Dynamic remodeling of membrane proteins for temperature adaptation in oysters

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ABSTRACT

Rising ocean temperatures due to global warming pose severe threats to marine organism survival. Membrane proteins perform important biological functions and serve as sensitive environmental stress sensors. However, the characterization of membrane protein content in response to temperature adaptation remains poorly understood in marine organisms. Here, we performed reciprocal transplantation experiments between the northern/cold and southern/warm natural habitats of two allopatric but closely related oyster species, *Crassostrea gigas* and *C. angulata*. Comparative membrane proteomics combined with transcriptomic analysis revealed ABC transporters, Notch signaling, biosynthesis of unsaturated fatty acids, and aquaporins as key molecular components of temperature adaptation in oysters. ABC transporters and key endoplasmic reticulum enzymes for unsaturated fatty acid synthesis (SCD, FADS2, and ELOVL4) exhibited consistently higher protein abundance in northern environments, indicating their involvement in cold adaptation. The Notch signaling pathway may serve as a crucial mechanism for sensing temperature fluctuations and coordinating regulation of antioxidant defense and apoptosis. Additionally, we identified that aquaporins function as both key heat- and cold-adapted proteins, suggesting their crucial role in temperature fluctuation responses. A nonsynonymous mutation identified in the AQUAPORIN-8 coding region may modulate tetrameric water channel formation, thereby altering transport efficiency and potentially contributing to differential temperature tolerance between the two oyster species. This study is the first to characterize dynamic changes of membrane protein in oysters, which reveals its critical role in ambient temperature adaptation and provides new insights into predicting the adaptability of marine organisms to future climate changes.

Key words: Global warming; Temperature adaptation; Membrane protein; Oysters

INTRODUCTION

In the context of global warming, oceans have absorbed most of the excess heat from anthropogenic warming, driving a persistent upward trajectory in sea temperatures (Mcculloch et al., 2024; Oh et al., 2024). Global ocean surfaces warmed exceptionally during 2023-2024, averaging 0.25 ℃ above the 2015-2016 peak, which is a 1-in-512-year extreme climatic event (Terhaar et al., 2025). Temperature governs core physiological processes including enzyme kinetics (Wang and Swartz, 2024), immune competence (Karl et al., 2011), and reproductive performance (Munns and Millar, 2023), which are critical determinants of organismal development and growth. The persistently rising ambient temperatures pose severe threats to marine organisms, a phenomenon that has received mounting attention (De Luzinais et al., 2024). For instance, during the 2023 marine heatwaves (MHWs), New Zealand experienced peak temperatures 4.4 ℃ above historical averages, resulting in >50% mortality of sponge at sampled sites (Bell et al., 2024). Studies have shown that Atlantic coral cover exhibited a fourfold decline from 1977 to 2020 due to increasing intensity and frequency of MHWs (Walker et al., 2024). Annually during summer months, mass mortality events occur in farmed large yellow croaker populations due to heat stress and secondary diseases, resulting in substantial economic losses across China's aquaculture industry (Wu et al., 2022). Therefore, rising sea temperatures and increasing frequency of extreme climatic events have caused substantial impacts on marine ecosystems and aquaculture industries (Masanja et al., 2024; Monteiro et al., 2023). Understanding the molecular basis of temperature adaptation in marine organisms has become crucial for enhancing their resilience to elevated temperatures.

Biological membranes serve as both a natural cellular barrier and a sensitive environmental stress sensor (Ernst et al., 2016). Their critical functions, including energy conversion, selective molecular transport and signal transduction, are predominantly mediated by proteins embedded within the lipid bilayer (Jafurulla et al., 2019; Sych et al., 2022; Zhang et al., 2015). Therefore, dynamic remodeling of

membrane protein composition is a sensitive mechanism for perceiving and adapting to ambient temperature variations (Permyakov et al., 2016; Xu et al., 2025). Due to the hydrophobic nature of the lipid bilayer, most hydrophilic solutes require specialized transport mechanisms to cross cell membranes, a process mediated by transmembrane transport proteins (Özkan et al., 2024; Stieger et al., 2021). Transport membrane proteins can be divided into channel proteins, carrier proteins, and ATP-powered pumps based on their transport mechanisms and energy requirements, enabling the transport of various substances to maintain internal homeostasis (Mazhab-Jafari et al., 2016; Nishiguchi et al., 2023; Thompson, 2022). By maintaining ion homeostasis and osmoregulation, transport proteins play a crucial role in regulating stress response mechanisms, such as temperature stress (Kamal et al., 2020; Vishwakarma et al., 2019). Biological membranes also serve as attachment sites for enzymes that catalyze essential reactions, exemplified by: lipid-synthesizing and glycogen-metabolizing enzymes on the smooth endoplasmic reticulum (Zibrín et al., 2005), electron transport chain complexes on the inner mitochondrial membrane (Kühlbrandt, 2015), and fatty acid beta-oxidation enzymes on peroxisomal membranes (Visser et al., 2007). Additionally, membrane proteins function as signal receptors, sensing ambient temperature changes and initiating downstream signaling cascades, such as the GPCR pathway, MAPK pathway and NF-κB pathway, to activate cellular defense mechanisms against temperature damage (Ren et al., 2023; Traylor-Knowles et al., 2017; Wang et al., 2024c). However, the extraction of membrane proteins remains challenging owing to their low abundance and hydrophobicity, leading to limited studies on their abundance characterization (Jiang et al., 2024; Young, 2023). The advances in detergent and mass spectrometry technologies have significantly improved membrane protein extraction and identification, which has laid the groundwork for investigating the adaptive mechanisms underlying changes in membrane protein composition during temperature adaptation (Chan et al., 2022; Ehsan et al., 2018).

Oysters inhabit the highly heterogeneous intertidal zone, experiencing drastic ambient temperature fluctuations, making them sentinels for sensing ocean temperature changes (Judge et al., 2018; Zhang et al., 2012). The extreme selective pressures of habitat drive oysters to develop high phenotypic plasticity, making them as ideal model for studying marine biotic responses to temperature change (Li et al., 2021; Wang et al., 2023a). Additionally, oysters are the largest cultivated marine organism globally, holding significant economic and ecological value (Du et al., 2024; Longmire et al., 2021; Wang et al., 2024b). However, mass summer mortality has emerged as a major constraint on global oyster aquaculture productivity, with well-documented cases causing substantial economic damage in major producing nations including China(Yang et al., 2021), the U.S. (Ashton et al., 2020) and Japan (Ventilla, 1984). Existing research indicates that high temperatures act as a key abiotic factor, which can significantly amplify the effects of other factors on physiological processes in oysters (Bozinovic et al., 2011; Liu et al., 2023b). Therefore, given the high economic value and significant ecological role of oysters, elucidating the molecular mechanisms underlying temperature adaptation is crucial and urgent in the context of global warming.

Crassostrea gigas and *C. angulata* are dominant cultured oyster species, naturally inhabiting the relative-cold northern and relative-warm southern coastal regions of China (Wang et al., 2024a; Wang et al., 2010). Habitat-specific temperature has driven the divergence of temperature tolerances in these allopatric but closely related *Crassostrea* oysters, which have undergone 2.7 million years of divergence (Ren et al., 2010). Therefore, these closely related oyster species can serve as an ideal model for investigating the molecular mechanisms of temperature adaptation in marine organisms (Du et al., 2025; Wang et al., 2023b). Previous studies revealed divergent and complex molecular mechanisms underlying temperature adaptation between two oyster species through proteomic and protein phosphorylation analyses of short-term heat stress responses (Wang et al., 2023a). However, there is a lack of

comprehensive characterization of membrane proteins in oysters that are more sensitive to temperature perception. Additionally, most studies on temperature responses focused on laboratory-based temperature stress experiments, which primarily reflected short-term acclimation (Liu et al., 2021; Poirier et al., 2014). In contrast, comparative studies of long-term temperature adaptation at ecological scales would better reveal the physiological mechanisms underlying organismal adaptation (Fuchs et al., 2025).

In this study, we conducted a comparative proteomic analysis of membrane proteins between *C. gigas* and *C. angulata* through reciprocal transplantation experiments in their northern and southern natural habitats. This study presents the first comprehensive characterization of the oyster membrane proteome and identifies key pathways and membrane proteins involved in temperature adaptation, which will provide novel insights into molecular mechanisms underlying dynamic reorganization of membrane proteins and prediction of the adaptive potential for marine organisms under global warming.

MATERIALS AND METHODS

Oysters collection and experimental design

Wild adult oysters of *C. gigas* were collected from intertidal habitats along the northern coast of China (Qingdao, 35°44′ N, 120°24′ E), and *C. angulata* were collected from the southern coast (Xiamen, 24°33′ N, 118°43′ E) to serve as broodstock for subsequent experimental procedures. The broodstock oysters were transported to Qingdao for a one-generation common garden experiment to minimize the influence of environmental variability. Artificial breeding procedures, including broodstock maturation induction, fertilization, and larval culture, were carried out in a hatchery at 22-26 ℃ using sand-filtered seawater with a salinity of 31±1‰. The spawning protocol followed our established methodology (Li et al., 2021). For each species, gametes from 30 males and 30 females were used to generate F_1 progeny. To ensure equal genetic representation, we pooled eggs from all females and distributed

them equally among 30 fertilization chambers, each containing sperm from a single male. Oyster larvae were reared in 70-liter plastic tanks and fed different microalgae at each developmental stage: *Isochrysis galbana* (D-stage), *Nitzschia closterium* (umbo stage), and *Platymonas subcordiformis* (metamorphosis). Eight-month-old oysters were divided into two groups and transported to northern (Qingdao) and southern (Xiamen) sites for subsequent cultivation. After experiencing two months (From February to March) of maximal temperature differential between sites, oysters were sampled for subsequent experiments. To validate the coding region variants, wild *C. gigas* and *C. angulata* were sourced from coastal waters near Tangshan (Hebei Province, 39°43′ N, 118°17′ E) and Quanzhou (Fujian Province, 24°22′N, 118°67′ E), representing their natural distribution ranges in northern and southern China, respectively.

