



Metabolomic profiling reveals that the heterogeneity of microhabitats can assist intertidal mollusks in surviving extreme cold events

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Abstract

Microhabitat heterogeneity results in significant variations in the thermal environment on a small spatial scale, leading to different intensities of cold stress during extreme low-temperature events. Investigating variations in body temperature and metabolomic responses of organisms inhabiting different microhabitats emerges as an important task for understanding how organisms respond to more frequent extreme low-temperature events in the face of climate change. In the present study, we measured substrate temperature, air temperature, wind speed, light intensity, and body temperature to evaluate the relative importance of drivers that affect body temperature in different microhabitats, and determined the metabolomic responses of intertidal snails *Littorina brevicula* and limpets *Cellana toreuma* from different microhabitats (snail: exposed vs. shaded rock; limpet, rock vs. tidal pool) during extreme low-temperature event in winter. Results showed that microhabitat type, substrate temperature, air temperature, wind speed, and light intensity contribute notably to the body temperatures. During extreme low-temperature events, mollusks collected from different microhabitats exhibited microhabitat-specific metabolomic responses that are associated with cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation. These metabolic pathways were highly induced in the more exposed areas (exposed rock for snails and rocky environment for limpets). Notably, in different microhabitats, the metabolites enriched from these pathways showed significant correlations with microclimate environmental variables (i.e., substrate temperature, wind speed, and body temperature). Overall, these findings highlight the importance of microhabitat heterogeneity for intertidal species surviving extreme cold events and are essential for understanding cold adaptation of intertidal species in the context of climate change.

Keywords Body temperature · Environment factor · Intertidal snail · Limpet · Metabolomic response · Microhabitat

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Introduction

Increases in the frequency and magnitude of extreme low-temperature events have been recognized as a notable manifestation of climate change (Chikoore et al. 2024; Currie-Olsen et al. 2023; Mirza 2003; Stott et al. 2016). An extreme low-temperature event typically refers to a region experiencing abnormally low temperatures during winter, with daily minimum air temperatures falling below the 10th percentile threshold of air temperatures recorded over the past 10 years or longer (Horton 1995; Klein Tank et al. 2009). The events can last for a few hours, days, or even longer, potentially leading to severe freezing conditions (Grotjahn et al. 2016; Newman 2023; Paçal et al. 2023). The outbreak of extreme low-temperature events has serious detrimental effects on marine organisms, especially those in the intertidal zone (Currie-Olsen et al. 2023). Organisms living in the intertidal zone are exposed to air during low tides, and extreme

temperatures can pose a serious threat to their survival and metabolic performance (Sun et al. 2023). For instance, oysters, mussels and barnacles experienced mass mortality after the severe winter of 1962 in Britain (Currie-Olsen et al. 2023). In addition, mass bleaching of the intertidal red algae *Chondrus crispus* Stackhouse (Gigartinales) and *Corallina officinalis* Linnaeus (Corallinales) has been observed following extreme low-temperature events in Atlantic Canada (Scrosati and Cameron 2023).

The habitat heterogeneity hypothesis, a central pillar of ecological theory, suggests that the spatial heterogeneity of abiotic and biotic conditions expands ecological niche dimensions, enabling coexistence among organisms and thereby increasing biodiversity (Heidrich et al. 2020; Kostylev et al. 2005; Stein et al. 2014). This perspective from the habitat heterogeneity hypothesis has been applied to disturbed environments (Cramer and Willig 2005; Greenberg et al. 1995). Nevertheless, it remains unclear whether this hypothesis is applicable to environmental stress induced by extreme low-temperature events, and the adaptive metabolic responses of intertidal organisms in different habitats remain poorly understood.

Intertidal rocky shores support a variety of habitats with different degrees of heterogeneity, and organisms experience varying levels of environmental stress among each habitat patch, resulting in the formation of patchy microhabitats (Aguilera et al. 2014; Bauer et al. 2024; Kostylev et al. 2005). The high spatial and temporal heterogeneity of temperatures creates varied thermal environments for intertidal species among microhabitats (Dong 2023; Foulk et al. 2024; Helmuth 1998; Helmuth and Hofmann 2001; Ma et al. 2024; Seabra et al. 2011). During low tides in winter, the body temperatures of intertidal species inhabiting exposed microhabitats routinely reached much larger temperature fluctuation than their counterparts attached to shaded surfaces (Seabra et al. 2011). Therefore, it is essential to examine the responses of intertidal species living in various microhabitats and experiencing different thermal conditions.

The body temperature of intertidal organisms is not simply equivalent to the ambient temperatures (air temperature or water temperature) but is driven by multiple environmental factors, including environmental temperature, wind, waves, tides, Solar radiation, and others (Ma et al. 2024; Wethey 2002; Wethey et al. 2011). Then, it is important to clarify the relative importance of each environmental factor on body temperature during extreme low-temperature events among microhabitats.

Different intensities of cold stress among microhabitats can lead to divergent metabolomic responses. Metabolites are small molecules that provide cells with energy, structural constituents, and the materials necessary for the synthesis of other macromolecules, such as DNA or proteins

(Aderemi et al. 2021; Sun et al. 2023), and are central to the interrelation between cellular changes and phenotypes that directly reflect organisms physiological state (Baker and Rutter 2023; Liu et al. 2021). The metabolism of organisms is controlled at the cellular, tissue, and systemic levels to meet the demands of a wide range of biological processes under changing environmental conditions (Koyama et al. 2020; Metallo and Vander Heiden 2013; Sun et al. 2023). Diverse thermal environments among microhabitats can induce divergent metabolomic responses. For instance, the diel cycle of metabolomic responses of the limpet *Cellana toreuma* was correlated with microhabitats (Sun et al. 2023). Clarifying the microhabitat-specific metabolomic response is crucial for understanding the physiological impacts of habitat heterogeneity.

The supralittoral high-intertidal periwinkle *Littorina brevicula* and mid-intertidal limpet *C. toreuma* are widely distributed along China's coastline and occupy various microhabitats (Chiba et al. 2016; Sun et al. 2023; Takada 2003). The periwinkle occupies microhabitats ranging from exposed rock surfaces to shaded rock surfaces on the high intertidal zone and splash zone, and limpets occupy microhabitats ranging from rock surfaces to tidal pools in the middle intertidal zone. Therefore, these two species are ideal for studying the metabolomic responses of intertidal mollusks to microhabitat heterogeneity. In this study, we measured the in-situ body temperature during extreme low-temperature events and analyzed the metabolomic responses of periwinkles and limpets living in different microhabitats. The objectives were to (1) investigate the patterns of body temperature of intertidal mollusks across various microhabitats and identify the main environmental drivers and (2) determine the metabolomic profiles of intertidal mollusks in differently exposed microhabitats, clarifying the impacts of microhabitat heterogeneity on the metabolic responses of snails and limpets. This information is essential for understanding the metabolomic response to extreme low-temperature events of intertidal mollusks and for determining the role of favorable microhabitats on the population dynamics of intertidal species in the context of climate change.

Materials and methods

Determination of extreme low-temperature events

Hourly air temperature data were obtained from the National Centers for Environmental Prediction Climate Forecast System Version 2 (CFSv2), which has a spatial resolution of $0.205^\circ \times 0.204^\circ$ (ref. NCEP Climate Forecast System Version 2 (CFSv2) Selected Hourly Time-Series Products) (Saha et al. 2011). We first identified coastal grid points on the seaward side, then extracted hourly air temperatures for

the nearest grib based on Euclidean distance calculation. We found that the 10th percentile of air temperature from 2014 to 2023 is 0.668 °C. In addition, the daily minimum air temperatures from December 16 to 22, 2023, in Qingdao, Shandong, China (36.06° N, 120.40° E) varied between −4 and −11 °C, which was below the threshold of 0.668 °C for extreme low-temperature events. Therefore, this study recognized December 16 to 22, 2023, as an extreme low-temperature event in Qingdao.

