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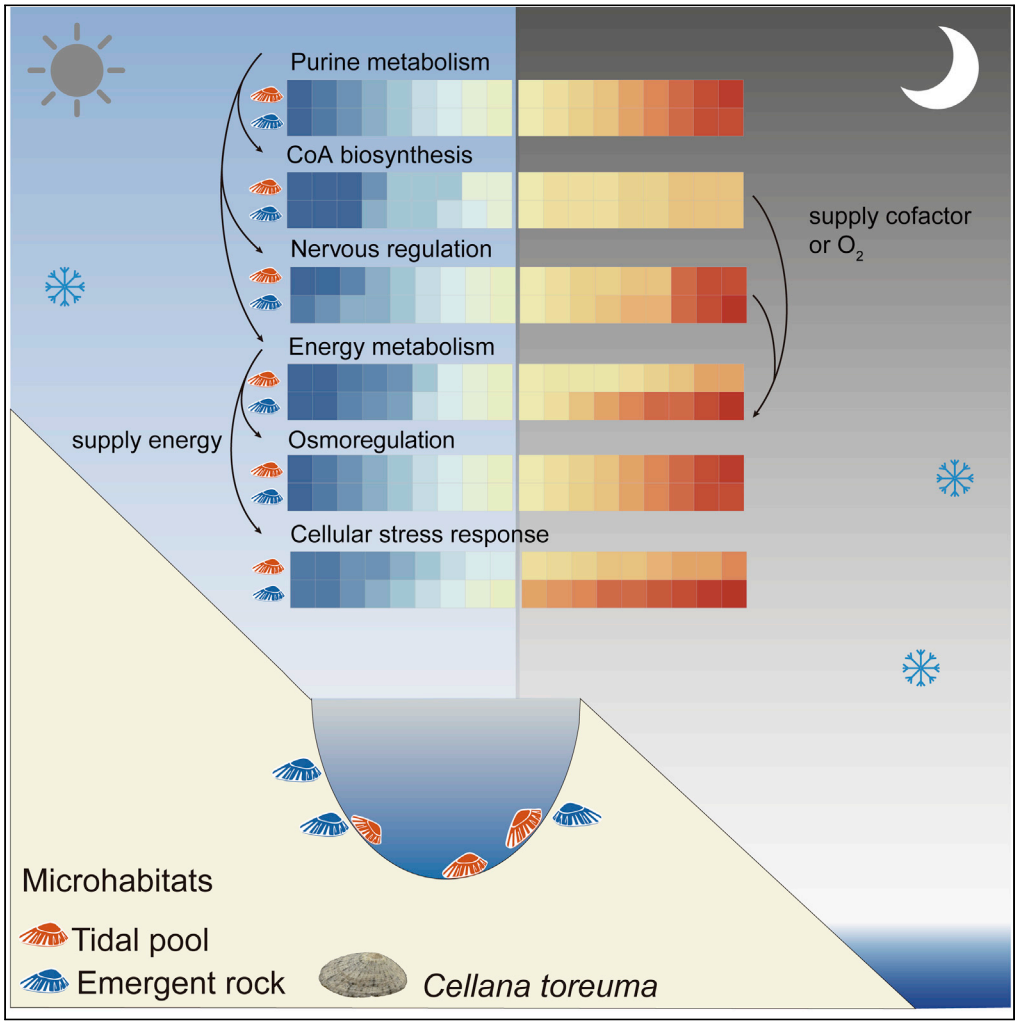

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Highlights

Limpets on emergent rock suffer harsher cold stress during emersion in winter

A series of metabolomic responses exhibited coordinated diurnal changes in winter

Limpets between microhabitats exhibited divergent diurnal metabolic responses

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Microhabitat-specific diurnal metabolomic responses of the intertidal limpet *Cellana toreuma* to winter low temperature

Yong-Xu Sun,[1] Li-Sha Hu,[2,*] and Yun-Wei Dong[2,3,4,*]

SUMMARY

High-throughput determination of circadian rhythms in metabolic response and their divergent patterns in various microhabitats are crucial for understanding how organisms respond to environmental stresses. A mid-intertidal limpet *Cellana toreuma* was collected at various time points across both daytime and nighttime in winter during low tide for investigating the diurnal metabolomic responses to cold stress and elucidating the divergent metabolic responses to temperature variations across microhabitats. Temperatures of emergent rock microhabitats were lower than the tidal pool and even aggravated at night. A series of metabolomic responses exhibited coordinated diurnal changes in winter. Metabolic responses which were associated with cellular stress responses and energy metabolism of emergent rock microhabitat individuals were highly induced compared to the tidal pool ones. This study shed light on the diurnal patterns of metabolomic responses of intertidal molluscs in the field and emphasized the variations in metabolic responses between microhabitats.