Membrane proteins extraction and identification

Gill tissue membrane protein extraction:

Membrane proteins were extracted from the gill tissues of four oyster sample types (northern/southern *C. gigas* and *C. angulata*) using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Scientific, USA). There were four experimental groups: North *C. gigas* (NG) and *C. angulata* (NA) cultured in Qingdao, and South *C. gigas* (SG) and *C. angulata* (SA) cultured in Xiamen. In this study, we systematically compared membrane protein abundance variations (i) between environmental conditions for each species (NG vs. SG for *C. gigas*; NA vs. SA for *C. angulata*), and (ii) between species under identical environments (NG vs. NA in northern; SG vs. SA in southern). Gill tissues (0.2-0.3 g) were vortexed in cell wash solution, then minced with micro-scissors and transferred to a 2 mL Kimble™ Kontes™ Dounce homogenizer (Thermo Scientific, USA) containing 1 mL permeabilization buffer. Tissues were homogenized until a uniform suspension was obtained. An additional 1 mL of permeabilization buffer was added, followed by continuous mixing and incubation at 4 ℃ for 10 minutes. The sample was centrifuged

at 16,000 ×g (4 ℃, 15 min) to remove the supernatant containing cytoplasmic proteins. The pellet was resuspended in 1 mL solubilization buffer and incubated at 4 ℃, for 30 min with continuous mixing. Membrane proteins were obtained in the supernatant after centrifugation at 16,000 ×g for 15 min at 4 ℃.

Then, the samples were immediately processed using Pierce™ Detergent Removal Spin Columns (Thermo Scientific, USA) to eliminate detergents that could interfere with subsequent mass spectrometric analysis. The spin column storage solution was removed by centrifugation at 1,000 ×g for 2 minutes, followed by two washing steps with equilibration buffer under identical centrifugation conditions. The membrane protein solution was gently layered onto the top of the compact resin bed and incubated at room temperature for 2 minutes. The detergent-free membrane protein fraction was collected by centrifugation at 1,000 ×g for 2 minutes.

Membrane protein trypsin digestion:

The mass spectrometry identification analysis of membrane proteins was completed by Applied Protein Technology (Shanghai, China). The membrane proteins were digested with trypsin using the filter-aided sample preparation (FASP) methodology as referenced in (Wisniewski et al., 2009). The resulting peptides were desalted using C18 cartridges, lyophilized, and reconstituted in 40 µL of 0.1% formic acid solution supplemented with appropriate iRT peptide standards.

Mass spectrometry analysis:

The resulting membrane peptides were separated via nanoscale chromatography using a Vanquish Neo system (Thermo Scientific) and subsequently analyzed with an Astral high-resolution mass spectrometer (Thermo Scientific). Full-scan MS1 spectra (380-980 *m/z*) were acquired at 240,000 resolution (at 200 *m/z*) with a normalized AGC target of 500% and maximum injection time of 5 ms. For data-independent acquisition (DIA), MS2 spectra were collected using 299 variable windows (2 *m/z* isolation width) with 25 eV HCD collision energy, 500% normalized AGC target, and 3 ms maximum injection time. DIA data were processed using DIA-NN software with

the following parameters: enzyme specificity set to trypsin (maximum 1 missed cleavage), fixed modification of Carbamidomethyln (C), and variable modifications including Oxidation (M) and Acetyl (Protein N-term). Protein identification required passing a 1% false discovery rate (FDR) threshold. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD065552.

Protein annotation and membrane quantification:

Raw data were analyzed using DIA-NN v1.8.1 based on the available genomes of *C. gigas* as references (BioProject Accession: PRJNA598006) (Qi et al., 2021). The software parameters are set as follows: the maximum missed cleavages was 1, and at least one unique peptide per identified protein during the database search. Proteins identified by database searches must pass the set filter parameter FDR < 1%. Protein identification was performed using the annotated non-redundant (NR) and Swiss-Prot (SP) databases from the reference genome (Qi et al., 2021). Proteins meeting the criteria of fold change >1.5 and *P*-value < 0.05 (t-test) were defined as differentially expressed. Functional annotation was performed using Blast2GO software (v.2.8.0+) for Gene Ontology (GO) classification, while KEGG pathway analysis was conducted with KOBAS software (v.3.0). Enrichment significance was calculated using Fisher's Exact Test, with *P*-values converted to $-\log_{10}$ scale for statistical interpretation. The calculation of protein molecular weight, isoelectric point, and other related information was performed using ProtParam (https://web.expasy.org/protparam/). Transmembrane domain prediction for membrane proteins was performed using TMHMM v.2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/).

Phylogenetic and transcriptome data analysis

All human protein sequences used in this analysis were retrieved from the NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed using the maximum likelihood method implemented in PhyloSuite

v1.2.2 (Zhang et al., 2020). Protein sequence evolution was modeled using the LG+I+G4 substitution matrix, identified as optimal by ModelFinder through Bayesian Information Criterion comparison. Phylogenetic node confidence was evaluated with 5,000 conventional bootstrap iterations. Protein domain architecture was analyzed using NCBI's Conserved Domains Database, while functional motifs were identified through MEME Suite (https://meme-suite.org/meme/) with the following parameters: maximum motif width = 200 aa, minimum sites = 10, and E-value < 1×10^{-5}. The phylogenetic tree was visualized and annotated using the Interactive Tree of Life (https://itol.embl.de/). Protein domains and motifs were visualized using TBtools software (version 2.310).

Raw transcriptome sequencing data from oysters under different tissue types and temperature stress conditions were obtained from the NCBI Sequence Read Archive (accessions: SRP014559 and SRP019967). Sequencing data was aligned to the reference genome using the established pipeline described in previous research (Wang et al., 2024c). Heatmap was generated using the OmicShare tools (https://www.omicshare.com/).

Screening and validation of variant sites in the AQUAPORIN-8 coding region

The resequencing data for *C. gigas* and *C. angulata* were obtained from previous studies (Qi et al., 2023). Variant sites within AQUAPORIN-8 coding region were extracted using bcftools (version 1.19), followed by functional annotation with snpEff (version 4.3). Genetic variants identified through whole-genome resequencing were subsequently validated using Sanger sequencing. Total genomic DNA was isolated from wild *C. gigas* and *C. angulata* specimens employing the TIANamp Marine Animals DNA Kit (TIANGEN BIOTECH, Beijing). Nucleic acid integrity was assessed via NanoDrop 2000 spectrophotometer, where samples demonstrating 260/280 nm absorbance ratios of 1.8-2.0 were considered protein-free. The variant regions were amplified using the Phanta Max Super-Fidelity DNA Polymerase Kit (Vazyme Biotech, China). Then, the PCR amplicons were subjected to Sanger

sequencing at Tsingke Biotechnology, followed by genotyping analysis based on sequencing chromatograms. The primers were listed in Supplementary Table S1.

AQUAPORIN-8 RNAi experiment

siRNA injection in oysters:

Small interfering RNA (siRNA) was synthesized by GenePharma (Shanghai, China). The target sequences were provided in Supplementary Table S1. *C. gigas* were cultured in a 500-L tank for a one-week acclimation period to mitigate potential environmental effects before the experiments commenced. Then, oysters were anesthetized by immersion in a solution containing 500 g of $MgCl_2$ dissolved in a 1:1 mixture of seawater and freshwater (total volume: 10 L) for 12 h, as previously described (Du et al., 2025). Following anesthesia-induced valve gape, 200 μL of siRNA or negative control (1 OD, dissolved in DEPC-treated water) was injected into the adductor muscle. The gills of the oysters were sampled and placed in liquid nitrogen, and stored in a -80 ℃ freezer.

Semi-lethal heat stress assay:

C. gigas injected with siRNA or negative control were placed into semi-lethal temperature (42 ℃) seawater for 1 h, which was maintained by a variable frequency heating rod (Ghaffari et al., 2019). Subsequently, oysters were transferred back to the 500 L acclimation tank after heat stress. The water temperature was maintained at 15 ± 2 ℃ under continuous aeration. To assess cumulative mortality, dead oysters were removed promptly upon discovery, and survival was recorded daily over a 15-day period. Gill tissues were immediately collected from live oysters following heat stress for subsequent histological staining.

qRT-PCR analysis:

Total RNA was extracted from oyster gill tissue using TRIzol reagent (Vazyme Biotech). First-strand cDNA synthesis was performed with the HiScript III RT SuperMix for qPCR kit (Vazyme Biotech). Each individual was repeated three times, and finally the average was taken as the final expression amount of the individual.

The elongation factor-1 alpha (Ef-1α) gene served as the internal control for normalization. The sequences of all qRT- PCR primers used in this study were presented in Supplementary Table S1. Quantitative real-time PCR was carried out on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with Taq Pro Universal SYBR qPCR Master Mix (Vazyme Biotech). Gene expression quantification was conducted via the $2^{-\Delta\Delta CT}$ method, and a comprehensive comparative analysis between the two species has been described (Li et al., 2019).

Tissue section staining and analysis:

Gill tissues from oysters subjected to semi-lethal temperature treatment, including four experimental groups (negative control and siAQU-8 under both indoor temperature and heat stress, n=5), were analyzed using hematoxylin-eosin (HE) staining and TdT-mediated dUTP nick end labeling (TUNEL) assays to evaluate histological morphology and apoptosis. The tissue sections were fixed for 15 minutes using a universal tissue fixative (Servicebio, China), followed by preparation of paraffin-embedded sections. Subsequently, the tissues were stained with hematoxylin and eosin using an H&E High-Definition Staining Kit (Servicebio), following the manufacturer's protocol. The stained sections were mounted with neutral balsam and then imaged using an upright optical microscope (Nikon ECLIPSE E100, Japan). Apoptosis in gill tissues was detected using a TUNEL assay kit (Servicebio, China). Briefly, fixed tissue sections were treated with proteinase K for antigen retrieval. The sections were then incubated with a reaction mixture containing TdT enzyme, dUTP, and buffer from the kit at 37 ℃ for 1 hour. After incubation, the samples were washed three times with PBS (pH 7.4) for 5 minutes each. Following PBS removal, DAPI staining solution was applied to the sections, which were incubated at room temperature for 10 minutes in the dark. Finally, the sections were mounted with an Anti-fluorescence quenching tablets (Servicebio) and imaged under a fluorescence microscope (Nikon ECLIPSE C1, Japan). The Aipathwell software (Servicebio, China) was employed to calculate integrated optical density (IOD) measurements, where

apoptotic cells are distinctly identified by red coloration.

Validation of AQUAPORIN-8 function in HEK293T cells

Cell overexpression:

HEK293T cells were cultured in 6 well plates at 37 ℃, 5% CO_2, using 1ml DEEM Medium (Viva Cell Biosciences, China) supplemented with 10% FBS (Biological Industries, israel). The details of plasmid construction were described in the previous study (Wang et al., 2023b). Briefly, the open reading frames (ORFs) of CgAQU8 were cloned into the pCMV-N-Flag vector (Beyotime Biotechnology, China). The site mutant plasmids were constructed using Mut Express II Fast Mutagenesis Kit V2 (Vazyme Biotech). Primer sequences were listed in Supplementary Table S1. The pCMV-CgAQU8 plasmid was transfected into HEK293T cells using Lipofectamine 3000 (Thermo Fisher Scientific). Transfection was performed one day after seeding, at which point the cells had reached approximately 80% confluence, using 2500 ng of plasmid per well.