Environmental data measurement

Thermochron iButton data loggers (DS1922L, Analog Devices, Norwood, USA) with the range of −40 to 85 °C were used to monitor the substrate temperatures as described in a previous study (Marshall et al. 2015). A total of nine loggers were deployed in three types of microhabitats with three replicates in Qingdao:

- (1) Exposed rock microhabitat: Loggers were deployed on emergent rocky surfaces in full sunlight where the snails and limpets usually occupy;
- (2) Shaded rock microhabitat: Loggers were deployed on rocky surfaces that are completely sheltered by other rocks where the snails and limpets usually occupy;
- (3) Tidal pool microhabitat: Loggers were deployed in the middle of tidal pools where the limpets usually inhabit.

The measuring accuracy of iButton loggers was set to 0.5 °C, with a monitoring interval of 10 min during an extreme low-temperature event from December 16 to 22, 2023.

To record meteorological data, including air temperature, wind speed, and light intensity, a portable meteorological station (FT-BQX5, Fengtu S&T Co., Weifang, China) was placed on the rock during low tides and set to record data every five minutes.

Infrared thermal imaging was used to characterize the thermal properties of different microhabitats during low tides from December 16 to 22, 2023, using a TiX660 infrared camera (Fluke, Washington, USA) as described in a previous study (Lathlean and Seuront 2014).

Animal collection, body temperature measurement, and metabolomic profiling

The snails *L. brevicula* from exposed and shaded microhabitats and limpets *C. toreuma* from the rock and tidal pool microhabitats were randomly collected at a rocky shore in Qingdao during an extreme low-temperature event in December 2023.

Animals collected from December 20 (day 1), December 21 (day 2), and December 22 (day 3) were used for

untargeted metabolomics analysis. 30 snails (shell length, 8–10 mm) and 12 limpets (shell length, 17–20 mm) were collected from each microhabitat during low tides, and each animal's collection time and body temperature were recorded. Body temperatures (i.e., temperatures of foot tissue) were measured with a thermal couple (54IIB, Fluke, Washington, USA). A standardized measurement protocol was established based on the characteristics of the two species: For snails, a thermal couple was inserted through the operculum into the foot tissue for body temperature measurement. For limpets, a small gap was created between the shell and adhering substrate with an anatomical tweezer, allowing the foot tissue to fully wrap around the probe for measurement. To eliminate any human thermal interference, direct hand contact with these samples was strictly avoided throughout the procedure. Then, those samples were frozen in liquid nitrogen immediately and stored at −80 °C for metabolomic profiling.

Snails and limpets adhere to the substrate using their foot, making foot tissue one of the first to be affected by low temperatures (Holland et al. 1991). To satisfy the requirement of untargeted metabolomics analyses, the foot tissues of four snails with similar body temperatures were mixed for one measurement.

Biotree (Shanghai, China) performed the untargeted metabolomics analysis, with seven replicates conducted each day in each microhabitat. Metabolites of snails and limpets were extracted using an extraction solution (composed of acetonitrile: methanol = 1:1, containing isotopically-labeled internal standard mixture), and metabolites were detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analysis was performed using an ultra-high-performance liquid chromatography (UHPLC) system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to a QExactive HFX mass spectrometer (Orbitrap MS, Thermo). The merged UHPLC-MS data (positive mode and negative mode) were log-transformed, converted to mzXML format by ProteoWizard (version 3.0.21229), and processed by R package XCMS (version 3.2) to generate a data matrix consisting of retention time (RT), mass-to-charge ratio (m/z) value, and peak abundance. R package CAMERA (version 3.16) was used for peak annotation (Han et al. 2024). Metabolites with MS/MS matching were included for subsequent analysis, with a total of 2084 annotated metabolic compounds.

Statistical analyses

Environmental data analysis

Because substrate temperature, air temperature, wind speed, light intensity, and body temperature data did not conform to

the assumption of homogeneity of variance (Levene's test, $P < 0.05$) and meet a normal distribution (Shapiro–Wilk test, $P < 0.05$), differences in substrate temperature and body temperature were analyzed by two-way factorial Kruskal–Wallis tests with date and microhabitat type as fixed-effect categorical factors, and differences in air temperature, wind speed, and light intensity among dates were analyzed with Kruskal–Wallis test. These analyses were carried out in R v4.4 (R Core Team 2024) with the packages *dplyr* and *PMCMRplus* (Slazak et al. 2021; Zhang et al. 2024).

Classification Random Forest analysis was conducted to analyze the contribution of each environmental variable (substrate temperature, air temperature, wind speed and light intensity) on body temperatures (Belgiu and Drăguț 2016; Delgado-Baquerizo et al. 2016) by evaluating the decrease in prediction accuracy, i.e., increase in the mean square error (MSE) between observations and predictions, when the data for that predictor was randomly permuted. This decrease was averaged over all trees to produce the final measure of importance. The accuracy importance measure was computed for each tree and averaged over the forest (5,000 trees). All environmental variables were selected based on a variance inflation factor (VIF) < 10 (Li et al. 2023). These analyses were carried out in R v4.4 (R Core Team 2024) with the packages *randomForest* and *rfPermute* (Delgado-Baquerizo et al. 2016; Li et al. 2022; Probst et al. 2019).

Metabolomic data analysis

The MetaboAnalyst 6.0 website (<https://www.metaboanalyst.ca/>) was used to perform statistical analysis (normalization, generalized logarithm transformation, auto-scaling, and cluster analysis) and pathway enrichment (Cambiaghi et al. 2017). Multivariate statistical analysis for temporal metabolic variation among microhabitats was performed by unsupervised principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). To obtain differentially expressed metabolites for snails and limpets in different microhabitats on each day, a student's *t*-test ($P < 0.05$) and orthogonal partial least squares discriminant analysis (OPLS-DA) (VIP score > 1) were performed (Su et al. 2024). To clarify the differences in metabolomic response between different microhabitats, metabolic pathway enrichment analyses were performed in the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>). Significant enriched pathways were determined with *P*-value calculated from hypergeometric tests (Sun et al. 2023). Circular heatmap was used to visualize the abundance of enriched metabolites in different microhabitats.

To detect the effects of the date, microhabitat type, and the interaction between date and microhabitat type on the variations of metabolites, a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations

was conducted in R v4.4 (R Core Team 2024) with packages *vegan* (Anderson 2017).

Shared pathways of snails and limpets differentially expressed between microhabitats were selected to visualize the metabolic pathways network. Mantel correlations between enriched metabolites and environmental variables and Spearman correlation between environmental variables were carried out in R v4.4 (R Core Team 2024) with packages *linkET* (Sunagawa et al. 2015). Environmental variables used for the Mantel analysis were filtered to remove the effects of multicollinearity by setting the correlation coefficient threshold to less than 0.7 (Cramer and Willig 2005).

To further explore the relationship between metabolic modules and environmental variables, a weighted gene co-expression correlation network analysis (WGCNA) was performed using the *WGCNA* package (Langfelder and Horvath 2008) in R v4.4 (R Core Team, 2024) on KEGG-annotated metabolites and environmental variables. The correlation coefficient threshold between the environmental variables was set to less than 0.7 to eliminate the effect of multicollinearity (Cramer and Willig 2005). After identifying modules significantly correlated with environmental factors ($P < 0.05$), metabolites within these modules were intersected with those involved in Mantel analysis to obtain key metabolites highly correlated with environmental variables.