INTRODUCTION

As responses to daily environmental change, many biological functions such as physiological and behavioral processes exhibit rhythmic fluctuations that refer to circadian rhythm,[1] which serves as an adaptation for daily changes by allowing organisms to anticipate and prepare for daily external environmental changes.[2,3] Because responses to abiotic stresses are energetically costly, circadian rhythm can temporally restrict the response pathways to abiotic stresses when environmental conditions are not stressful enough.[4] The timing of physiological responses is coordinated with environmental changes. The diurnal biological responses are crucial for maximizing the efficiency of energy use during diurnal environmental changes and ultimately affect the resistance to environmental stresses.[4–8]

Metabolites are essential components of cellular regulatory and metabolic processes, and changes in metabolite levels can provide indicators of the overall integrated responses of an organism.[9,10] Previous studies have shown that seasonal changes in metabolites increased the resistance to environmental stress for organisms.[11–13] For instance, plants have adapted to seasonal temperature variations by adjusting their metabolism during autumn, increasing their content of a range of cryo-protective compounds to maximize their cold tolerance.[12] In the diurnal changes of the environment, a series of metabolites also exhibited coordinated changes and allowed species to adapt their physiological performance to daily environmental variations.[14,15] Therefore, detailed knowledge of the coordinated changes in metabolism to the diurnal cycle is essential for understanding how organisms cope with stress.

Recently, an increasing number of studies have emphasized the important roles of small-scale spatial variations (e.g., microhabitat scale) in assessing the vulnerability of organisms to environmental stress.[16–19] Small-scale variations in habitats can produce dramatically different microclimates and exhibit different diurnal environmental variations.[20,21] In a long-term record of intertidal animals' body temperature in different microhabitats; for instance, the daily temperature trajectories between sun-exposed and shaded microhabitats, exhibited large differences, which were larger than the variability associated with the seasons.[22,23] Therefore, environmental small-scale variation should be considered for determining physiological adaptations to diurnal environment changes.[24]

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The rocky intertidal habitat is a model system for exploring the physiological adaptations to variable and harsh environmental conditions.[17,25–29] There, a high degree of topographic complexity offers varieties of microhabitats and microclimates.[30,31] The variations of thermal conditions between microhabitats can largely exceed those between latitudes.[30,32,33]

Among these intertidal microhabitats, emergent rock and tidal pool microhabitats are common microhabitats.[34,35] Body temperatures of organisms in emergent rock microhabitats are driven by multiple environmental factors including solar radiation, air temperature, relative humidity, wind speed, and cloud cover.[36] Organisms in tidal pools are continually submerged and hence are not subject to the same environmental factors as on freely draining rock microhabitats.[37] During low tides, these microhabitats provide different microclimates for living organisms.[26] In general, tidal pools are considered as refuges for organisms to escape environmental pressures, which is of great significance for enhancing the population persistence of intertidal organisms.[34,38]

Localized extremely cold winter temperatures have significant impacts on the intertidal ecosystem by limiting foraging opportunities, resulting in mortality, and setting the range of species.[39–43] When organisms encounter low temperatures, a series of metabolites are produced to protect them from cryoinjuries.[10,44–47] To resist cold stress, for instance, intertidal mussels accumulated osmolytes to increase freeze tolerance.[10,48] Consequently, metabolic adaptations are crucial for resisting cold stress during dynamic environmental changes.

In this context, field studies are crucial because they provide *in situ* information related to the physiological changes in intertidal organisms under natural conditions.[49,50] The intertidal limpet *Cellana toreuma* is an important grazer on rocky intertidal shores and is widely distributed in China, Korea, and Japan.[51–53] In winter, this species occupies a microhabitat range from rock surfaces to tidal pools and exhibited a high incline to stay in the original microhabitat in a short term. The widespread distribution in different microhabitats could be attributed to factors such as food availability, temperature variation, and predation pressure.[19,54,55] Based on LC-MS/MS metabolomics, we performed a field experiment designed to assess the metabolomic responses of *C. toreuma* to diurnal environmental fluctuations. Compiling these environmental and metabolomic data allows us to test the following hypothesis: limpets between microhabitats exhibited divergent patterns of diurnal metabolomic responses in the face of winter low temperatures.

RESULTS

Environmental temperature of tidal pool and emergent rock microhabitats

Thermal infrared images gave a visual representation of the difference in temperature between tidal pools and rock surfaces (Figure 1A). The orange color of the tidal pools indicated a relatively warm microhabitat, and the rest of the emergent rock microhabitat was blue in color, associated with a much colder temperature. From November 2021 to February 2022, the temperatures ranged from −4.1 to 29.4°C in the emergent rock microhabitat and ranged from −0.3 to 22.5°C in the tidal pool microhabitat (Figure 1B). The daily thermal variation in the emergent rock microhabitat (13.28 ± 4.94°C) was significantly higher than that in the tidal pool microhabitat (5.39 ± 1.68°C) (t-test, $p < 0.001$). During the low tides, the temperature in the emergent rock microhabitat was lower than that in the tidal pool (Figure 1C). The minimum temperature during the sampling period (December 12th, 2021) in the emergent rock (−2.73 ± 1.25°C) was significantly lower than that in the tidal pool microhabitat (2.34 ± 0.71°C) (t-test, $p < 0.001$).