Cell apoptosis and viability detection:

The heat stress group was exposed to a culture temperature of 42 ℃ and 5% CO_2 for 1 hour before the experiments. Apoptotic and necrotic cell death were assessed with an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Solarbio Life Sciences, China). Cell staining and measurement were performed on a FACSAria II flow cytometer (Becton Dickinson, USA), and resulting data were processed with FlowJo software (Version 10.8.1). Cell Counting Kit-8 (CCK-8) Assay was performed using the Enhanced Cell Counting Kit-8 (Beyotime Biotechnology) following the manufacturer's protocol. Absorbance was measured at 450 nm with a Varioskan Flash multimode reader (Thermo Fisher Scientific).

Western blotting:

The transfected HEK293T cells were lysed with Western and IP-compatible cell lysis buffer supplemented with protease inhibitors (Beyotime Biotechnology). After centrifugation, the supernatant proteins were denatured by heating at 95 ℃ for 5

minutes in the presence of 4X protein loading buffer (GenScript Biotech, China). Subsequently, the protein samples were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane featuring 0.45 nm pores (Millipore, USA) utilizing an eBlot™ L1 wet transfer system (GenScript Biotech). The membrane was blocked for 10 minutes using QuickBlock™ Western Blocking Buffer (Beyotime Biotechnology), followed by an overnight incubation with the primary antibody diluted in TBST and incubated with a secondary antibody for 2 hours at room temperature. Protein bands were visualized using the Omni-ECL™ Femto Light Chemiluminescence Kit (Epizyme, China) and imaged with the Molecular Imager® Gel Doc™ XR System (Bio-Rad, USA). The antibodies used were as follows: Flag-tag (ZENBIO, 390002), beta Actin (ZENBIO, 200068-8F10), Anti-Mouse/Rabbit IgG(H + L) (Epizyme, LF102).

Molecular docking

The AQUAPORIN-8 protein sequence was submitted to AlphaFold3 software for structural prediction of its tetrameric complex conformation. Then, the obtained protein-protein complex was also manually subjected to optimization operations such as dewatering and hydrogenation using AutoDockTools-1.5.7. Finally, pymol was used to predict protein-protein interactions and generate protein-protein interaction maps. The tetramer binding energy was predicted using PDBePISA (https://www.ebi.ac.uk/pdbe/pisa/).

Statistical analyses

Multiple group comparisons were performed using analysis of variance (ANOVA) in GraphPad Prism 9 (version 9.4.1). Prior to parametric analysis, data normality was assessed using the Shapiro-Wilk test (α=0.05). For non-normally distributed datasets, non-parametric comparison was conducted via the Mann-Whitney U test. Significant differences between groups were marked with "*" for $P < 0.05$, "**" for $P < 0.01$, "***" for $P < 0.001$, "****" for $P < 0.0001$.

RESULTS

Overview of membrane proteomic data

The experimental design for reciprocal transplantation was schematically presented in Figure 1A. Proteomic analysis identified 10,499 unique proteins corresponding to 186,799 peptide spectra across all samples. Complete protein identification and quantification results for all experimental samples were provided in Supplementary Table S2. The iRT retention time, average full width at half maximum (FWHM), and peptide length distribution all confirmed that our results have high reliability and meet quality control requirements (Supplementary Figure S1). Additionally, the distribution of protein quantification values within samples indicated better quantitative reproducibility (Supplementary Figure S2). Principal component analysis revealed clear separation of four experimental groups, with PC1 and PC2 explaining 35.95% and 30.95% of the total variance, respectively (Figure 1B). High reproducibility was confirmed by Pearson correlation coefficients (Supplementary Figure S3). Protein identification overlaps between experimental groups were visualized using a Venn plot (Supplementary Figure S4), revealing both shared and group-specific protein profiles.

Differentially abundant membrane proteins across all comparison groups were visually summarized in Figure 1C, with complete datasets provided in Supplementary Table S3. In the NG vs. SG comparison group, a total of 1,009 showed increased abundance while 1,128 proteins demonstrated decreased levels. In the NA vs. SA comparison group, there were 1,117 proteins that showed increased abundance, while 1,323 proteins demonstrated decreased levels. In the NG vs. NA comparison group, 910 proteins showed increased abundance while 844 proteins demonstrated decreased levels. In the SG vs. SA comparison group, 888 proteins showed increased abundance while 787 proteins demonstrated decreased levels.

Gene ontology and KEGG pathway enrichment analyses of differentially abundant membrane proteins

To elucidate the functional mechanisms underlying temperature adaptation

421 mediated by differential membrane proteins, we performed enrichment analysis of
422 significantly regulated proteins across all comparison groups.
423 In the GO enrichment analysis, both environmental comparisons (SG vs. NG and
424 SA vs. NA) showed significant enrichment in molecular function (MF) categories
425 including transporter activity, ATP-dependent activity, and molecular transducer
426 activity (Figure 2A, B, Supplementary Table S4). Few biological processes (BP) or
427 cellular components (CC) showed statistically significant enrichment in our analysis.
428 In species comparisons (NA vs. NG and SA vs. SG), ATP-dependent activity and
429 molecular transducer activity were significantly enriched in both contrasts, while
430 transporter activity showed enrichment only in the SA vs. SG comparison (Figure 2C,
431 D, Supplementary Table S4). Analysis of CC revealed no overlapping enriched terms
432 across the four comparison groups. Subsequently, we focused our functional
433 interpretation primarily on BP categories. In the environmental comparison SG vs.
434 NG group, significant enrichment was observed for ribosome biogenesis, cellular
435 component biogenesis, and ion transport/homeostasis-related processes
436 (Supplementary Figure S5). In the SA vs. NA group, anion transport and ion transport
437 were similarly enriched. In the species comparison under northern conditions (NA vs.
438 NG), significant enrichment was observed for transition metal ion transport and
439 homeostasis processes (Supplementary Figure S5). The southern species comparison
440 (SA vs. SG) showed no significant enrichment of ion transport pathways. Instead,
441 DNA replication, animal organ development, and DNA metabolic processes were
442 prominently enriched.
443 KEGG pathway analysis identified ATP-binding cassette (ABC) transporters as
444 the most significantly enriched pathway in both environmental comparisons (SG vs.
445 NG and SA vs. NA, Figure 3A, B; Supplementary Table S5). In species comparisons,
446 ABC transporters pathway was exclusively enriched in the NA vs. NG group, while
447 steroid hormone biosynthesis was the most significantly enriched pathway (Figure
448 3C). Similar to GO enrichment results, the SA vs. SG group exhibited distinct

449 pathway enrichments including nitrogen metabolism, base excision repair, and two-
450 component system, which were not observed in other comparisons (Figure 3D).
451 Therefore, the ABC transporters pathway may be a key pathway for temperature
452 adaptation in oysters. Additionally, KEGG enrichment analysis also revealed
453 significant overrepresentation of metabolic pathways, particularly those related to
454 lipid metabolism including: steroid hormone biosynthesis, biosynthesis of unsaturated
455 fatty acids, and fatty acid biosynthesis (Figure 3A-D). Genes involved in the
456 biosynthesis of unsaturated fatty acids, including stearoyl-CoA desaturase (SCD),
457 fatty acid desaturase 2 (FADS2), and elongation of very long-chain fatty acids protein
458 4 (ELOVL4), exhibited significantly higher protein abundance in northern
459 environments in both oyster species (Supplementary Figure S6). We found significant
460 enrichment of the critical cell signaling pathway, the Notch signaling pathway, in both
461 comparison groups: SA vs. NA and SA vs. SG.

462 **Key pathway for temperature adaptation: ABC transporters**

463 Combining GO and KEGG analyses, the ATP-dependent transporter ABC
464 transporter family may represent an important pathway in temperature adaptation.
465 Enrichment analysis identified 29 genes in ABC transporters pathway, with
466 comprehensive physicochemical characterization (molecular weights, theoretical pI,
467 and instability index) detailed in Supplementary Table S6. To better characterize
468 functional divergence among the 29 ABC transporter genes, we constructed a
469 maximum-likelihood phylogenetic tree using their full-length protein sequences with
470 human orthologs as evolutionary anchors (Figure 4A). Phylogenetic analysis coupled
471 with motif and domain prediction classified the 29 ABC transporter genes into five
472 subfamilies: ABCA (7 genes), ABCB (7 genes), ABCC (9 genes), ABCD (3 genes),
473 and ABCG (3 genes, Fig. 4B). The classification details of each ABC transporters
474 subfamily were presented in Supplementary Table S7.

475 We observed significantly higher ABC transporters protein abundance in oysters
476 cultured under northern environmental conditions compared to southern counterparts,

with the exception of specific proteins including CgABCA1-1, CgABCA1-7, CgABCC1/2-like-3, CgABCC1/2-like-9, and CgABCG1/2-like-2 (Figure 4C). The overall trend suggested that relatively cold northern environments may require higher ABC transporters protein abundance. Transcriptomic analysis revealed no consistent tissue-specific expression pattern across ABC transporters family genes, with individual members showing distinct expression profiles among different tissues (Figure 4D). Under controlled short-term temperature stress, ABCA, ABCB, and ABCD subfamilies exhibited a clear increase trend at lower temperatures (5-15 ℃, Figure 4E). These findings aligned with the long-term temperature adaptation patterns observed in field populations, confirming the crucial role ABC transporters played in cold adaptation across oyster species. However, ABCC subfamily members responded to high temperature (25-35 ℃), while ABCG transporters showed no temperature-dependent expression changes (Figure 4E).

Identification of heat and cold adaptation membrane proteins

We considered membrane proteins that were present in higher content under the warm southern environment in both oyster species (SG > NG, SA > NA) and were also more abundant in the relatively heat tolerant *C. angulata* compared to *C. gigas* (NA > NG, SA > SG) as heat adapted membrane proteins, while conversely as cold adapted membrane proteins. The information of the screened heat and cold adapted membrane proteins was presented in Supplementary Table S8. Through transmembrane domain prediction to verify membrane localization and remove false positives, we ultimately identified 5 heat and 6 cold adapted membrane proteins (Figure 5A). We observed that AQUAPORIN family members were represented in both heat-adapted (CgAQUAPORIN-8) and cold-adapted (CgAQUAPORIN-11) membrane protein group. Subsequently, a phylogenetic tree was constructed based on human orthologs, and combined with motif and domain prediction results, the functional classification of aquaporins was determined (Figure 5B). The protein content of CgAQUAPORIN-8 and CgAQUAPORIN-11, as mentioned above,

exhibited significant differences across different environments and species comparisons (Figure 5C). Transcriptomic analysis showed both CgAQUAPORIN-8 and CgAQUAPORIN-11 were highly expressed in gills, which were sensitive to environmental changes (Figure 5D). CgAQUAPORIN-11 also showed specifically high expression in labial palps. However, CgAQUAPORIN-8 and CgAQUAPORIN-11 did not respond to short-term temperature stress (Figure 5D). The expression levels were higher under non-stressful temperatures (15-25 ℃), but decreased when exposed to temperature stress.