Results

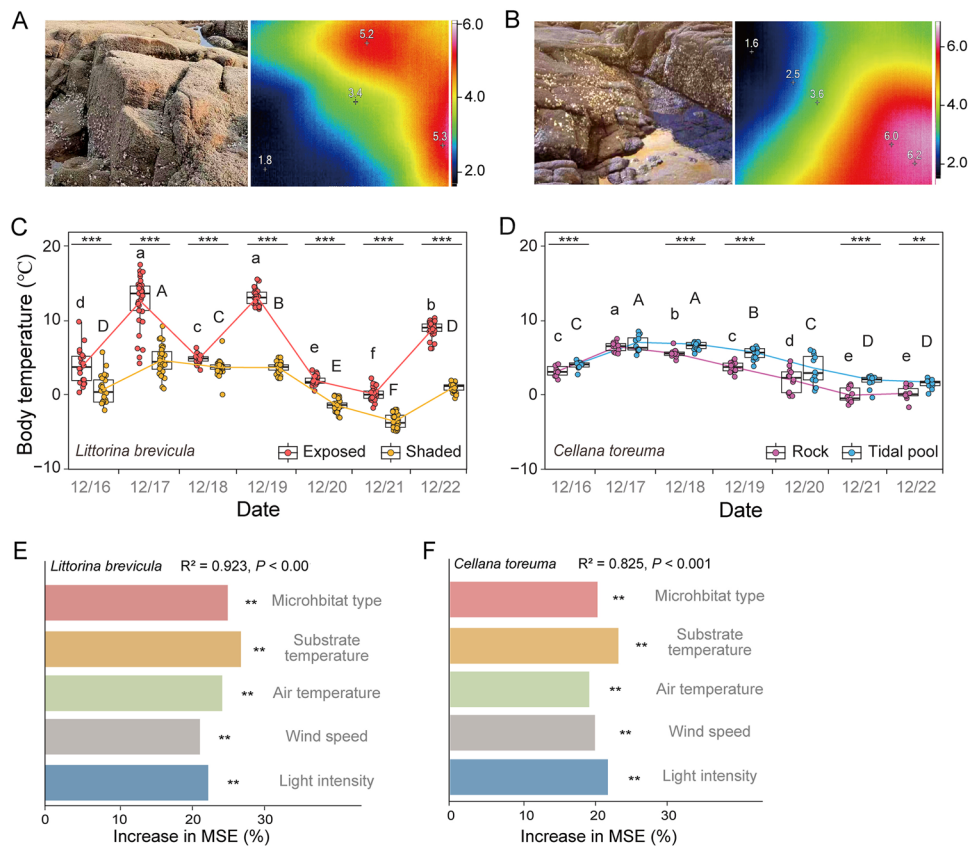
Patterns and drivers of body temperature during an extreme low-temperature event

During the extreme low-temperature event, substrate temperatures in the three microhabitats (exposed rock, shaded rock, and tidal pool) varied with date and were significantly influenced by microhabitat type (Table 1). Thermal infrared images showed a high degree of spatial heterogeneity among microhabitats (Fig. 1A, B). From December 16 to 22, substrate temperatures in exposed rocks (ranged from -10.95 to 23.81 °C) exhibited significantly larger daily fluctuations (16.622 ± 1.016) than in shaded rocks (ranged from -3.29 to 8.51 °C, 7.191 ± 0.361) and tidal pools (ranged from -2.95 to 8.53 °C, 7.583 ± 0.433) (one-way ANOVA, shaded rocks,

Table 1 Two-way factorial Kruskal–Wallis test results for substrate temperatures across date and microhabitat type

Source	Substrate temperature	
	df	<i>P</i> value
Date	6	$< 2e-16$
Microhabitat type	2	$< 2e-16$
Date $\times$ microhabitat type	20	< 0.001

Fig. 1 Different thermal conditions and body temperature of mollusks. Thermal images in the **A** exposed and shaded rock surface of *Littorina brevicula* and **B** rocky environment and tidal pool of *Cellana toreuma*. Body temperatures of **C** *Littorina brevicula* and **D** *Cellana toreuma* in different microhabitats. Lowercase/capital letters indicate significant differences between dates in the same microhabitat. Random Forest predictor importance (% of increase of MSE) of environmental drivers on body temperatures for the **E** *Littorina brevicula* and **F** *Cellana toreuma*



$P < 0.001$; tidal pools, $P < 0.001$), with no significant difference between shaded rocks and tidal pools (one-way ANOVA, $P = 0.934$) (Fig. S1A).

During low tides, the air temperature ($H_{6, 293} = 207.351$, $P < 0.001$; Fig. S1B), wind speed ($H_{6, 293} = 190.233$, $P < 0.001$; Fig. S1C), and light intensity ($H_{6, 293} = 159.619$, $P < 0.001$; Fig. S1D) of the meteorological station varied significantly with date. Low air temperature, high wind speed, and low light intensity were recorded in December 20 to 21, 2023 (Fig. S1B–D).

Body temperature of mollusks in different microhabitats

Body temperatures of snails and limpets varied significantly with date and were significantly influenced by microhabitat type (Table 2). On each day, snails' body temperatures in the exposed rocks were significantly higher than that in the shaded rocks (Kruskal–Wallis test, all $P < 0.001$; Fig. 1C). Limpets' body temperatures in the tidal pools were significantly higher than those in the rocky environments on December 16, 18, 19, 21, and 22 (Kruskal–Wallis test, all $P < 0.001$; Fig. 1D).

The snails' body temperatures in different microhabitats varied significantly each day from December 19 to 22 (Kruskal–Wallis test, all $P < 0.05$), following a trend

Table 2 Two-way factorial Kruskal–Wallis test results for body temperatures of mollusks across date and microhabitat type

Source	<i>Littoraria brevicula</i>		<i>Cellana toreuma</i>	
	df	P value	df	P value
Date	6	$< 2e-16$	6	$< 2e-16$
Microhabitat type	1	$< 2e-16$	1	0.029
Date $\times$ microhabitat type	13	$< 2e-16$	13	$< 2e-16$

of downward and then upward variation, with the lowest body temperature recorded on December 21 (Fig. 1C). From December 18 to 22, the body temperatures of limpets in different microhabitats also varied significantly each day (Kruskal–Wallis test, all $P < 0.05$), showing a downward and then slightly upward trend, with the lowest body temperatures recorded on December 21 and 22 (Fig. 1D).

The Random Forest model explained 92.3 and 82.5% of the environmental variables related to the body temperatures of snails and limpets, respectively (Fig. 1E, F). Among these environmental variables, microhabitat type (snails, $P = 0.010$; limpets, $P = 0.010$), substrate temperature (snails, $P = 0.010$; limpets, $P = 0.010$), air temperature (snails, $P = 0.011$; limpets, $P = 0.010$), wind speed (snails, $P = 0.011$; limpets, $P = 0.010$), and light intensity (snails, $P = 0.010$; limpets, $P = 0.011$) significantly influenced the

body temperature of mollusks. Among them, microhabitat type, substrate temperature, air temperature, and light intensity showed a positive correlation with the body temperature (Fig. S2A–C, E), while wind speed showed a negative correlation with the body temperature (Fig. S2D).

Metabolites identification and multivariate statistical analysis of mollusks

A total of 2084 annotated metabolites involving 23 categories were identified in mollusks, with a high number of organoheterocyclic compounds (20.25%), lipids and lipid-like molecules (15.45%), benzenoids (12.04%), organic acids and derivatives (9.98%) (Fig. 2A). PERMANOVA results showed that the metabolites of mollusks varied significantly with date and were significantly affected by microhabitat type (Table 3).