Metabolomics analysis

Limpets were collected at both tidal pool and emergent rock microhabitats after the tide had receded (just emerged, D0 at 12:00, N0 at 0:00), when the tide faded to the lowest (emerged for 3 h, D3 at 15:00, N3 at 3:00), and just before submergence by the returning tide (emerged for 5 h, D5 at 17:00, N5 at 5:00), respectively. For limpets collected at the time points at daytime (D0, D3, D5) and nighttime (N0, N3, N5) during low tides, the partial least squares discrimination analysis (PLS-DA) showed the samples from different time points were separated clearly in both the tidal pool and emergent rock microhabitats (Figure 2A).

As the results of the permutational multivariate analysis of variance (PERMANOVA) showed metabolites were significantly affected by sampling times ($F = 19.8857$, $p < 0.001$) and microhabitats ($F = 4.8461$, $p = 0.020$), but the interaction effect between sampling times and microhabitats was not significant ($F = 1.2746$, $p = 0.271$).

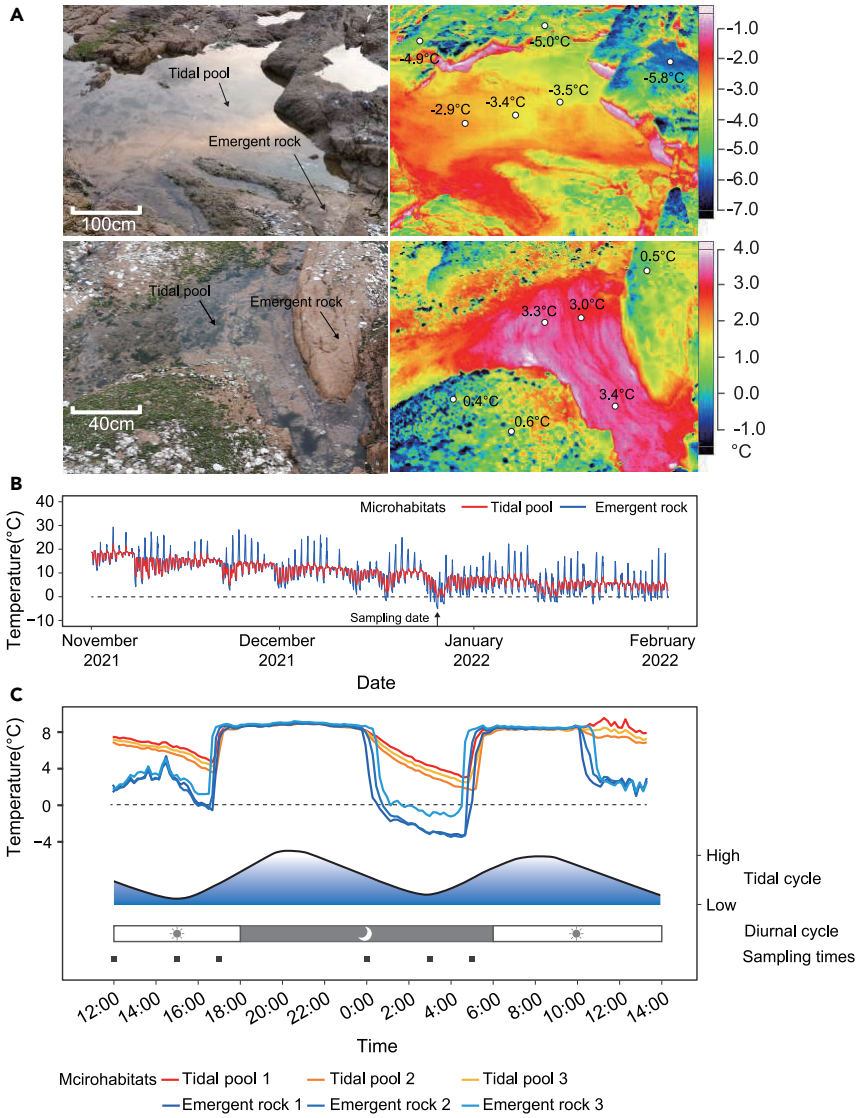

Figure 1. Different thermal conditions of tidal pool and emergent rock microhabitats

(A) Thermal images in the tidal pool and emergent rock microhabitats.

(B) Temperatures recorded in tidal pool and emergent rock microhabitats.

