Mitigation of low temperature stress by increased salinity is associated with multiple physiological responses in the gills of *Takifugu fasciatus*

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Introduction

Studies have shown that the combination of low salinity and low temperature results in high mortality in fish from brackish water habitats. There have not been many studies on the effects of changing salinity on gills in a low temperature water environment, especially for euryhaline anadromous fish. Thus, we aimed to study the combined effects of low temperature and salinity changes on the morphological, oxidative, cold stress, osmotic, and apoptotic responses of *T. fasciatus* gills, aiming to reveal the effects of different combinations of environmental factors as well as provide a preliminary molecular and physiological study reference for anadromous fish under a double-factor experiment.

Methods and Materials

- Experimental Fish Preparation, Experimental Design and Sample Collection: four temperatures were 25, 21, 17, and 13 °C and three salinities were as follows: 0, 10, and 20 ppt .
- Gill Microscopy: Paraffin section and Hematoxylin-eosin staining
- Oxidation-related Enzyme Activity Detection: Catalase (CAT), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px) activities were measured using commercial kits.
- Total RNA Extraction, cDNA Synthesis, and Qrt-PCR: Total RNA was extracted using High Purity RNA Fast Extract Reagent and purified RNA was reverse-transcribed into cDNA using HiScript® Reverse Transcriptase, then immediately stored at -20℃ for subsequent quantitative real-time PCR (qRT-PCR). (The cold stress-related genes cold-inducible RNA-binding protein (*cirp*), *manganese superoxide dismutase* (*sod2*), *heat shock protein 90* (*hsp90*). *Osmosis-related genes Na+, K+-ATPase* (*nka*), *sodium chloride co-transporter* (*ncc*), *sodium kalium chloride co-transporter* (*nkcc*). Apoptosis-related genes *B-cell lymphoma-2* (*bcl-2*), *cysteinyl aspartate specific proteinase 3* (*caspase-3*), and *cysteinyl aspartate specific proteinase 9* (*caspase-9*) were used to evaluate the stress response levels of *T. fasciatus* gills in different environments. β-actin was used as a housekeeping gene.

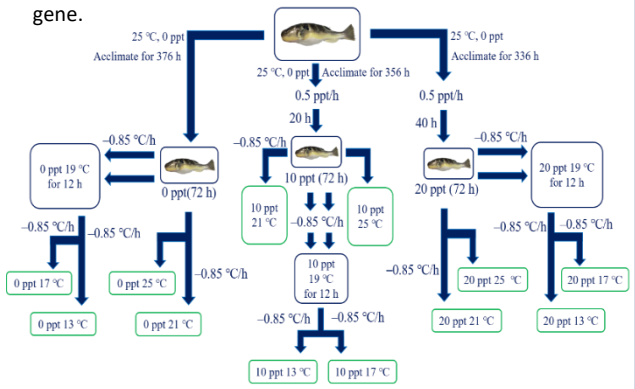

Fig 1. Experiment design for *Takifugu fasciatus* under cold and salinity stresses.

Table 1. Primers used in this research

Forward Primer	Sequence, 5′ to 3′
***tfβactin*-F**	AAGCGTGCGTGACATCAA
***sod2*-F**	GCCCACATTATCCATACCATC
***hsp90*-F**	TTTGGTGTGGGATTTTACTCAGCCTAC
***caspase-9*-F**	CACCTGTCATCCCAGTTCCC
***caspase-3*-F**	GACAACAGTCGGGTTCGTCT
***bcl-2*-F**	CATCACTCCTGACACGGCTT
***cirp*-F**	CTGCCTCTGGAATAACCTCT
***nka*-F**	CGGTCTTTGTCGCTGTAGTC
***ncc*-F**	GGTTCGGTCCACCATAGA
***nkcc*-F**	ATAAGACGCCAGGAAGAAG
Reverse Primer	**Sequence, 3′ to 5′**
***tfβactin*-R**	TGGGCTAACGGAACCTCT
***sod2*-R**	GCAATACAAAGCCTTCCAGTC
***hsp90*-R**	TTGTCCGTCCTGACTGTAAATGAACCT
***caspase-9*-R**	TACGATCGTTCAGCTCGCTC
***caspase-3*-R**	CCGAGGCTCGAGAACACTTT
***bcl-2*-R**	CAAACCAGTTGGCTCGCATC
***cirp*-R**	GCGATGAACGGAAAGTCT
***nka*-R**	GCAACGGTTTCCCTCTTC
***ncc*-R**	ACCAACGGAAAGGTCAAA
nkcc-R	ATACGGCAAGAACAACGA