To validate the critical role of the AQUAPORIN family in temperature adaptation, RNA interference (RNAi) targeting CgAQUAPORIN-8 was performed in oysters. In pilot experiments, both siRNAs "siAQU8-37" and "siAQU8-167", effectively suppressed the expression level of CgAQUAPORIN-8. Based on the observed alterations in gene expression, the "siAQU8-167" was identified as the more effective siRNA and an interference duration of 24 hours was determined to be optimal (Supplementary Figure S7). Subsequently, we conducted a semi-lethal temperature (42 ℃) heat stress experiment to compare the morphological changes of oyster tissues between negative control and CgAQUAPORIN interference group. HE staining indicated that elevated temperatures induced significant structural alterations in the gill tissues of oysters. However, no notable differences were observed between the control group and the siAQU-8 group (Supplementary Figure S8). Additionally, survival curve analysis revealed that oysters in the siAQU-8 group exhibited a higher mortality rate after heat stress compared to the control group (Figure 6A), along with an increased apoptosis rate in the gill tissues (Figure 6B, C).

Genetic variations of AQUAPORIN mediate alterations in transport channels to adapt to temperature changes

To investigate the potential link between AQUAPORIN family genes and temperature adaptation, we employed genetic approaches to identify protein variants between two oyster species with divergent temperature tolerance. Comparative

resequencing data uncovered a conserved nonsynonymous variant Val183Ile in the AQUAPORIN-8 coding sequence between 108 *C. gigas* and 47 *C. angulata* (Table 1). To validate the accuracy and conservation of this variant, we performed Sanger sequencing on 44 wild *C. gigas* and 48 *C. angulata* (Table 1). The nonsynonymous mutation Val183Ile was evolutionarily conserved between *C. gigas* and *C. angulata* and located in the fifth transmembrane domain, with the dominant genotype being Ile in *C. gigas* and Val in *C. angulata* (Figure 7A, B). Through literature review and structural prediction, we confirmed that AQUAPORIN-8 forms transport channels as a tetramer, with the variant site Val183Ile located inside the channel (Figure 7C). Structural analyses revealed that the protein mutation significantly altered tetramer stabilization. The dominant *C. angulata* genotype exhibited stronger binding affinity (-34.8 kcal/mol) compared to *C. gigas* (-31.3 kcal/mol, Fig. 7D, E). Additionally, we constructed mutant plasmids for *C. gigas*/Ile and *C. angulata*/Val and validated the association between the variation sites and temperature sensitivity in the cellular model. The results demonstrated that the *C. angulata* AQUAPORIN-8 mutant exhibited enhanced cell viability (Figure 8A) and reduced apoptosis (Figure 8B, C, Supplementary Figure S9) after heat stress, indicating its stronger heat tolerance.

DISCUSSION

The sustained rise in ocean temperatures poses significant threats to marine organisms' survival, compelling investigation into their molecular mechanisms of temperature adaptation under global warming (Mcculloch et al., 2024; Starko et al., 2024). However, the abundance characterization of membrane proteins remains poorly understood, which function as sensitive temperature sensors (Jafurulla et al., 2019; Wu et al., 2023). In this study, we conducted a comparative study on the changes in membrane protein content of *C. gigas* and *C. angulata* with divergent temperature tolerances due to long-term adaptation to different ambient temperatures in southern and northern regions. Our results revealed that ATP-dependent transporters, Notch signaling pathway, lipid metabolism-related pathways and

aquaporins may serve as membrane protein targets for further studies on temperature adaptation. Previous proteomic and phosphoproteomic analyses of oyster's gill tissue under acute thermal stress identified significantly enriched pathways in signal transduction, energy metabolism, protein synthesis, and cell survival/apoptosis, which primarily involved intracellular proteins rather than membrane-associated proteins (Wang et al., 2023a). However, the limitation of this study is that each experimental group only has three biological replicates.

ABC transporters family

GO and KEGG analyses revealed significant enrichment of ABC transporters pathway in both environmental and species comparisons, strongly suggesting their critical role in oyster temperature adaptation. ABC transporters are one of the largest protein families discovered in organisms, which utilize energy from ATP hydrolysis to transport diverse compounds across membranes and regulate vital physiological activities (Davidson et al., 2008; Theodoulou and Kerr, 2015). ABC transporters are classified into eight major subfamilies (A-H), each specialized for transmembrane transport of distinct substrates (Broehan et al., 2013; Singh et al., 2024). In this study, we identified ABC transporter members belonging to five subfamilies: ABCA, ABCB, ABCC, ABCD, and ABCG. ABCA subfamily transporters mediated cellular lipid transport and played essential roles in lipoprotein biogenesis in mammals (Wenzel et al., 2007). However, their functional characterization remained limited in invertebrate systems. ABCB subfamily transporters participate in porphyrin transport and reactive oxygen species (ROS) regulation, mediating crucial cellular detoxification processes (Zutz et al., 2009). The ABCB family member P-glycoprotein (ABCB1) was found to be actively involved in the transport of inorganic mercury in sea urchins (Bosnjak et al., 2009). ABCC subfamily transporters demonstrated significant functional diversity, including transport of organic anions, translocation of cyclic nucleotides and cell signal transduction via sulfonylurea receptors (Deeley et al., 2006). ABCD subfamily transporters executed the ATP-dependent translocation of fatty acids and fatty acyl-

CoAs into peroxisomes (Morita and Imanaka, 2012). Most members of the ABCG subfamily mediated mammalian lipid homeostasis by transporting internally synthesized and dietary lipids, such as cellular cholesterol efflux (Kusuhara and Sugiyama, 2007). The functional diversity and complexity of ABC transporters underscored their fundamental roles in biological processes, consistently suggesting their equally critical involvement in environmental adaptation mechanisms.

A majority of identified ABC transporter pathway genes consistently exhibited higher protein abundance in northern environments both in *C. gigas* and *C. angulata*, suggesting their potential involvement in cold adaptation. Short-term cold stress experiments further supported ABCA, ABCB and ABCD transporters' responsiveness to low temperatures. ABC transporters was extensively demonstrated to play crucial roles in plant responses to cold stress, including maize (Yang et al., 2024), tea plants (Shen and Li, 2023), and *Pyropia yezoensis* (Tian et al., 2024). Additionally, temperature-responsive regulatory elements were widely identified upstream of ABC transporter genes in bacteria, providing direct evidence for their temperature-dependent expression changes (Tong et al., 2024). Studies on temperature-responsive ABC transporters in marine animals were notably lacking, whereas their involvement in acclimatization to salinity stress (Honorato et al., 2017; Shi et al., 2023) and heavy metal exposure (Contreras et al., 2010; Morcillo et al., 2018) was documented. Therefore, this study is the first to report in marine organisms that the important membrane protein ABC transporter family is associated with temperature adaptation and actively responds to low temperatures by increasing protein content. Gene family analysis revealed significant expansion of ABC transporter genes in bivalves, with 64, 67, and 58 members identified in *P. yessoensis*, *C. farreri*, and *C. gigas* respectively, compared to only 48 in humans (Wang et al., 2021). The expansion of the ABC transporter family in bivalves also indicated its important function, enhancing the organism's adaptability to better cope with environmental changes (Sandve and Fjellheim, 2010; Wang et al., 2021).

Notch signaling pathway

Temperature variations can activate cell-surface thermoreceptors, initiating downstream signaling cascades that orchestrate adaptive responses to environmental changes (Sharma et al., 2024). Our study identified Notch signaling pathway as playing a critical role in oyster temperature adaptation, which was a highly conserved intercellular communication mechanism fundamental to most organisms (Cai et al., 2016). In vertebrates, Notch signaling was demonstrated to participate in key biological processes, including inflammatory responses (Cai and Dai, 2025), oxidative stress regulation (Chiappara et al., 2024), and cell apoptosis (Zeng et al., 2016). High temperatures induce inflammatory responses and oxidative stress in marine organisms, posing significant threats to their physiological homeostasis, such as coral (Tang et al., 2024), shrimp (Guo et al., 2020) and sea cucumber (Ji et al., 2008). Additionally, previous studies documented that high temperatures induced apoptosis in oysters, accompanied by increased oxidative stress markers malondialdehyde (MDA) and reactive oxygen species (ROS) (Wang et al., 2023a). Therefore, oxidative stress and cell apoptosis were key mechanisms of thermal-induced cellular damage in marine organisms (Tang et al., 2024). Thermal stress-activated Notch signaling may confer heat resistance in oysters through coordinated regulation of antioxidant defenses and apoptotic pathways (Ishio et al., 2015; Nomura et al., 2022). However, mechanistic explorations of the Notch signaling pathway and its potential role in temperature adaptation remained scarce in marine organisms. We can only speculate about its role under high temperatures based on its functions in vertebrates, and further research is still needed.