(C) Temperatures recorded in the tidal pool and emergent rock microhabitats during sampling periods. During low tides on December 12th, 2021, limpets were sampled at 12:00 (D0), 15:00 (D3), and 17:00 (D5) in the day and 0:00 (N0), 3:00 (N3), and 5:00 (N5) at night, respectively. The arrow represents the sampling date. The dotted lines represent the temperature of 0°C.

Cluster analyses showed that the top 25 metabolites were significantly different among sampling times ($p < 0.05$) and were grouped into four clusters in both tidal pool and emergent rock microhabitats (Figure 2B). The levels of metabolites in the Cluster 1 decreased from D0, D3 to D5 time points and were maintained at low level from N0, N3 to N5 time points. In the Cluster 2, the concentrations of metabolites in the tidal pool microhabitat were maintained at a low level during the day (D0, D3 to D5 time points) and decreased during the night (N0, N3 to N5 time points), but were maintained at high levels during the day and low levels during the night in the emergent rock microhabitats. The metabolites in Cluster 3 and Cluster 4 were maintained at low levels during the day and accumulated during the night. These differentially expressed metabolites (DEMs) were related to purine metabolism, TCA cycle, amino acids metabolism, sugar metabolism, and cellular stress response (Figure 3).

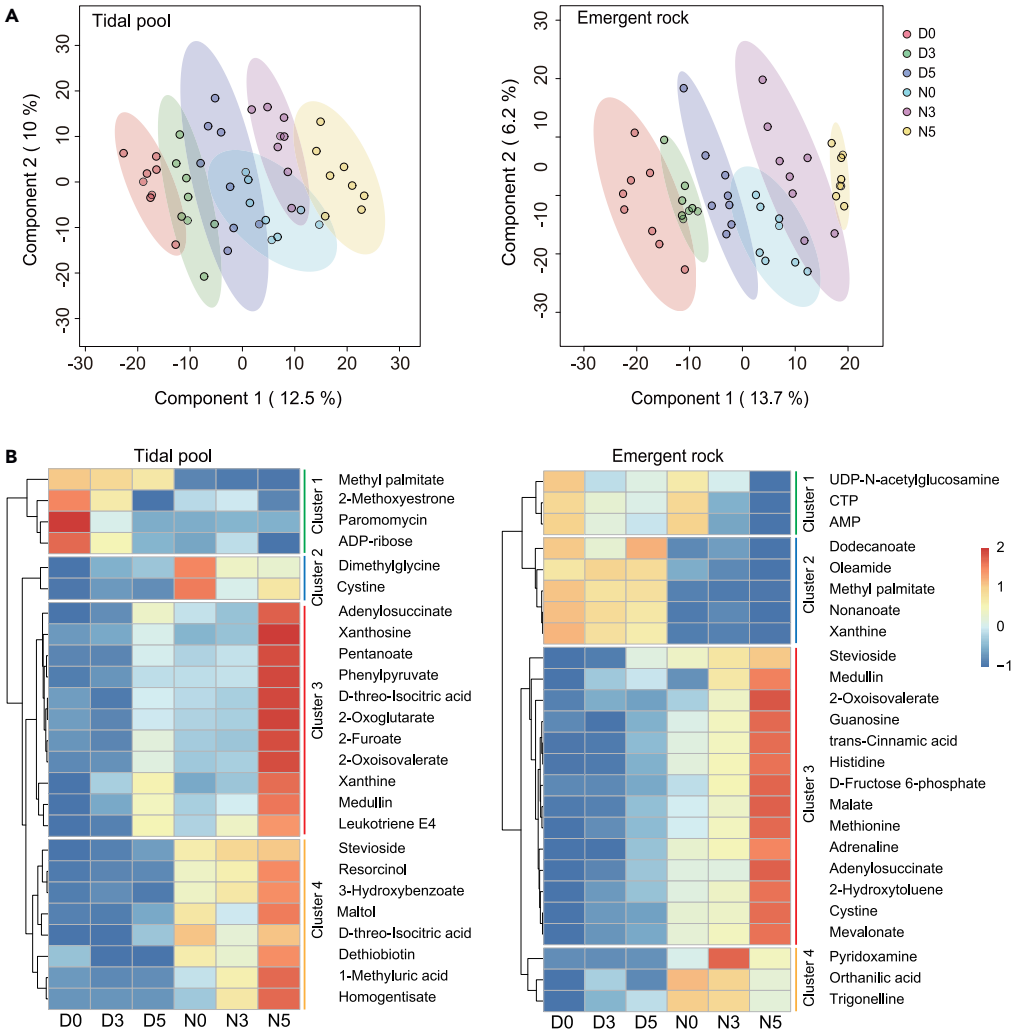

Figure 2. The partial least squares discrimination analysis (PLS-DA) and heatmap analysis of metabolites across sampling times

(A) The PLS-DA of metabolites among sampling times for tidal pool and emergent rock microhabitats, respectively.