PCA and PLS-DA results showed that the snails were clearly separated between microhabitats, while the limpets were separated between dates (Fig. 2B–E). For snails, the first principal component (PC1) separated animals from different microhabitats with a variance contribution of 25.4%, and the second principal component (PC2) separated the snails from different dates with a variance contribution of 10.4% (Fig. 2B). The PLS-DA model with parameters of

Table 3 The permutational multivariate analysis of variance (PERMANOVA) results for metabolites of mollusks across date and microhabitat type

Source	<i>Littoraria brevicula</i>		<i>Cellana toreuma</i>	
	F	P value	F	P value
Date	1.854	0.044	2.217	0.023
Microhabitat type	16.568	0.001	3.025	0.003
Date × microhabitat type	1.672	0.095	1.709	0.069

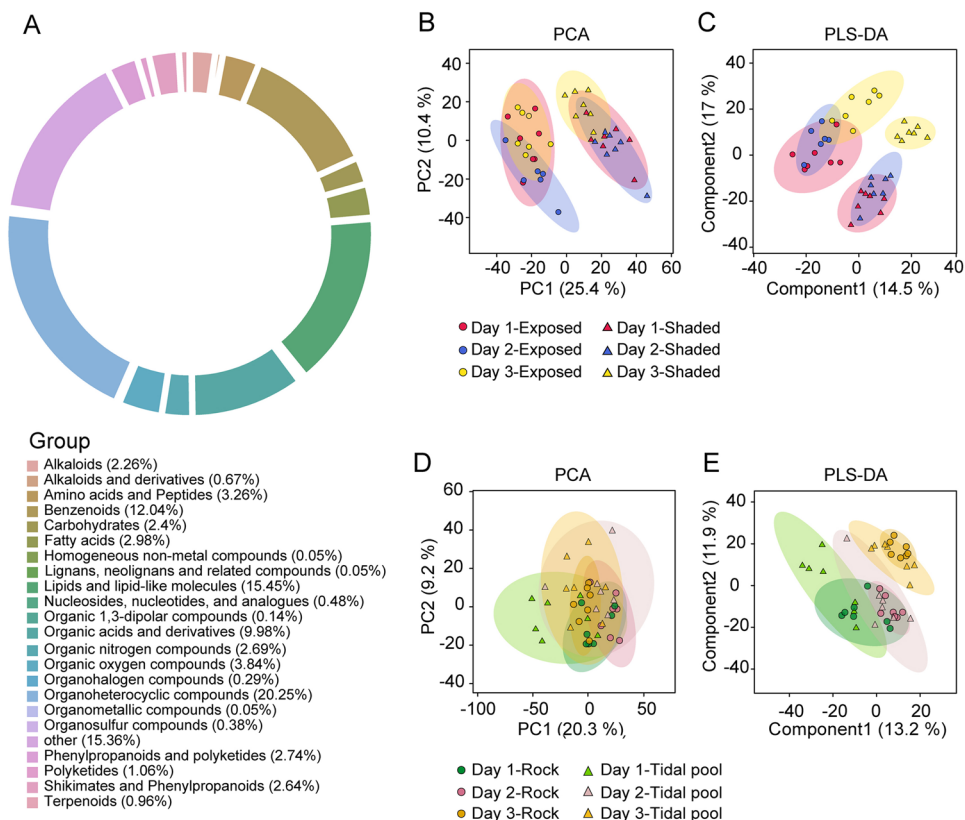
$R^2Y=0.999$ and $Q^2=0.477$ indicated high explanatory and general predictive rates (Fig. 2C).

For limpets, PC1 separated animals from different microhabitats with a variance contribution of 20.3% and PC2 separated limpets from different dates with a variance contribution of 9.2% (Fig. 2D). The PLS-DA model, with parameters $R^2Y=0.996$ and $Q^2=0.596$, indicated high explanatory and general predictive rates and can be used for further differential metabolite analysis.

Metabolomic responses of mollusks between microhabitats

On December 20 (day 1), December 21 (day 2), and December 22 (day 3), the exposed rock vs. shaded rock

Fig. 2 Metabolites identification and multivariate statistical analysis of mollusks. **A** Classification of metabolites identified in mollusks. **B** The principal component analysis (PCA) and **C** partial least squares discriminant analysis (PLS-DA) analysis of metabolites across different dates in the exposed and shaded rock of *Littoraria brevicula* ($n=7$). **D** The principal component analysis (PCA) and **E** partial least squares discriminant analysis (PLS-DA) analysis of metabolites across different dates in the rocky environment and tidal pool of *Cellana toreuma* ($n=7$)



group of snails had 249 differentially expressed metabolites ($VIP > 1$, $P < 0.05$), 260 differentially expressed metabolites ($VIP > 1$, $P < 0.05$) and 210 differentially expressed metabolites ($VIP > 1$, $P < 0.05$), respectively. Similarly, the rock vs. tidal pool group of limpets had 112 differentially expressed metabolites ($VIP > 1$, $P < 0.05$), 52 differentially expressed metabolites ($VIP > 1$, $P < 0.05$), and 44 differentially expressed metabolites ($VIP > 1$, $P < 0.05$), respectively.

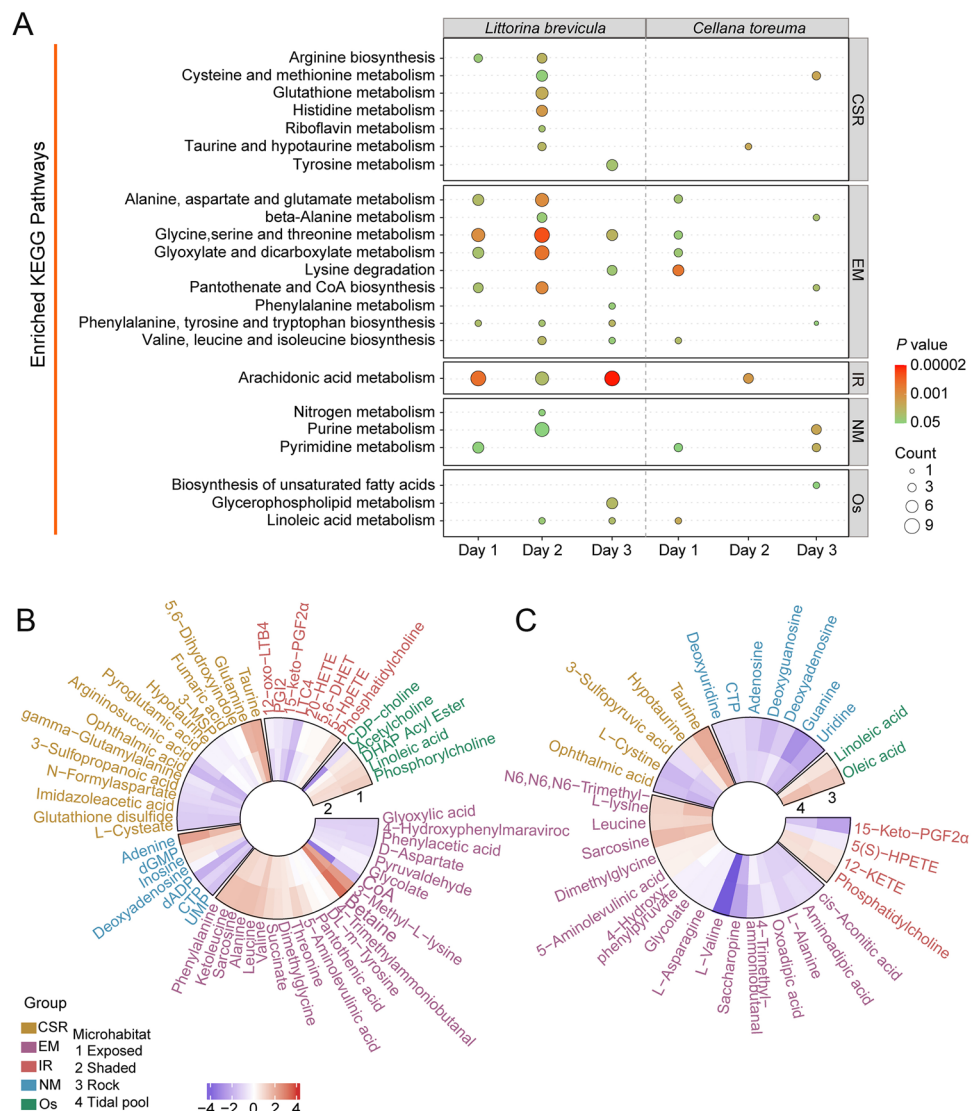
Metabolomic response of snails in different microhabitats

The exposed rock vs. shaded rock group of snails was found to be significantly enriched in 22 metabolic pathways across day 1, day 2, and day 3. These pathways were associated with crucial functions, including cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation (Fig. 3A).

On day 1, eight metabolic pathways were significantly enriched. Among these pathways, "arginine biosynthesis" ($P = 0.030$) is related to cellular stress response. Pathways including "alanine, aspartate and glutamate metabolism" ($P = 0.012$), "glycine, serine and threonine metabolism" ($P < 0.001$), "glyoxylate and dicarboxylate metabolism" ($P = 0.018$), "pantothenate and CoA biosynthesis" ($P = 0.016$), "phenylalanine, tyrosine and tryptophan biosynthesis" ($P = 0.014$) are associated with energy metabolism. "Arachidonic acid metabolism" ($P < 0.001$) is immune-related, and "pyrimidine metabolism" ($P = 0.044$) is nucleotide-related.