Results

- **Cold and Salinity Stress-inducing Gradients**
- **Gill Oxidative Responses Could be Alleviated by 10 ppt Salinity**

Table2. Gill lesion severity observed in *Takifugu fasciatus* from 12 treatments

Treatments	Va	Ct	Hp	Oe	Se
013	++	/	/	+++	+++
017	+	/	++	-	/
021	-	+	/	+	/
025	/	/	/	/	/
1013	+++	+++	+++	+++	/
1017	++	+	+++	/	/
1021	+	+	/	/	/
1025	-	/	++	/	/
2013	+++	+++	+++	+++	/
2017	+++	++	+++	++	/
2021	++	/	++	/	++
2025	++	++	/	++	/

/ refers to none or little, - refers to only one or two parts, + refers to a minority, ++ refers to nearly half, +++ refers to the majority, of all observable locations showing pathological signs.

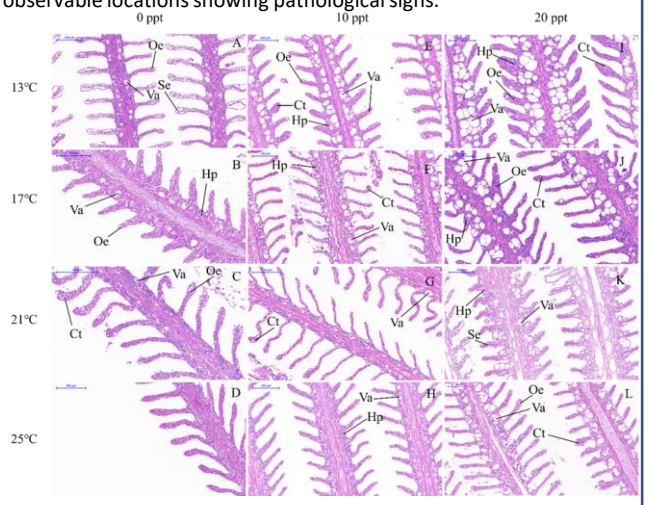

Fig 2. Gill morphology of Takifugu fasciatus under different cold and salinity stresses. The panels include (A) 0 ppt 13 °C, (B) 0 ppt 17 ℃, (C) 0 ppt 21 ℃, (D) 0 ppt 25 ℃, and (E) 10 ppt 13 ℃, (F) 10 ppt 17 ℃, G) 10 ppt 21 ℃, (H) 10 ppt 25 ℃, (I) 20 ppt 13 ℃, (J) 20 ppt 17 ℃, (K) 20 ppt 21 ℃, and (L) 20 ppt 25 ℃. The gills of (D) 0 ppt at 25 °C indicated a normal histology, while all treatment groups showed injuries comprising vacuoles (Va), clubbed tips (Ct), hyperplasia (Hp), oedema (Oe), and subepithelial oedema (Se). Scale bar is 100 μm.

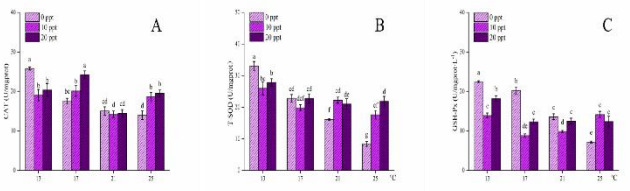

Fig 3. Oxidation-related enzyme activities of Takifugu fasciatus gills under different cold and salinity stresses. The enzyme activities of catalase (CAT, Fig. 3A), total superoxide dismutase (T-SOD, Fig. 3B), and glutathione peroxidase (GSH-Px, Fig. 3C) in the gills. The 12 treatments were compared using Tukey's test, and lowercase letters indicate significant differences ($P < 0.05$).