(B) Heat maps of the top 25 metabolites that differ significantly ($p < 0.05$) among sampling time points between tidal pool and emergent rock microhabitats, respectively. Ellipses on PLS-DA score plots represented a confidence interval of 95%. In the heat maps, sampling times were in columns (n = 8) and metabolites were in rows. The colors varied from deep blue to dark red representing data values ranged from very low (cold colors) to extremely high (hot colors). Metabolites were clustered in four clusters. Metabolites codes: CTP, cytidine triphosphate; AMP, Adenosine monophosphate.

According to their functions, these DEMs are divided into four groups (Figure 4). The metabolites in Group 1 were associated with purine metabolism, including guanosine diphosphate (GDP), inosine, adenosine monophosphate (AMP), hypoxanthine, and xanthine. The concentrations of GDP, inosine, and hypoxanthine were maintained at low levels during the day and increased from N0, N3 to N5. The concentration of xanthine increased from D0, D3 to D5, and also increased from N0, N3 to N5. The concentrations of GDP, inosine, and hypoxanthine at the sampling time point N5 were significantly higher than that at other time points ($p < 0.05$) (Table S1). The concentration of AMP was maintained at a high level during the day and decreased during the night.

Metabolites in group 2 were related to energy metabolism, including pantothenate, pantetheine, L-phenylalanine, L-adrenaline, citrate, isocitrate, 2-oxoglutarate, and malate. The contents of pantetheine decreased from D0 to D5 and exhibited a low level at night, and its content at sampling timepoint D0 was significantly higher than that at other time points ($p < 0.05$). The contents of phenylalanine, adrenaline,

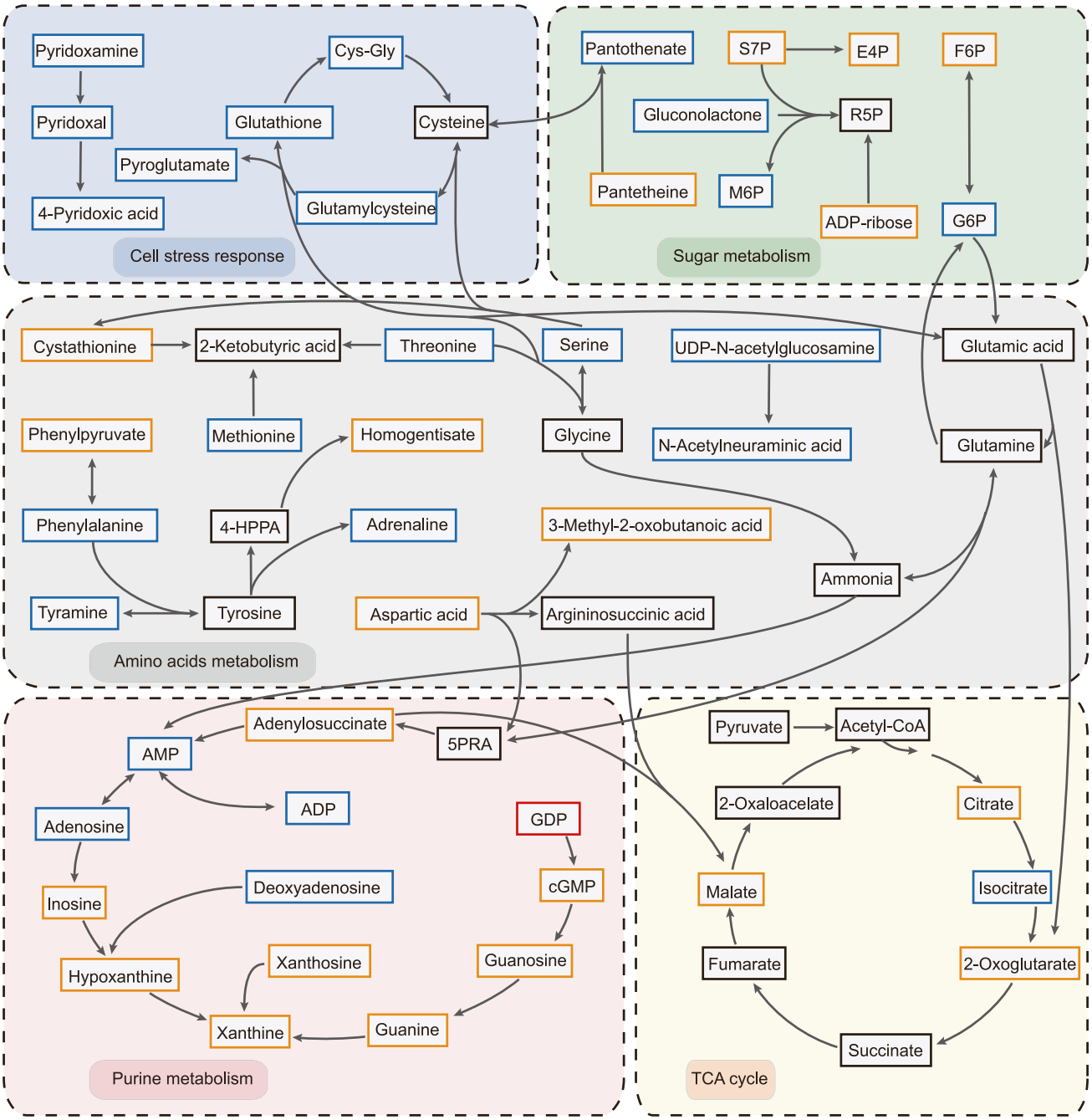

Figure 3. Pathways of differentially expressed metabolites in tidal pool and emergent rock microhabitats