Biosynthesis of unsaturated fatty acids

The fluid nature of biological membranes underpins their dual role as both structural barriers and functional platforms for vital cellular processes (Raj et al., 2023). Fluctuations in ambient temperature can sensitively affect the physical properties of membranes (fluidity), disrupt the ordered arrangement of membrane

lipids, and result in membrane dysfunction (Wu et al., 2023). The double bonds in unsaturated fatty acids can cause loose packing of lipid molecules and enhance membrane fluidity, which is an important mechanism for organisms to maintain membrane homeostasis, also known as homeoviscous adaptation (Wu et al., 2023). Our study identified three key membrane proteins involved in unsaturated fatty acid biosynthesis: SCD, FASD2 and ELOVL4. These three proteins are endoplasmic reticulum membrane-bound proteins, which serve as the primary intracellular site for the biosynthesis of lipids, carbohydrates, and proteins (Miyazaki and Ntambi, 2003). SCD and FADS2 served as key rate-limiting enzymes in fatty acid desaturation, catalyzing the biosynthesis of oleic acid and polyunsaturated fatty acids (PUFAs) that modulated membrane fluidity of oysters during cold adaptation in previous studies (Li et al., 2024; Wang et al., 2023b). ELOVLs were enzymes involved in very long-chain fatty acid (VLCFA) biosynthesis and responded to environmental stress in oysters (Liu et al., 2023a). The regulation of unsaturated fatty acid content to maintain membrane fluidity homeostasis represented a central research focus in marine organism temperature adaptation studies (Holm et al., 2022; Miliou et al., 2006). Global plankton lipidome surveys revealed that fatty acid unsaturation was fundamentally constrained by temperature, with cold-region organisms exhibiting three-fold higher unsaturated fatty acid content compared to warm-water counterparts (Holm et al., 2022). In this study, we observed significantly higher expression of these proteins in northern cold-adapted versus southern warm-adapted populations of both oyster species, suggesting their potential role in maintaining membrane fluidity via unsaturated fatty acid biosynthesis under cold conditions. Lipid metabolism serves as a fundamental biological process, and these three key lipid metabolic genes were also attracted significant research attention in other marine organisms (Dong et al., 2016; Monroig et al., 2012). As the primary site of membrane lipid synthesis, the temperature response and molecular regulatory mechanisms of the endoplasmic reticulum and its membrane-associated lipid metabolic genes still require further

673 investigation (Fagone and Jackowski, 2009).

674 **Key temperature adaptation membrane protein: AQUAPORIN**

675 Combining the conditions of environmental comparison and species comparison,
676 the more reliable heat and cold adaptive proteins were identified. AQUAPORIN-8 as
677 a heat-adaptive protein and AQUAPORIN-11 as a cold-adaptive protein showed
678 significant differences in protein content among different treatment groups, which
679 demonstrated the importance of functions in long-term temperature adaptation.
680 Additionally, AQUAPORINs showed tissue-specific overexpression in gills and labial
681 palps of oysters, which are known to be highly sensitive to environmental temperature
682 fluctuations. The AQUAPORIN family represents a diverse group of membrane
683 channel proteins that facilitate passive transport of water and small solutes (urea,
684 glycerol, H_2O_2, NH_3, and CO_2) driven by osmotic gradients across biological
685 membranes (Finn and Cerdà, 2015; Sansom and Law, 2001). Many studies have
686 established AQUAPORIN-8 as a dual-function channel mediating both water and
687 ammonia transport, with particularly high-water permeability observed in
688 mitochondrial membranes (Calamita et al., 2005; Geyer et al., 2013). AQUAPORIN-
689 11 belongs to the superaquaporin group based on sequence divergence from classical
690 aquaporins, retaining water transport capability despite its atypical NPA motifs
691 (Yakata et al., 2008). As essential water channel proteins, AQUAPORINs have been
692 demonstrated to be important targets of temperature adaptation in plants. Low
693 temperature reduced water mobility, AQUAPORINs maintained plant water
694 homeostasis and participated in cellular tolerance mechanisms by regulating
695 transmembrane water flux under cold stress (Dong et al., 2022). Elevated
696 AQUAPORIN expression represented an adaptive response to high-temperature stress
697 by mitigating evaporative water loss, a major cause of cellular damage under heat
698 conditions (Yooyongwech et al., 2008). In this study, we found functionally
699 specialized AQUAPORINs evolved divergent expression pattern for temperature
700 adaptation in oysters under ambient temperature selection pressures. RNAi

experiments demonstrated that AQUAPORIN-8 played a functional role in resisting heat damage. However, the role of AQUAPORINs in temperature adaptation remains poorly understood in marine organisms, with limited studies of how these channels mediated temperature responses at the molecular level.

In addition to differential protein abundance, genetic variations were identified within the coding region of AQUAPORIN-8. The nonsynonymous mutation Val183Ile exhibited conserved differentiation, being present in the two wild populations of *C. gigas* and *C. angulata*. During long-term thermal adaptation, the two closely-related oyster species accumulated numerous environmentally selected variants in non-coding regions, including promoter polymorphisms of lipid metabolism genes (SCD, ATGL, SREBP) that correlate with their differential expression patterns (Du et al., 2025; Li et al., 2021; Wang et al., 2023b). The nonsynonymous mutations in coding regions, which alter the amino acid sequence of proteins, may exert more direct structural impacts on their functional performance (Farhana et al., 2022). In this study, the Val183Ile variation was located within the AQUAPORIN-8 tetrameric channel, potentially affecting the stability of tetrameric protein assembly and the formation of the transport channel, thereby regulating the translocation of water and other small molecules to adapt to ambient temperature. We demonstrated that the Val183Ile site directly affected cell apoptosis and viability after heat stress at the cellular level. The identified genetic variants could be utilized in oyster breeding programs to enhance stress resistance traits. Compared to cytoplasmic proteins that maintain intracellular homeostasis, membrane proteins—exposed to more dynamic and selective extracellular environments—evolve faster and harbor more variant sites (Sojo et al., 2016). Therefore, taking AQUAPORIN-8 in this study as an example, we proposed that coding-region variations in membrane proteins may serve as potential targets for investigating temperature adaptation in marine animals. A schematic of molecular mechanisms underlying membrane protein-mediated temperature adaptation in *C. gigas* and *C. angulata* was shown in Fig. 8.

CONCLUSION

In conclusion, we conducted the first proteomic characterization of membrane protein composition changes during long-term temperature adaptation in two congeneric oyster species. Our analysis identified several key pathways associated with oyster temperature adaptation, including ABC transporters, Notch signaling, biosynthesis of unsaturated fatty acids, and aquaporins. ABC transporters, a gene family responsible for transmembrane transport of diverse compounds, showed consistently higher protein abundance in northern environments, suggesting their involvement in cold adaptation. The Notch signaling pathway may serve as a key membrane protein pathway that sensed temperature fluctuations and coordinating regulation of antioxidant defense and apoptosis mechanisms. The rate-limiting enzymes for unsaturated fatty acid biosynthesis in the endoplasmic reticulum (SCD, FADS2, and ELOVL4) showed higher expression in northern environment, potentially maintaining membrane fluidity through increased unsaturated fatty acid production. Aquaporins were membrane channel proteins that mediated osmotically driven transport of water and small solutes, serving as key temperature adapted membrane proteins, with AQUAPORIN-8 functioning as a heat-adapted protein and AQUAPORIN-11 as a cold-adapted protein. Nonsynonymous mutations in the AQUAPORIN-8 coding region may modulate tetrameric water channel assembly, thereby altering transport efficiency and potentially driving divergent temperature tolerance between the two oyster species. This study provides the first characterization of membrane proteome dynamics under temperature adaptation in oysters, identifying key temperature-responsive pathways and candidate targets for future research, which offers novel insights for predicting adaptive potential in marine organisms and establish a genetic foundation for precision molecular breeding in aquaculture.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD065552.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Li Li and Guo-Fan Zhang conceived the study. Ming-Yang Du, and Chao-Gang Wang participated in the data analysis, and drafted the manuscript. Ming-Yang Du, Chao-Gang Wang, Mei-Qian Pang, Jin-Cheng Chen, Zhu-Xiang Jiang, Min Wang, carried out the field and laboratory work, collected the oyster samples. Ri-Hao Cong and Wei Wang contributed to cultural management. Ming-Yang Du, Chao-Gang Wang, Li Li and Guo-Fan Zhang revised the manuscript. All authors read and approved the final version of the manuscript.

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Table 1. Statistics of Genotype Frequency of nonsynonymous mutations in AQUAPORIN-8.

Methods	Species	GG	GA	AA	Chi-square Test *P*-value
Resequencing	***C. gigas***	56	44	8	<0.0001
	C. angulata	45	2	0	
Sanger sequencing	***C. gigas***	17	18	9	<0.0001
	C. angulata	45	2	1	

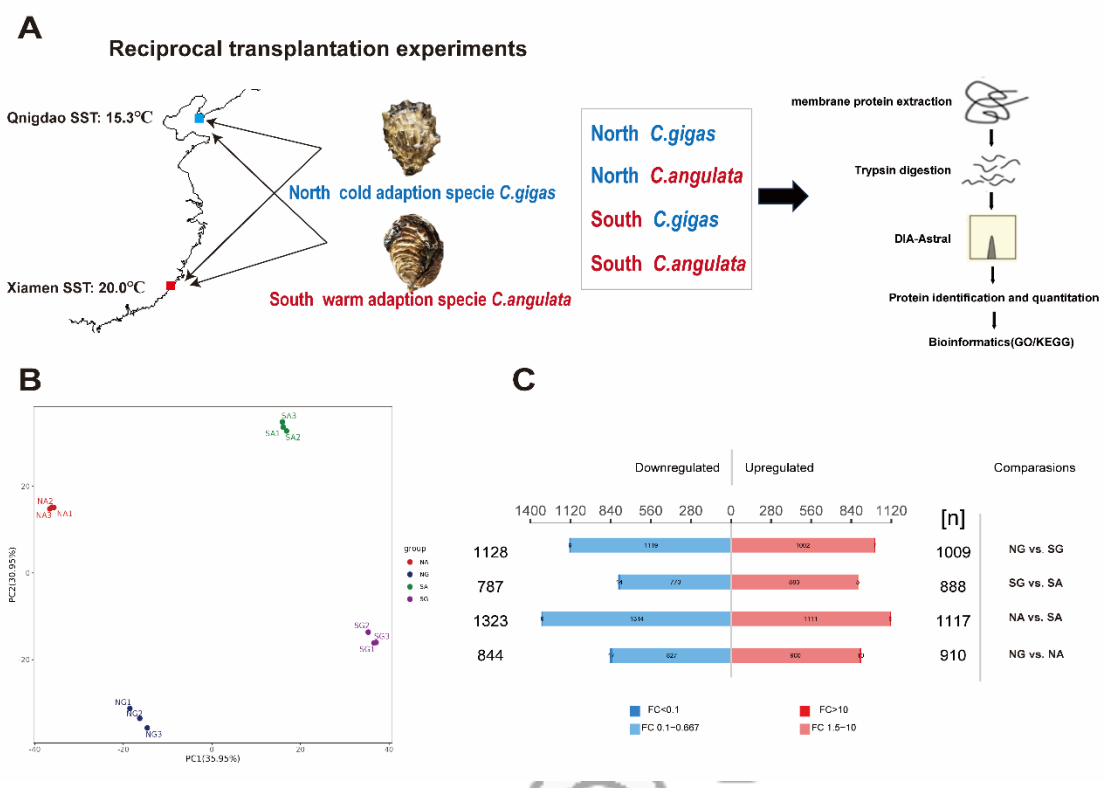


Figure 1 Characterization of the oyster membrane proteome during long-term temperature adaptation in natural northern and southern habitats. A: The details of reciprocal transplantation experiment design. B: Principal component analysis (PCA) of four treatment groups (North *C. gigas*, North *C. angulata*, South *C. gigas*, South *C. angulata*) with three biological repetitions (n = 3). C: Number of differentially expressed membrane proteins in environmental comparison (NG vs. SG and NA vs. SA) and species comparison (NG vs. NA and SG vs. SA).