On day 2, 17 metabolic pathways were significantly enriched. Among these pathways, "arginine biosynthesis" ($P = 0.007$), "cysteine and methionine metabolism" ($P = 0.041$), "glutathione metabolism" ($P = 0.004$), "histidine metabolism" ($P = 0.002$), "riboflavin metabolism" ($P = 0.019$), "taurine and hypotaurine metabolism"

Fig. 3 The metabolomic responses of mollusks in different microhabitats. **A** KEGG metabolic pathways between microhabitats of *Littorina brevicula* and *Cellana toreuma* on different dates. The bubble size represents the count of enriched differential metabolites, and the bubble color indicates the degree of significance from the highest (red) to the lowest (green). Metabolites enriched by **B** *Littorina brevicula* and **C** *Cellana toreuma* in different microhabitats. **CSR** cellular stress response, **EM** energy metabolism, **IR** immune response, **NM** nucleotide metabolism, **Os** osmoregulation



($P=0.009$) are related to the cellular stress response. Pathways involving "alanine, aspartate, and glutamate metabolism" ($P<0.001$), "beta-alanine metabolism" ($P=0.032$), "glycine, serine and threonine metabolism" ($P<0.001$), "glyoxylate and dicarboxylate metabolism" ($P=0.018$), "pantothenate and CoA biosynthesis" ($P<0.001$), "phenylalanine, tyrosine and tryptophan biosynthesis" ($P=0.019$), and "valine, leucine and isoleucine biosynthesis" ($P=0.009$) are associated with energy metabolism. "Arachidonic acid metabolism" ($P=0.013$) is immune-related. "Nitrogen metabolism" ($P=0.044$) and "purine metabolism" ($P=0.050$) is nucleotide-related. Pathway of "linoleic acid metabolism" ($P=0.031$) is involved in osmoregulation.

On day 3, nine metabolic pathways were significantly enriched. Within these pathways, "tyrosine metabolism" ($P=0.020$) is related to cellular stress response. Pathways including "glycine, serine and threonine metabolism" ($P=0.007$), "lysine degradation" ($P=0.025$), "phenylalanine metabolism" ($P=0.035$), "phenylalanine, tyrosine and tryptophan biosynthesis" ($P=0.008$), and "valine, leucine and isoleucine biosynthesis" ($P=0.035$) are associated with energy metabolism. "Arachidonic acid metabolism" ($P<0.001$) is immune-related. Pathways

of "glycerophospholipid metabolism" ($P=0.011$) and "linoleic acid metabolism" ($P=0.013$) are involved in osmoregulation.

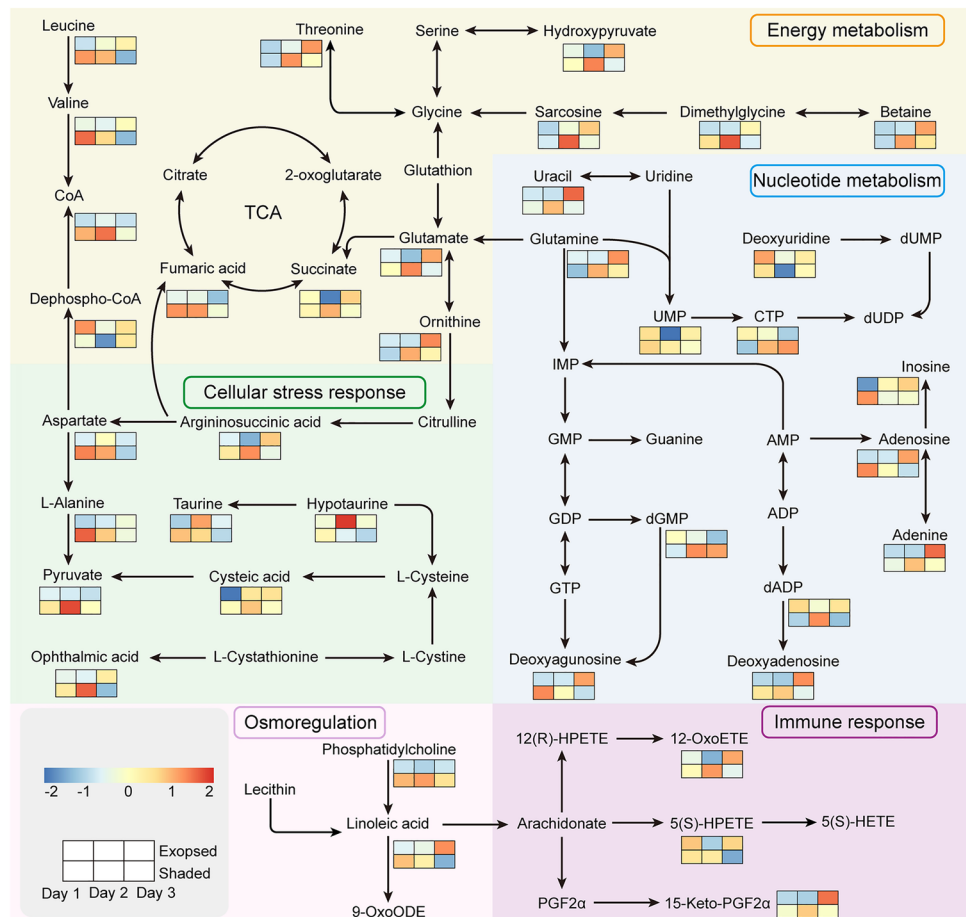
The metabolites enriched in these pathways associated with cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation exhibited higher abundances in snails inhabiting exposed rock compared to those in shaded rock (Fig. 3B).

Metabolomic response of limpets in different microhabitats

Limpets in the group of rock vs. tidal pool exhibited significant enrichment in 15 metabolic pathways across day 1, day 2, and day 3. These pathways were associated with cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation (Fig. 3A).

On day 1, seven metabolic pathways were significantly enriched. Among these pathways, "alanine, aspartate and glutamate metabolism" ($P=0.020$), "glycine, serine and threonine metabolism" ($P=0.032$), "glyoxylate and dicarboxylate metabolism" ($P=0.027$), "lysine degradation"

Fig.4 Schematic overview of shared metabolic pathways that the differentially expressed metabolites involved in different microhabitats of *Littorina brevicula* ($n=7$). In the heatmap, each row represents a microhabitat, and each column represents the different dates. Color indicates the values of metabolites from the highest (red) to the lowest (blue)



($P < 0.001$), "valine, leucine and isoleucine biosynthesis" ($P = 0.012$) are related to energy metabolism. "Pyrimidine metabolism" ($P = 0.048$) is nucleotide-related. Pathway of "linoleic acid metabolism" ($P = 0.004$) is associated with osmoregulation.

On day 2, a total of two pathways were found to be significantly enriched. "Taurine and hypotaurine metabolism" ($P = 0.004$) is associated with cellular stress response, and "arachidonic acid metabolism" ($P = 0.002$) is related to immune response.

On day 3, seven pathways were significantly enriched. Within these pathways, "cysteine and methionine metabolism" ($P = 0.003$) is related to cellular stress response. Pathways including "beta-alanine metabolism" ($P = 0.017$), "pantothenate and CoA biosynthesis" ($P = 0.015$), "phenylalanine, tyrosine and tryptophan biosynthesis" ($P = 0.039$) are associated with energy metabolism. "Purine metabolism" ($P = 0.004$) and "pyrimidine metabolism" ($P = 0.006$) are nucleotide-related. "Biosynthesis of unsaturated fatty acids" ($P = 0.046$) is related to osmoregulation.