These metabolites were related to purine metabolism, TCA cycle, amino acids metabolism, sugar metabolism, and cellular stress response. Metabolites in the blue and red boxes were metabolites whose relative concentrations differed significantly only in rock and tidal pool microhabitats, respectively. The yellow box indicated metabolites whose relative concentrations differed significantly both in rock and tidal pool microhabitats. The black box indicated metabolites that were related to the pathways mentioned above, but the relative concentrations are not significantly different in rock or tidal pool microhabitats. Metabolites codes: Cys-Gly, L-cysteinyl glycine; S7P, Sedoheptulose 7-phosphate; E4P, Erythrose 4-phosphate; F6P, Fructose 6-phosphate; R5P, Ribulose 5-phosphate; M6P, Mannose-6-phosphate; G6P, glucose-6-phosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; 5PRA, 5-phosphoribasylamine; GDP, guanosine diphosphate; cGMP, cyclic adenosine monophosphate.

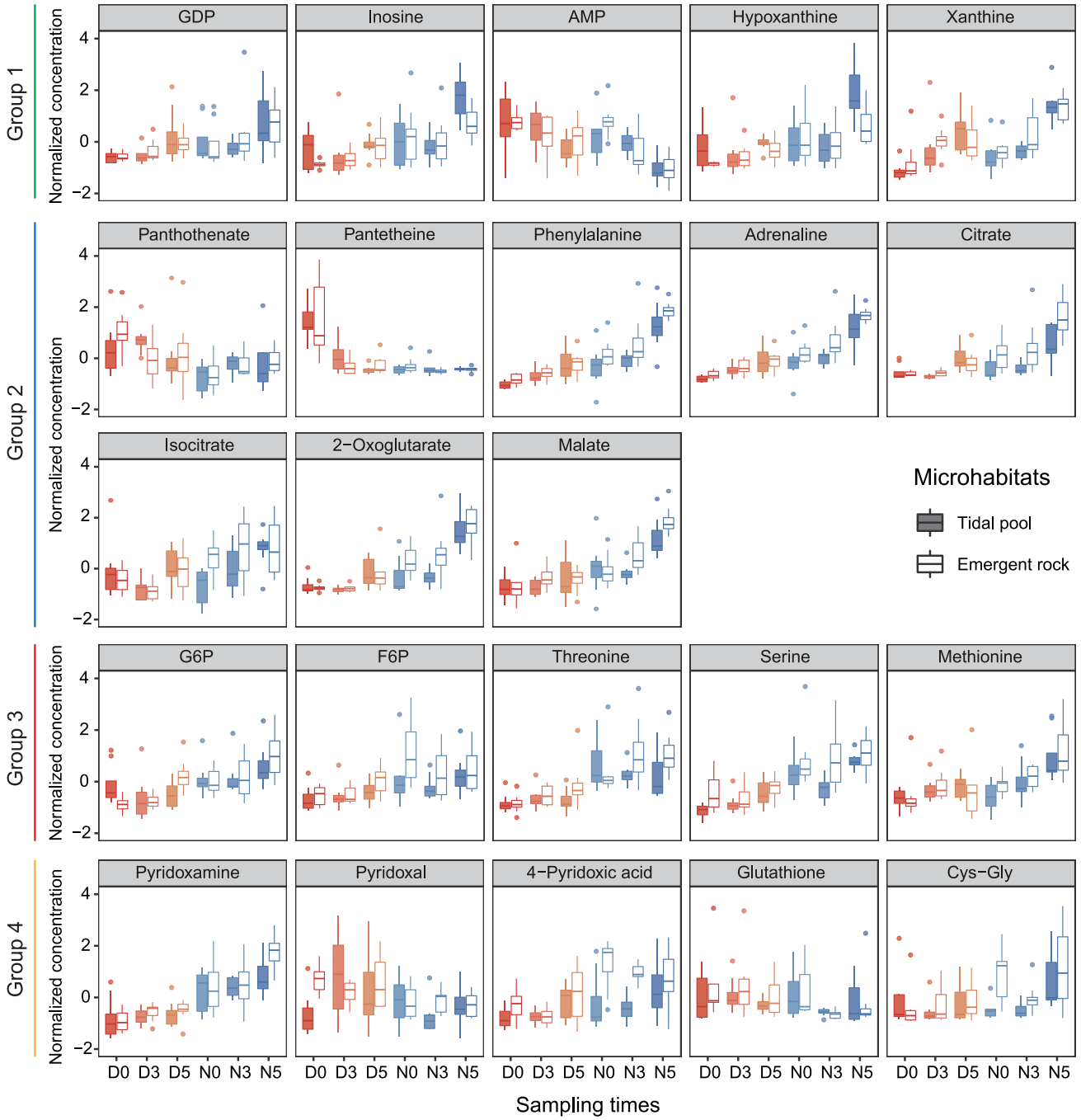

Figure 4. Variation in relative concentrations of key metabolites

The metabolites in the Group 1, Group 2, Group 3 and Group 4 were related to purine metabolism, energy metabolism, osmoregulation, and cellular stress response, respectively. For statistical analysis, tests for normality distribution and variance homogeneity were assessed with the Shapiro-Wilk test and Levene's test, respectively. Data were log-transformed for normality and homogeneity and presented as mean ± SEM (n = 8). Metabolites codes: GDP, guanosine diphosphate; AMP, adenosine monophosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; Cys-Gly, L-cysteinyl glycine.