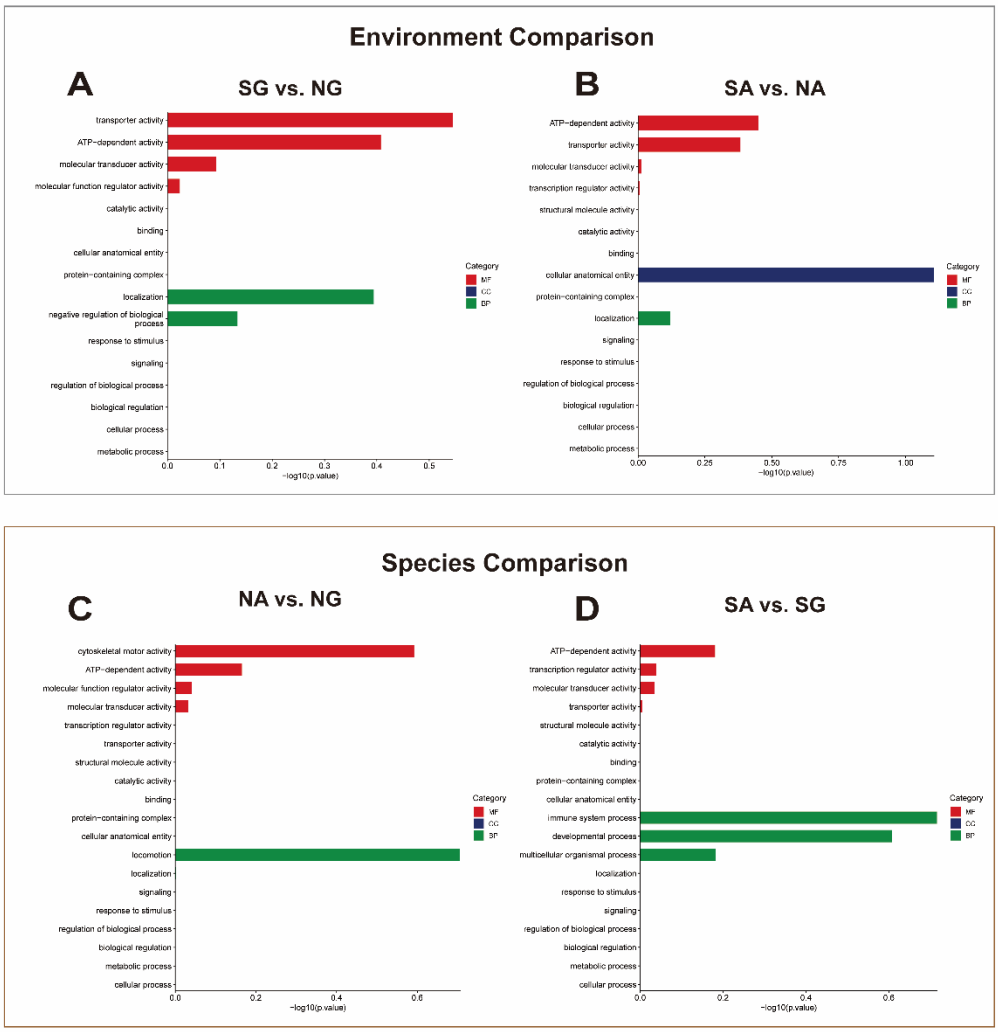


Figure 2 GO enrichment of differentially abundant membrane proteins. A,B: GO enrichment analysis of differentially expressed proteins identified in north and south comparison. (A) South *C. gigas* vs. North *C. gigas*, (B) South *C. angulata* vs. North *C. angulata*. C,D: GO enrichment analysis of differentially expressed proteins identified in *C. gigas* and *C. angulata* comparison. (C) North *C. angulata* vs. North *C. gigas*, (D) South *C. angulata* vs. South *C. gigas*. The red modules represented molecular functions (MF), the blue modules represented cellular components (CC), and the green modules represented biological processes (BP). The size of the horizontal axis corresponded to the -$\log_{10}$ (*p*-value).

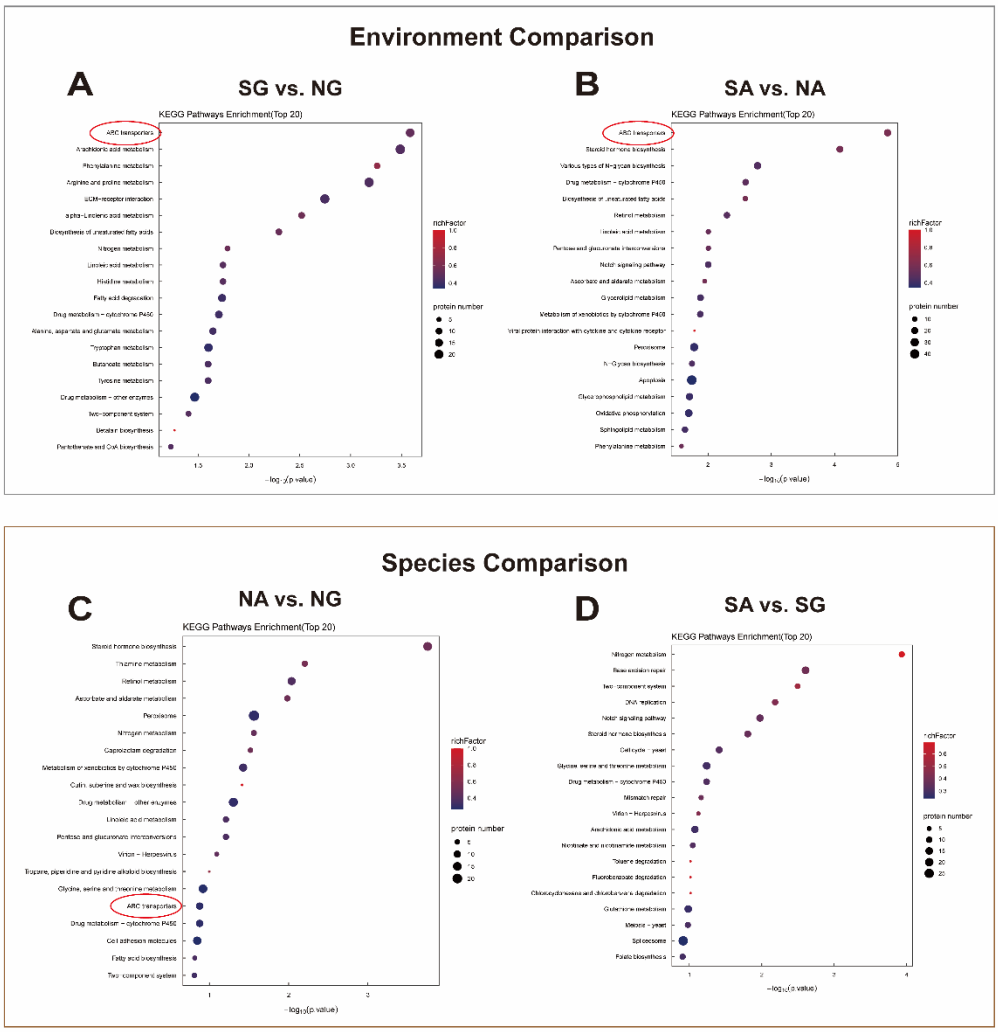


Figure 3 KEGG pathway enrichment of differentially abundant membrane proteins. A,B: KEGG pathway enrichment analysis of differentially expressed proteins identified in north and south comparison. (A) South *C. gigas* vs. North *C. gigas*, (B) South *C. angulata* vs. North *C. angulata*. C,D: KEGG pathway enrichment analysis of differentially expressed proteins identified in *C. gigas* and *C. angulata* comparison. (C) North *C. angulata* vs. North *C. gigas*, (D) South *C. angulata* vs. South *C. gigas*. Color corresponded to the rich factor, size represented gene counts, and the length of the horizontal axis corresponded to the -$\log_{10}$ (*p*-value).