Moreover, the abundance of metabolites enriched in pathways related to cellular stress response, energy metabolism,

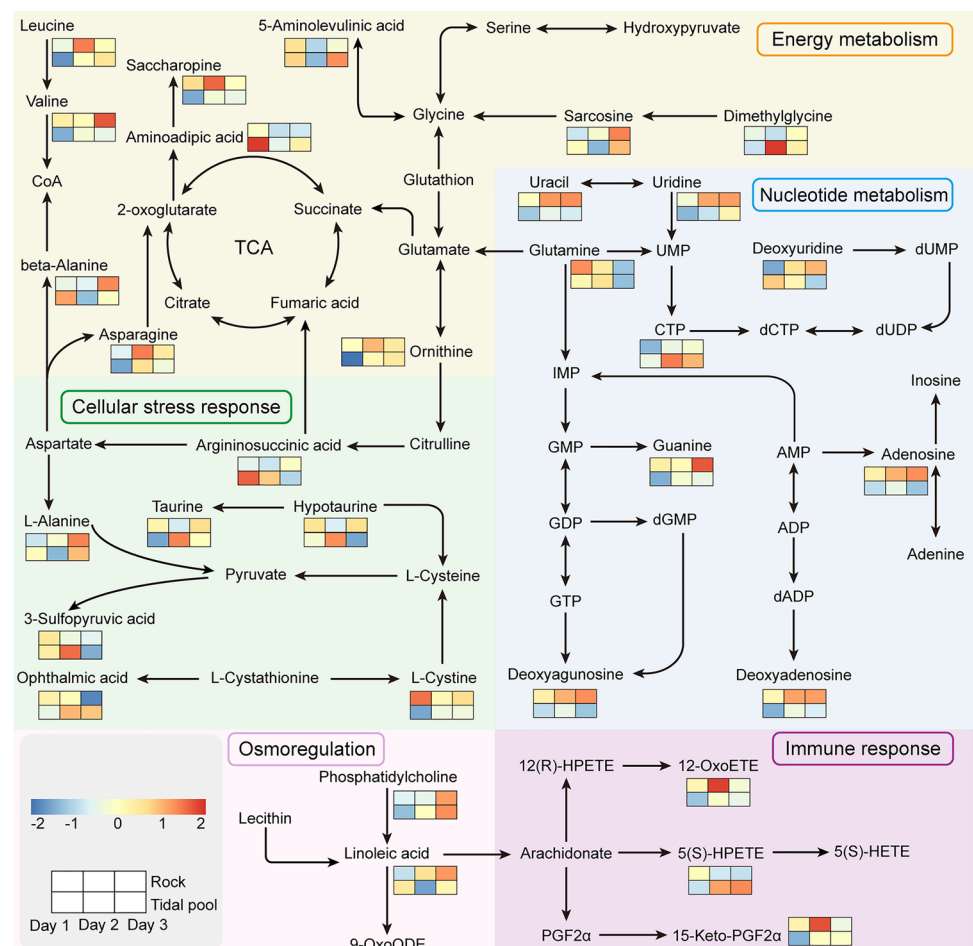
immune response, nucleotide metabolism, and osmoregulation was generally higher in limpets inhabiting the rocky environment compared to those in the tidal pool (Fig. 3C).

Metabolic network of mollusks

Metabolic networks were created based on the shared metabolic pathways of mollusks to show the differences in metabolism among microhabitats (Figs. 4, 5). The metabolic networks of snails and limpets were composed of various pathways, such as "cysteine and methionine metabolism", "taurine and hypotaurine metabolism", "alanine, aspartate and glutamate metabolism", "beta-alanine metabolism", "glycine, serine and threonine metabolism", "glyoxylate and dicarboxylate metabolism", "lysine degradation", "pantothenate and CoA biosynthesis", "valine, leucine and isoleucine biosynthesis", "arachidonic acid metabolism", "purine metabolism", "pyrimidine metabolism", and "linoleic acid metabolism".

Categories of these pathways included cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation. There were 43 and

Fig.5 Schematic overview of shared metabolic pathways that the differentially expressed metabolites involved in different microhabitats of *Cellana toreuma* ($n = 7$). In the heatmap, each row represents a microhabitat, and each column represents the different dates. Color indicates the values of metabolites from the highest (red) to the lowest (blue)



32 enriched differentially expressed metabolites in the metabolic network of snails and limpets, respectively. These metabolites exhibited significant differences in abundance across different microhabitats (Student's *t*-test, all $P < 0.05$). Within the networks of snails and limpets, glutamate, argininosuccinic acid, and linoleic acid were identified as key metabolites that interacted in multiple metabolic pathways.

Relationship between metabolites and environmental variables between microhabitats

Mantel test results in mollusks exhibited significant correlations between the enriched metabolites and environmental variables (Fig. 6). The metabolites of snails in the exposed and shaded rock microhabitats showed a significant negative correlation with substrate temperature (exposed rock: $r = -0.116$, $P = 0.014$; shaded rock: $r = -0.280$, $P = 0.001$), and body temperature (exposed rock: $r = -0.211$, $P = 0.001$; shaded rock: $r = -0.421$, $P = 0.001$), while they were positively correlated with wind speed (exposed rock: $r = 0.169$, $P = 0.004$; shaded rock: $r = 0.184$, $P = 0.001$) (Fig. 6A).

Similarly, the metabolites of limpets in the rocky environment and tidal pool microhabitats exhibited significant negative correlations with substrate temperature (rocky environment: $r = -0.566$, $P = 0.001$; tidal pool: $r = -0.717$, $P = 0.001$), and body temperature (rocky environment: $r = -0.223$, $P = 0.014$; tidal pool: $r = -0.303$, $P = 0.001$). However, there was no significant correlation with wind speed (rocky environment: $r = -0.021$, $P = 0.297$; tidal pool: $r = -0.025$, $P = 0.735$) (Fig. 6B).

Correlation between metabolic modules and environmental variables

Metabolites exhibiting similar expression patterns (co-expressed metabolites) were grouped into cohesive modules. The multiple metabolic modules of mollusks significantly correlated with environmental variables (Fig. 7).

For the snail *L. brevicula*, in the exposed rock microhabitats, the salmon module exhibited a significant positive correlation with wind speed ($r = 0.490$, $P = 0.030$); the pink module showed a significant negative correlation with body temperature ($r = -0.470$, $P = 0.040$), while turquoise module exhibited a significant positive correlation with body temperature ($r = 0.500$, $P = 0.020$) (Fig. 7A). In the shaded rock microhabitats, the blue, magenta, and turquoise modules showed significant positive correlations with substrate temperature (blue: $r = 0.740$, $P = 1e-04$; magenta: $r = 0.830$, $P = 4e-06$; turquoise: $r = 0.600$, $P = 0.004$); the turquoise module exhibited a significant negative correlation with wind speed ($r = -0.450$, $P = 0.040$); the blue, magenta, and turquoise modules showed significant positive correlations with body temperature (blue: $r = 0.780$, $P = 3e-05$; magenta:

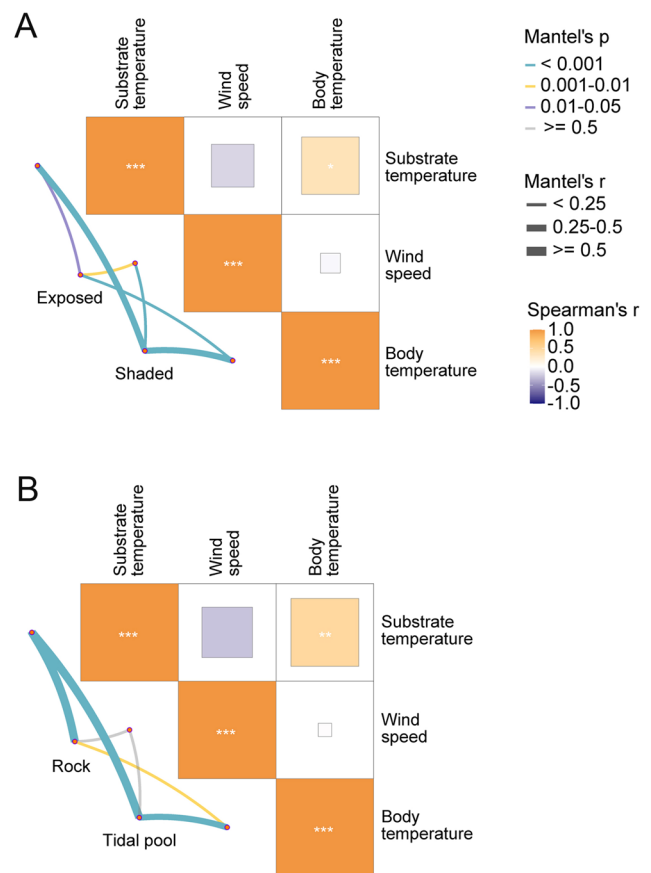


Fig. 6 Mantel's test for the correlation of environmental variables and metabolites enriched in different microhabitats of **A** *Littorina brevicula* and **B** *Cellana toreuma*. Color gradient denoting Spearman's correlation coefficients. Edge width corresponds to Mantel's *r* statistic for the corresponding distance correlations

$r = 0.720$, $P = 2e-04$; turquoise: $r = 0.530$, $P = 0.010$) (Fig. 7A).