citrate, isocitrate, 2-oxoglutarate, and malate increased from D0 to N5, and increased significantly at time point N5 ($p < 0.05$). In addition, the contents of adrenaline, citrate, isocitrate, 2-oxoglutarate, and malate in emergent rock microhabitat were significantly higher ($p < 0.05$) than that of tidal pool microhabitat at time-point N3.

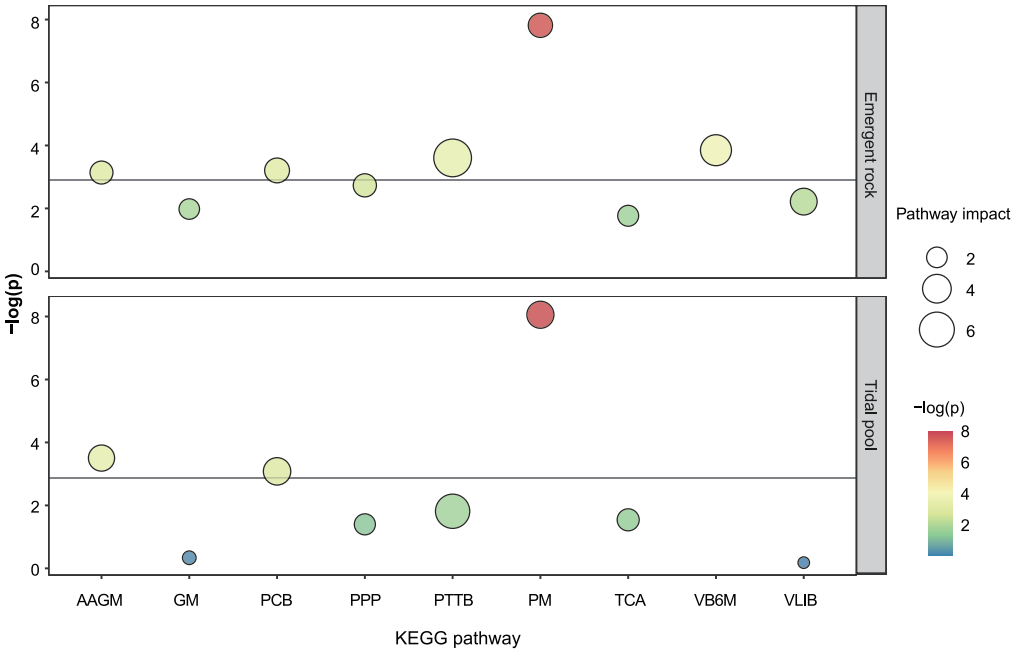

Figure 5. KEGG metabolic pathway analysis of metabolites for which content significantly differs among sampling times for tidal pool or emergent rock microhabitats, respectively

The abscissa presents the name of each metabolic pathway; the ordinate represents the degree of difference between groups for each pathway. The degree of difference is explained by the negative log-transformed p-value. The gray lines represent p values of 0.05, and significantly enriched pathways were shown above the lines. The color of each circle is based on the p value and the size is based on the pathway impact value. AAGM, alanine, aspartate and glutamate metabolism; GM, glutathione metabolism; PCB, Pantothenate and CoA biosynthesis; PPP, Pentose phosphate pathway; PTTB, Phenylalanine, tyrosine and tryptophan biosynthesis; PM, Purine metabolism; TCA, TCA cycle; VB6M, Vitamin B6 metabolism; VLIB, Valine, leucine and isoleucine biosynthesis.

Metabolites in group 3 were related to osmoregulation, including glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), threonine, serine, and methionine. These metabolites increased from N3 to N5 sampling time points and exhibited high levels at night.