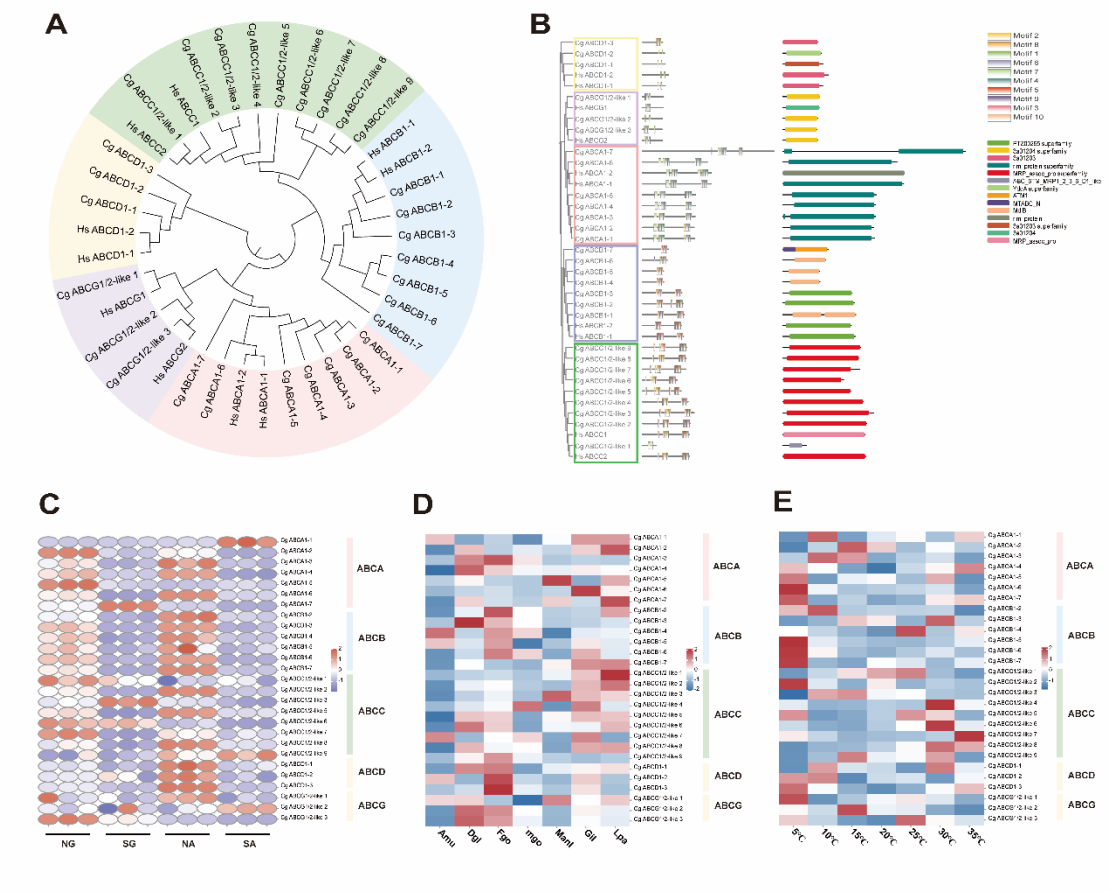


Figure 4 Phylogenetic analysis and expression changes of key pathway for temperature adaptation ABC transporters. A: Phylogenetic tree of identified ABC transporters. The tree was constructed with the maximum likelihood (ML). Hm, *Homo sapiens*; Cg, *Crassostrea gigas*. B: The motif and domain prediction of identified ABC transporters. The right section represented domain information, while the middle section represented motifs. C: The protein content of ABC transporters in four group. North *C. gigas* (NG); South *C. gigas* (SG); North *C. angulata* (NA); South *C. angulata* (SA). The color corresponded to the variation in content levels. D: The gene expression profiles of ABC transporters in different tissues. Amu, Adductor; Dgl, Digestive gland; Fgo, Female gonad; Mgo, Male gonad; Man, Mantle; Gil, Gill; Lpa, Labial palp. The color corresponded to the variation in expression levels. E: The gene expression profiles of ABC transporters under different temperature stress. The color corresponded to the variation in expression levels.

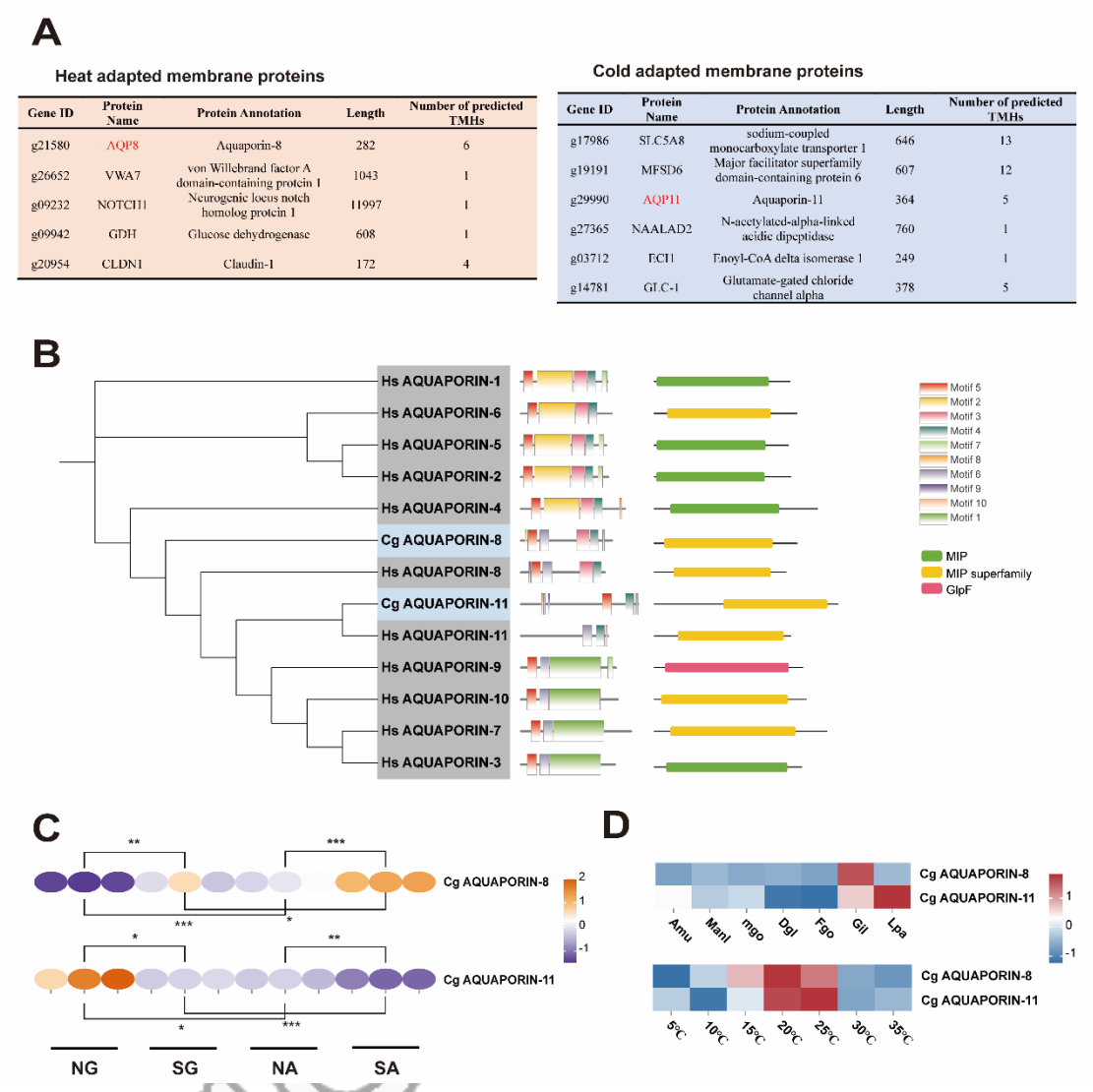
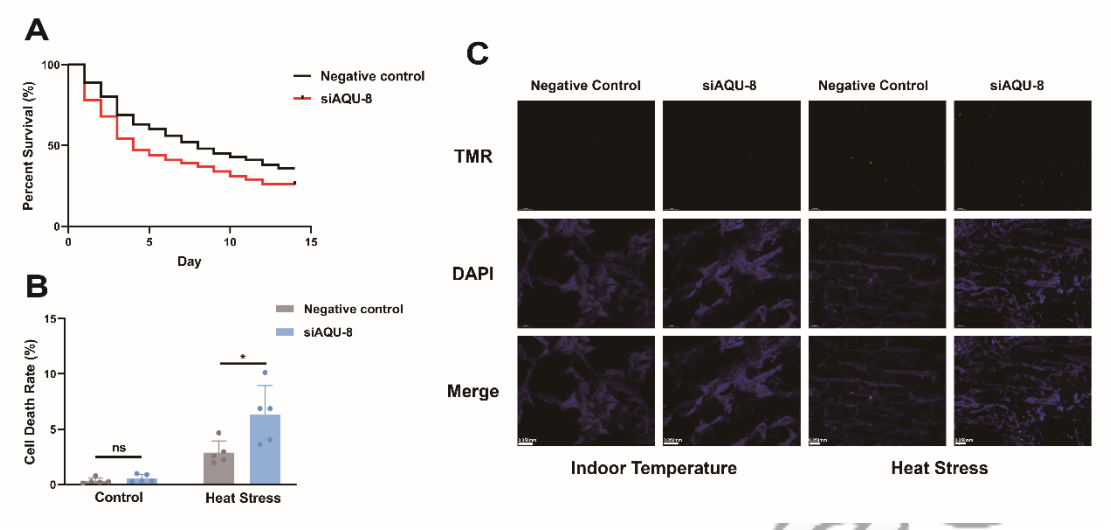


Figure 5 Identification of key heat and cold adapted membrane proteins AQUAPORIN. A: Statistics of heat and cold adapted membrane proteins. B: Phylogenetic tree of AQUAPORINs. The tree was constructed with the maximum likelihood (ML). Hm, *Homo sapiens*; Cg, *Crassostrea gigas*. C: The protein content of AQUAPORINs in four group. North *C. gigas* (NG); South *C. gigas* (SG); North *C. angulata* (NA); South *C. angulata* (SA). The color corresponded to the variation in content levels. D: The gene expression profiles of ABC transporters in different tissues and temperature stress treatment. Amu, Adductor; Dgl, Digestive gland; Fgo, Female gonad; Mgo, Male gonad; Man, Mantle; Gil, Gill; Lpa, Labial palp. The color corresponded to the variation in expression levels.



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1206 **Figure 6 Functional validation of the key heat adapted membrane protein**
1207 **AQUAPORIN-8 in oysters.** A: Kaplan–Meier survival curves of oysters in heat-
1208 lethal experiments after AQUAPORIN-8 RNA interference experiments. B: The cell
1209 apoptosis rate results of gill tissues in control and siAQU-8 group under indoor
1210 temperature and heat stress. C: Effects of heat stress on cellular death in gill tissues
1211 (TUNEL analysis) between control and siAQU-8 group.

