in this study

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10499-023-01106-7>.

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**Author contributions** Zhenlu Wang: Writing – original draft, preparation. Guowei Liao: Software, Validation. Bing Chen: Visualization, Investigation, and Editing. Lanfen Fan: Methodology, Writing – review & editing, Supervision.

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**Data availability** All the data in the article are available from the corresponding author upon reasonable request.

## Declarations

**Ethical approval and consent to participate** Not applicable.

**Human and animal ethics** All studies were authorized by the animal care and use committee and were carried out in accordance with the South China Agricultural University's Guidelines for Experimental Animals.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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