For the limpets, in the rocky environments, the modules in black and pink colors were positively correlated with wind speed (black: $r = 0.460$, $P = 0.040$; pink: $r = 0.480$, $P = 0.030$), while the module in blue color was negatively correlated with wind speed ($r = -0.630$, $P = 0.002$) (Fig. 7B). In the tidal pool microhabitats of limpets, the module in cyan color was positively correlated with wind speed ($r = 0.590$, $P = 0.005$), while purple module was negatively correlated with wind speed ($r = -0.530$, $P = 0.010$); the modules in blue and turquoise colors were positively correlated with body temperature (blue: $r = 0.490$, $P = 0.020$; turquoise: $r = 0.530$, $P = 0.010$) (Fig. 7B).

Several key metabolites for snail *L. brevicula* and limpet *C. toreuma* were highly correlated with environmental variables. In the exposed rock for snails and rocky environment for limpets, the key metabolites that were highly correlated with environmental variables included pyruvaldehyde, 15-keto-prostaglandin F2alpha and 12-oxo-ETE. In

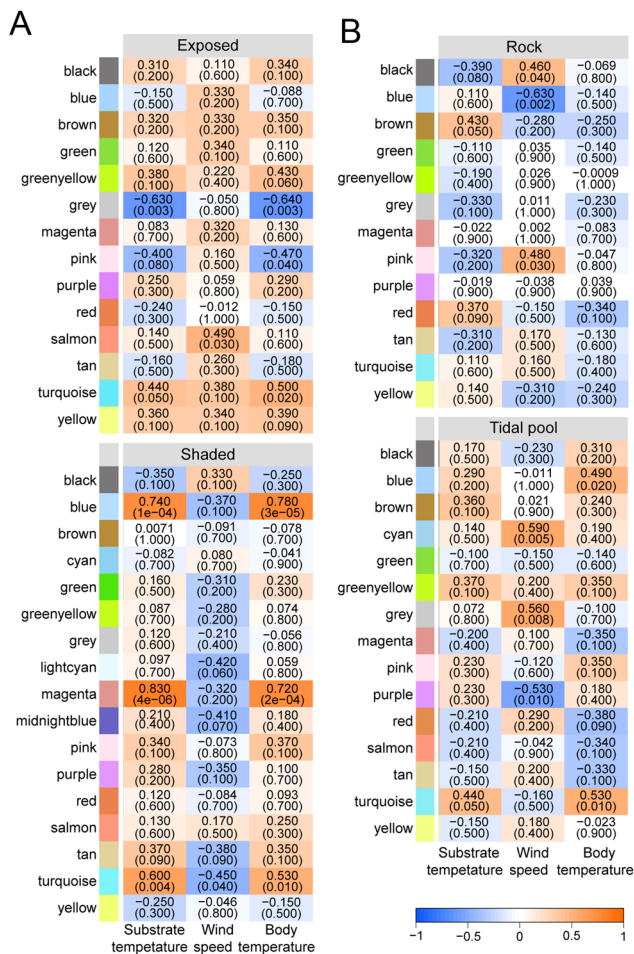


Fig. 7 WGCNA analysis of metabolic modules and environmental variable correlations in different microhabitats of **A** *Littorina brevicula* and **B** *Cellana toreuma*. The distinct modules identified are indicated by color. Each row corresponds to a module metabolite, and each column to an environmental variable, respectively. Each cell contains the corresponding correlation and *P*-value. The cell is color-coded by correlation according to the color legend

the shaded rock for snails and tidal pool for limpets, the key metabolite that was highly correlated with environmental variables was uracil.

Discussion

Extreme weather events can cause substantial short-term ecological impacts, while the variety of microhabitats may help mitigate these threats. In the present study, microhabitat type, substrate and air temperatures, wind speed, and light intensity notably influenced the body temperature of intertidal mollusks. Small-scale fluctuations in body temperature can potentially protect populations from the impacts of extreme low-temperature events, thereby promoting physiological diversity and

physiological plasticity. During the extreme low-temperature event, mollusks collected from different microhabitats exhibited microhabitat-specific metabolomic responses in various pathways associated with cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation. These pathways were highly induced in the more exposed microhabitats (i.e., exposed rock for snails and rocky environment for limpets). Moreover, the metabolites and metabolic modules in mollusks were significantly correlated with environmental variables, indicating that environmental factors play a crucial role in shaping the metabolic patterns of mollusks. The adaptive metabolomic responses of mollusks in different microhabitats ensure the relatively benign microhabitat can play the role of refugia against extreme low-temperature events, thereby favoring the conservation of biodiversity.

Thermal microhabitat heterogeneity during extreme low-temperature event

Body temperature is regulated by multiple environmental factors and can drop to freezing point during low tides in winter (Denny et al. 2011; Helmuth 1998; Ma et al. 2024; Miranda et al. 2019), and is a major driver of biological performance, affecting survival, cell homeostasis, energy distribution, and geographic boundaries of species distribution (Bozinovic et al. 2011; Han et al. 2013; Ma et al. 2024; Ng et al. 2017; Pörtner et al. 2006; Sokolova et al. 2012; Somero 2002).

Microhabitat heterogeneity causes intertidal animals to experience varying degrees of thermal stress, resulting in differences in body temperature. The differences in temperature among microhabitats may exceed those caused by distances of several thousand kilometers (Deák et al. 2021; Reid and Harley 2021). In the present study, microhabitat type significantly influenced the body temperatures of snails and limpets. During the extreme low-temperature event, animals on the exposed shores suffer from extremely low temperatures and high variations of temperature. Substrate temperature, air temperature, and light intensity can affect body temperature positively, while strong wind speed can dramatically decrease intertidal species' body temperature. This implies the complexity of environmental factors impacting the microhabitat thermal environment on the shore.

Freezing tolerance is an important physiological adaptation against low temperatures. Intertidal invertebrates are freeze-tolerant at low temperatures and can survive ice formation within their body cavity (Gill et al. 2024). The temperature at which body fluids spontaneously freeze (supercooling point) in intertidal mollusks varies among species, with the snail *L. brevicula* having a supercooling point of -6.60 ± 1.54 °C (Wang and Wang 2023) and the limpet *Patinigera Polaris* having a supercooling point of -10 °C (Hargens and Shabica 1973). In the present study,

the lowest body temperatures recorded of snails and limpets during the extreme low-temperature event were -4.9 and -1.7 °C, respectively, and neither of these species was frozen (Wang and Wang 2023).