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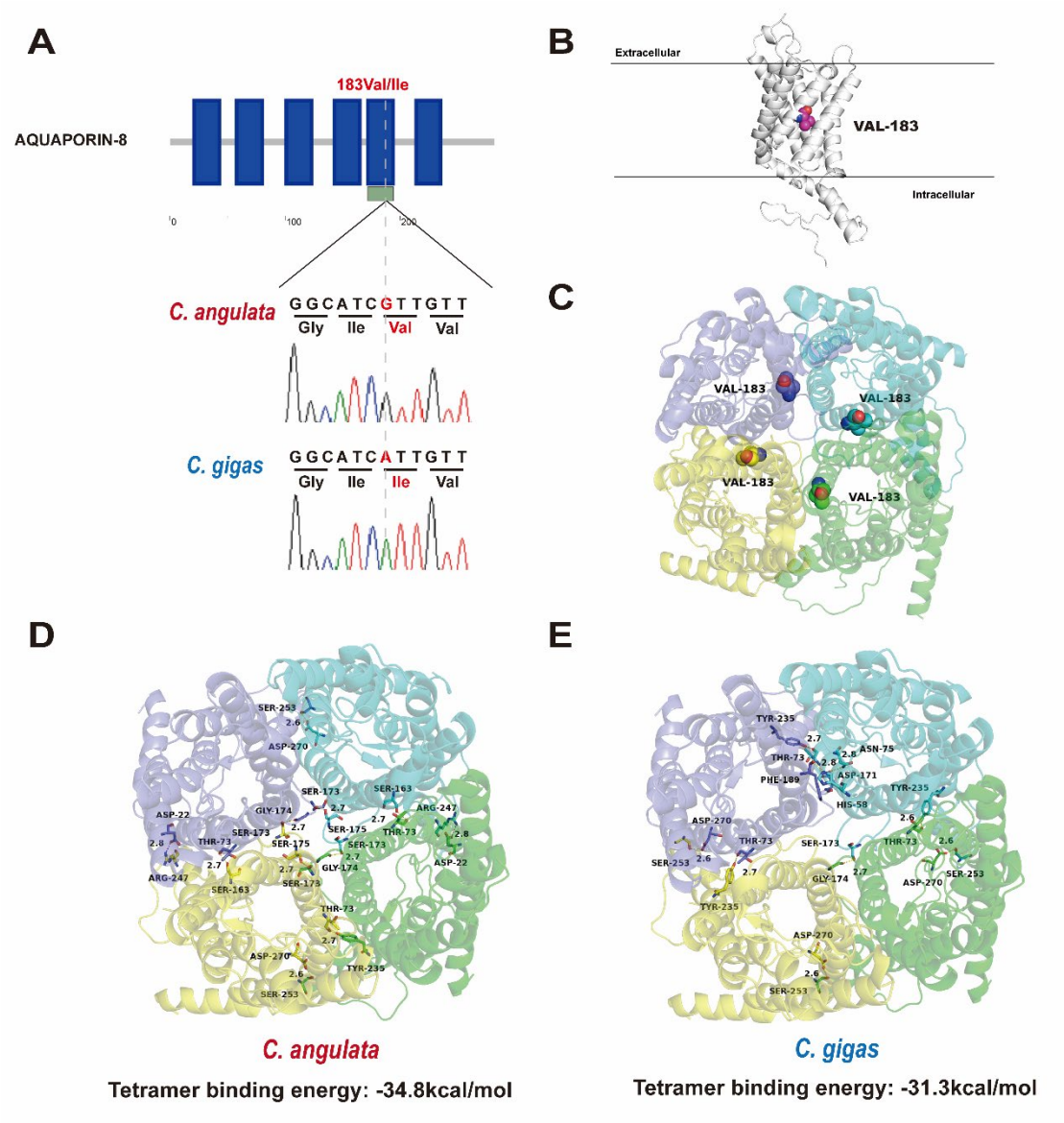

Figure 7 Genetic variations of AQUAPORIN-8 mediated structural changes in water channel tetramer conformation. A: The nonsynonymous mutation of AQUAPORIN-8 in *C. gigas* and *C. angulata*. The upper section represented protein structure prediction, with blue indicating transmembrane regions. The lower section showed the CDS sequence and amino acid variations in *C. gigas* and *C. angulata*. B: The protein structure of AQUAPORIN-8. The location of the nonsynonymous mutation was shown in the figure. C: The tetrameric structure of AQUAPORIN-8. The location of the nonsynonymous mutation was shown in the figure. D,E: The schematic of nonsynonymous mutations affecting AQUAPORIN-8 tetramerization in *C. gigas* (D) and *C. angulata* (E).

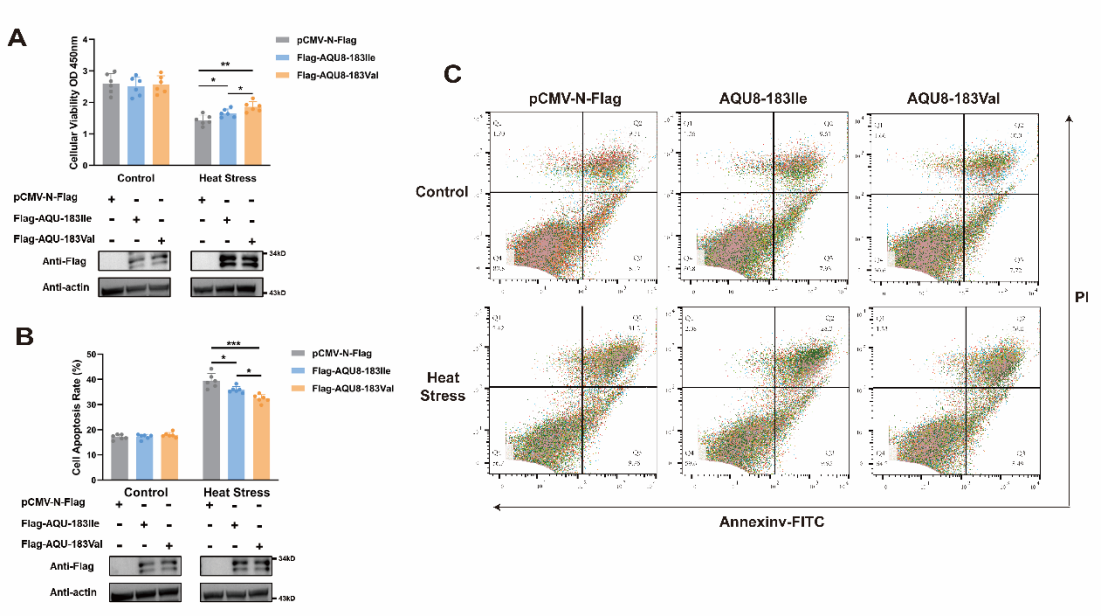

Figure 8 Genetic variations of AQUAPORIN-8 influence cellular thermal resistance. A: The CCK-8 assay for the cell viability (n=6). HEK293T cells transfected with Flag-AQU8-183Ile and 183Val under control and heat stress. The error bars represent SE. AQUAPORIN-8 protein expression was confirmed by western blotting. β-Actin served as a loading control. B: The cell apoptosis rate of HEK293T cells under control and heat stress (n=6). C: The cell apoptosis chart in each group. Each group displayed overlaid data from six biological replicates.

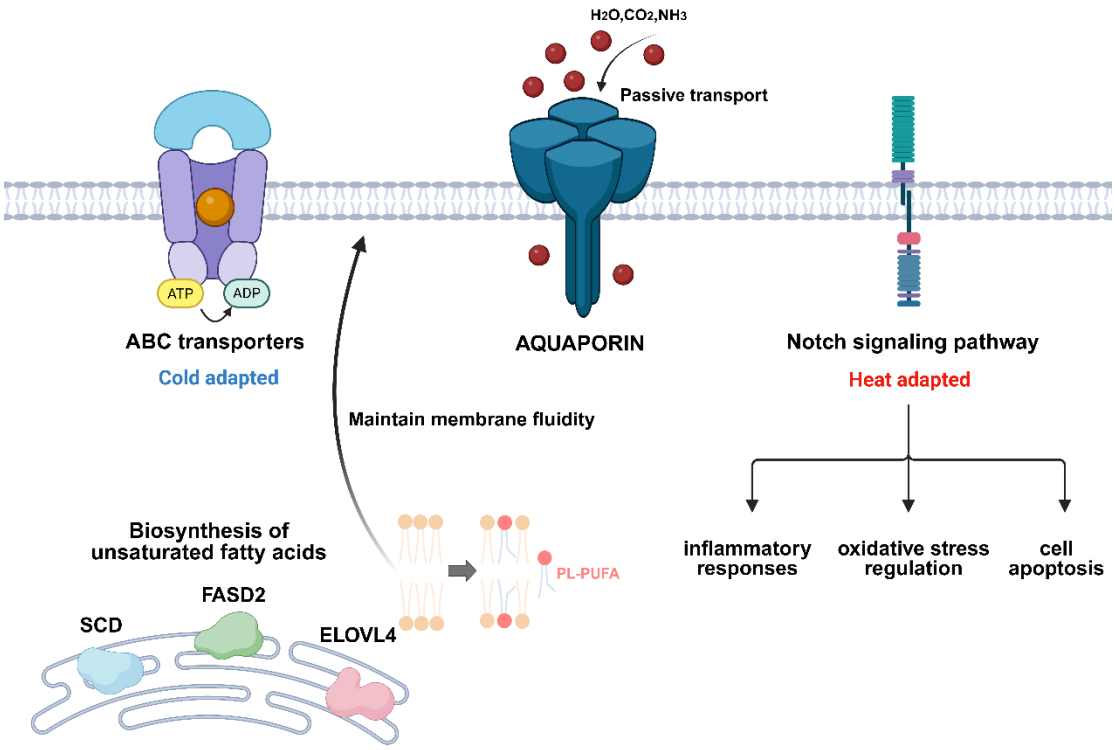

Figure 9 The molecular mechanisms of key membrane protein for temperature adaptation in oysters. ABC transporters utilized energy from ATP hydrolysis to transport various compounds across membranes. Aquaporins facilitated the passive transport of water and small solutes (urea, glycerol, H_2O_2, NH_3, and CO_2) across biological membranes along osmotic gradients. The Notch signaling pathway participated in inflammatory responses, oxidative stress regulation, and cell apoptosis to help oysters cope with high-temperature stress. The endoplasmic reticulum membrane proteins SCD, FADS2, and ELOVL4 were key enzymes in unsaturated fatty acid biosynthesis, which maintained membrane fluidity and normal membrane function.

膜蛋白在牡蛎温度适应中的动态重塑表征

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摘要

全球变暖导致的海洋温度上升对海洋生物生存构成严重威胁。膜蛋白作为敏感的环境压力感应器承担重要的生物学功能。然而，对膜蛋白在海洋生物响应响应环境温度的含量变化表征却有待阐明。本研究选取了两种异域分布但亲缘关系密切的牡蛎物种长牡蛎（*Crassostrea gigas*）与福建牡蛎（*C. angulata*），在它们的北方/寒冷与南方/温暖自然栖息地之间进行了对调移植实验。结合膜蛋白质组和转录组分析发现 ABC 转运蛋白、Notch 信号通路、不饱和脂肪酸生物合成以及水通道蛋白 AQUAPORIN 可能是牡蛎温度适应的关键分子组分。ABC 转运蛋白及不饱和脂肪酸合成的关键内质网酶（SCD、FADS2 和 ELOVL4）在北方环境中持续表现出更高的蛋白质含量，表明它们参与冷适应过程。Notch 信号通路可能作为一种感知温度波动、并协调抗氧化防御与细胞凋亡调控的关键机制。此外，我们发现水通道蛋白 AQUAPORIN 既是关键的热适应蛋白也是冷适应蛋白，表明其在响应温度波动中发挥重要作用。在 AQUAPORIN-8 蛋白编码区鉴定出了一个非同义突变，可能调控蛋白四聚体水通道的形成从而改变运输效率，并介导两种牡蛎物种间的差异温度耐受性形成。本研究首次系统表

征了牡蛎膜蛋白含量的动态变化，揭示了其在环境温度适应中的关键作用，并为预测海洋生物对未来气候变化的适应潜力提供了新见解。

关键词： 全球变暖；温度适应；膜蛋白；牡蛎