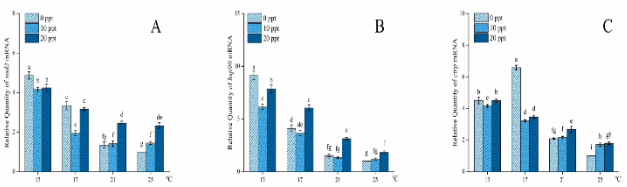

Fig 4. Cold stress-response gene expression levels in Takifugu fasciatus gills under different cold and salinity stresses. Manganese superoxide dismutase (sod2, Fig. 4A), heat shock protein 90 (hsp90, Fig. 4B), and cold-inducible RNA-binding protein (cirp, Fig. 4C) genes. The 12 treatments were compared using Tukey's test, and lowercase letters indicate significant differences ($P < 0.05$)

- **Gill Apoptosis Could be Alleviated by 10 ppt Salinity**

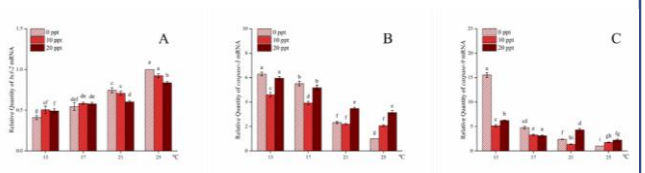

Fig 6. Apoptosis-related gene expression in *Takifugu fasciatus* gills under different cold and salinity stresses *B-cell lymphoma-2* (*bcl-2*, Fig. 6A), *cysteinyl aspartate specific proteinase 3* (*caspase-3*, Fig. 6B), and *cysteinyl aspartate specific proteinase 9* (*caspase-9*, Fig. 6C) genes. The 12 treatments were compared using Tukey's test, and lowercase letters indicate significant differences ($P < 0.05$).

- **Osmotic Regulation Could Be Enhanced when Cold and Salinity Stress Are Combined**

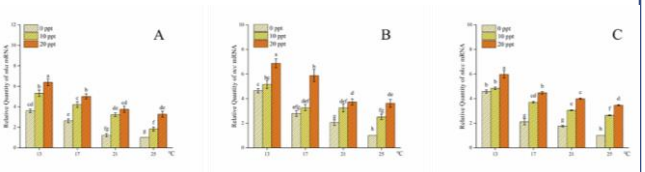

Fig 5. Osmosis-related gene expression levels in *Takifugu fasciatus* gills under different cold and salinity stresses. *Na+, K+-ATPase* (*nka*, Fig. 5A), *sodium chloride co-transporter* (*ncc*, Fig. 5B), and *sodium kalium chloride co-transporter* (*nkcc*, Fig. 5C) genes. The 12 treatments were compared using Tukey's test, and lowercase letters indicate significant differences ($P < 0.05$).

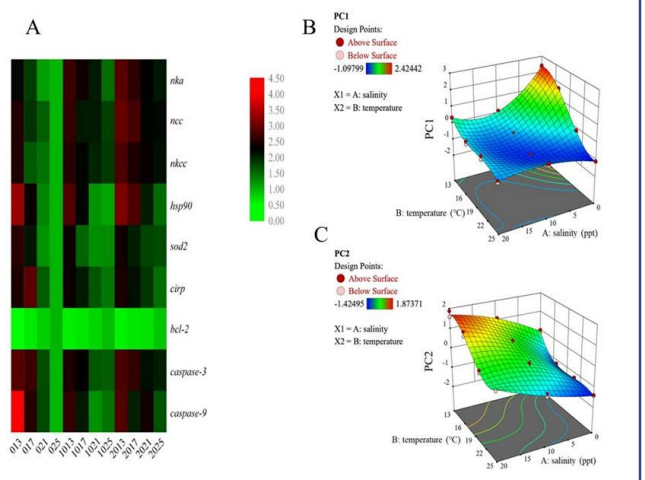

Fig 7. (A) Expression patterns of nine genes in *Takifugu fasciatus* gills under different cold and salinity stresses (the high relative expression of genes indicated by dark and red squares). (B) Response surface of PC1 under different cold and salinity stresses. (C) Response surface of PC2 under different cold and salinity stresses.

- **Mild Salt Treatment Alleviates Stress Responses**

Conclusions

This research is the first report of representative indicators of the stress responses that occur in the gills of *T. fasciatus*. Low temperature and salinity lead to the thickening of *T. fasciatus* gill filaments which was more frequently accompanied by obvious vacuoles and hyperplasia. Low temperature can aggravate the oxidative stress level and efficiently trigger cold stress, osmosis, apoptosis responses which threatens the survival of *T. fasciatus*. Interestingly, we noticed that the negative impacts on *T. fasciatus* gills under low temperature stress (13 °C or 17 °C) could be alleviated by the addition of 10 ppt salinity, which might be a helpful information for the culturing of *T. fasciatus*. However, further studies are needed to elucidate the accurate mechanisms.

Acknowledgements

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