Microhabitat-specific metabolomic responses of mollusks

Animals inhabiting different microhabitats exhibited differential metabolomic responses. Changes in gene expression and protein production caused by environmental stresses are influenced by regulatory controls and feedback mechanisms, which ultimately amplify at the metabolomic level (Lankadurai et al. 2013; van Ravenzwaay et al. 2007). Metabolomics can systematically identify metabolites, and the metabolomic responses of intertidal animals to various microclimates shed light on their adaptive mechanisms against environmental stresses (Dong 2023). These strategies confer the flexibility needed for organisms to survive in diverse environments, thereby preserving biodiversity and offering valuable insights for studying the impacts of climate change on organisms (Lavergne et al. 2010; Leeuwis and Gamperl 2022). In the present study, pathways related to cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation exhibited microhabitat-specific metabolomic responses. In addition, the metabolites in mollusks exhibited significant correlations with environmental variables, indicating that microhabitat heterogeneity and environmental conditions play a crucial role in shaping metabolic patterns.

A serious impact of extreme low-temperature events on the metabolism of mollusks was oxidative stress. Exposure to low temperatures can disrupt cellular redox balance, leading to an increase in reactive oxygen species (ROS) (González et al. 2020). Oxidative stress can cause excessive generation of free radicals inside the cells, resulting in damage to cell membranes, proteins, and nucleic acids, ultimately leading to apoptosis (Airaki et al. 2012; Chattopadhyay et al. 2011). Metabolites such as arginine, cysteine, glutathione, and histidine play an important role in maintaining oxidative homeostasis, and glutathione can serve as a substrate to eliminate H_2O_2 and lipid peroxides (Zhu et al. 2024). The high levels of cysteine, glutathione, and other metabolites in the exposed rock for snails and rocky environment for limpets indicated that the species in the more exposed microhabitats exhibited higher levels of antioxidant capacity.

The energy-related metabolism of intertidal animals is sensitive to environmental stresses, requiring them to expend a large amount of energy to cope with protein damage, maintain metabolic processes, and regulate body temperature (Dong and Zhang 2016). Energy production within cells primarily occurs through glycolysis, the tricarboxylic

acid cycle (TCA cycle), and oxidative phosphorylation (OXPHOS), involving many key metabolites such as fructose, mannose, galactose, and coenzyme A (CoA) (Dashty 2013; Rueda et al. 2016). Fructose and mannose can be converted into fructose-6-phosphate and mannose-6-phosphate, respectively, to participate in the glycolytic process (Boulanger et al. 2021). Coenzyme A (CoA) is involved in mitochondrial fatty acid oxidation and ketone body synthesis (van Rossum et al. 2016). The upregulation of CoA, succinate, oxoadipic acid, and other metabolites in exposed rock for snails and rocky environment for limpets revealed an enhanced energy requirement for somatic maintenance.

The immune response is a protective reaction of an organism to injury or external threats, serving as a defense mechanism of the organism (Kulkarni et al. 2016). Arachidonic acid (AA) is an important polyunsaturated fatty acid, and its metabolic pathway primarily involves four key enzyme systems: Cyclooxygenase, Lipoxygenase, Cytochrome p450 (CYP 450), and Anandamide pathways (Adam et al. 2017; Funk 2001). These systems convert arachidonic acid into various biologically active mediators, such as prostaglandins, leukotrienes, and hydroperoxyeicosatetraenoic acid (HpETE) (Funk 2001; Hanna and Hafez 2018). Prostaglandins increase the synthesis of anti-inflammatory bioactive lipids, and leukotrienes enhance macrophage phagocytosis (Das 2018). These mediators have immune functions and play a crucial role in inflammatory responses by activating inflammatory cells, thereby enhancing the immune response (Buczynski et al. 2009; Lie et al. 2016). In the present study, the compounds involved in arachidonic acid metabolism were strongly activated in exposed snails and rocky limpets, indicating that the two species would expend more energy to activate the immune response against extreme low-temperature events.

Nucleotides are essential to cell growth and survival, providing cells with building blocks for DNA and RNA, energy carriers, and cofactors. In addition, nucleotide metabolism is a crucial pathway for the production of purine and pyrimidine molecules (Siddiqui and Ceppi 2020). Purine metabolism, starting with phosphoribosylpyrophosphate (PRPP) and ending with inosine monophosphate (IMP), xanthine, and hypoxanthine play significant roles in purine metabolism as components of the cellular nucleotide pool (Pang et al. 2012). The pyrimidine metabolism de novo synthetic pathway assembles uridine monophosphate (UMP) from glutamine, aspartic acid and PRPP (Hatse et al. 1999). Moreover, the synthesis of purines and pyrimidines requires a nitrogen source. On the one hand, nitrogen enters the cell in the form of amino acids and is then utilized in nucleotide synthesis through transamination reactions or other processes (Zrenner et al. 2006). On the other hand, certain intermediates in nitrogen metabolism can be converted into precursors for nucleotide synthesis (Guo et al. 2024). The

disturbances in purine metabolism and pyrimidine metabolism could increase the burden of mutagenic deaminated nucleobases in DNA and interfere with gene expression and RNA function (Pedley and Benkovic 2017). In our study, the levels of metabolites such as deoxyuridine, CTP, and adenine in the exposed rock for snails and rocky environment for limpets were higher, suggesting a stronger nucleotide metabolism.

Cellular osmotic stress is closely tied to temperature changes, particularly under low temperature stress. Low temperatures can significantly disrupt membrane fluidity and stored lipids, affecting the activity of membrane-bound proteins and ATP production (de Mendoza and Cronan 1983; Van Dooremalen et al. 2011). To adapt to low temperature stress, animals typically increase the unsaturation of fatty acids in their bodies, such as the production of linoleic acid, to maintain fluidity (Neidleman 1987). Most of the intertidal invertebrates are freeze-tolerant (Gill et al. 2024; Kennedy et al. 2020). As a low molecular weight compatible osmolyte, Betaine can work synergistically with other osmotic agents to maintain osmotic balance both inside and outside the cell, thereby preventing cell dehydration (Gill et al. 2024). In this study, the metabolite of linoleic acid was at high expression in the exposed rock for snails and rocky environment for limpets.

These findings from the present study suggest that intertidal mollusks can adjust their metabolomic responses to different microhabitats. This flexibility is crucial for the adaptation and survival of intertidal animals, allowing them to maintain homeostasis during extreme low-temperature events. Furthermore, this microhabitat-specific metabolomic response may reflect the ecological niche differentiation of mollusks in the intertidal zone, which helps to enhance physiological resistance to environmental stress and enhance population resilience. Ultimately, these results support the habitat heterogeneity hypothesis and enrich this theory from the perspective of metabolomics.

Conclusions

During extreme low-temperature events, microhabitat significantly influences the body temperature of intertidal mollusks. Additionally, substrate temperature, air temperature, wind speed, and light intensity contribute notably to the body temperatures. The mollusks exhibited microhabitat-specific metabolomic responses, with these differences focusing on cellular stress response, energy metabolism, immune response, nucleotide metabolism, and osmoregulation. Notably, these metabolic pathways were highly induced in the more exposed microhabitats and the metabolites enriched from these pathways were significantly correlated with environmental variables across different microhabitats.

In addition, multiple metabolic modules and key metabolites are highly correlated with environmental variables. These findings highlight the importance of microhabitat heterogeneity. Therefore, it is essential to take full account of the environmental heterogeneity across microhabitats for assessing and predicting the effects of extreme weather events on intertidal organisms. Furthermore, in the face of future environmental challenges, protecting microhabitat complexity is essential for biodiversity conservation and the resilience of intertidal ecosystems.

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Author contributions Ning Zhang, Chen-Ming Lv, Xiao-Ning Zhang, Gianluca Sarà, and Yun-Wei Dong all contributed to the study. Yun-Wei Dong, Ning Zhang, Chen-Ming Lv, Xiao-Ning Zhang, and Gianluca Sarà designed the study. Ning Zhang, Chen-Ming Lv, and Xiao-Ning Zhang collected the samples. Ning Zhang and Chen-Ming Lv performed the analysis. Ning Zhang, Chen-Ming Lv, and Yun-Wei Dong wrote the manuscript. All authors approved the final version for submission.

Data availability All environmental data measured in this study can be downloaded from the links in this article. The metabolomic data and other information are accessible from the main text and supplementary information.

Declarations

Conflict of interest All authors declare that they have no conflict of interest in publishing this paper.

Animal and human rights statement No animal or human rights are involved in this article.

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