Metabolites in group 4 related to the cellular stress response, including pyridoxamine, pyridoxal, 4-pyridoxic acid, glutathione, and L-cysteinyl glycine (Cys-Gly). The concentrations of pyridoxamine were elevated at night and were significantly higher in the emergent rock microhabitat than in the tidal pool microhabitat at timepoint N5 ($p < 0.01$). The concentrations of 4-pyridoxic acid were maintained at a higher level in emergent rock microhabitats during the night and were significantly different between the tidal pool and emergent rock microhabitats in N0 and N3 time points ($p < 0.01$). The concentrations of glutathione decreased and were maintained at low level during the night.

These DEMs in both rock and tidal pool microhabitats were used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis (Figure 5). The results showed that there were five significantly enriched pathways in the emergent rock microhabitat ($p < 0.05$). The pathways related to alanine, aspartate and glutamate metabolism, pantothenate and CoA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, purine metabolism and vitamin B6 metabolism. In the tidal pool microhabitats, the significantly enriched pathways included alanine, aspartate and glutamate metabolism, pantothenate and CoA biosynthesis, and purine metabolism.

Relationship between metabolites and environmental variables

The redundancy analysis (RDA) results indicated that the environmental variables in the first two RDA dimensions provided 17.09% and 2.90% of the cumulative variance of metabolites composition, respectively (Figure 6). These metabolites were highly influenced by the diurnal cycle, exposure time, microhabitats, and environmental temperature. Most metabolites involved in cellular stress response, energy

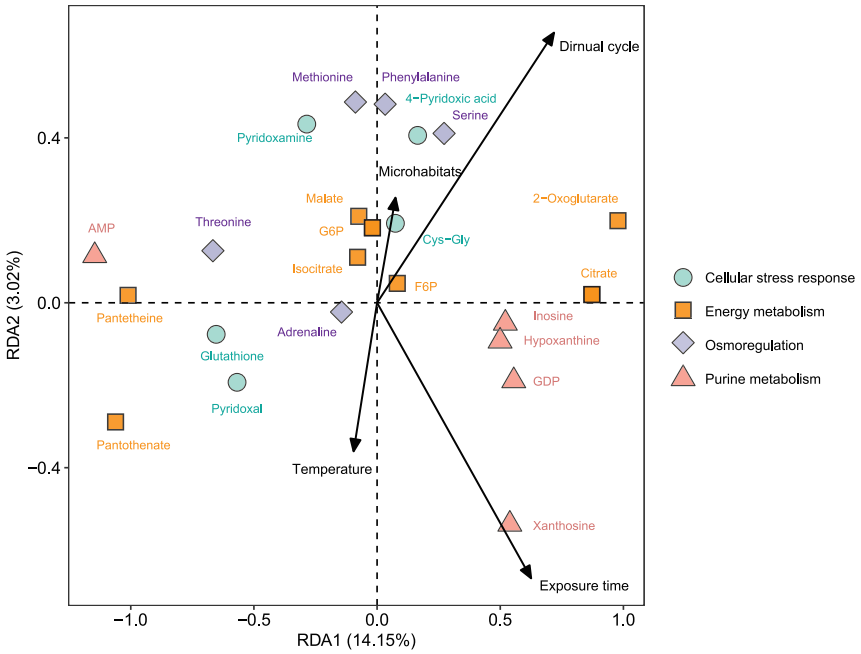

Figure 6. Metabolites-environment biplot diagrams based on redundancy analysis (RDA)

Correlations between environmental variables and RDA axes are represented by the length and angle of arrows. Metabolites were omitted for clarity. Metabolites codes: GDP, guanosine diphosphate; AMP, adenosine monophosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; Cys-Gly, L-cysteinyl glycine. The environmental temperatures were calculated by using the hourly mean values of the temperature recorded during the sampling periods at 12:00 (D0), 15:00 (D3), 17:00 (D5) in the day and at 0:00 (N0), 3:00 (N3), 5:00 (N5) at night on December 12th, 2021.

metabolism, osmoregulation, and purine metabolism were influenced by the diurnal cycle and exposure time. The metabolites related to energy metabolism and cellular stress response were highly correlated with microhabitats. Based on hierarchical partitioning, the variables which explained the highest part of these metabolites variability were diurnal cycle (R^2 = 48.25%), followed by exposure time (R^2 = 38.50%), temperature (R^2 = 7.00%), and microhabitat (R^2 = 6.30%).

DISCUSSION

The diurnal metabolomic response is an adaptive physiological response to the ambient environment. In winter, limpets experienced serious stress from low temperatures, especially during the night. A series of metabolic responses, including increases in purine and energy metabolism, accumulation of osmolytes, and metabolites protecting organisms from cellular stress was highly induced during the night and affected the survival of limpets in the changing environment. Moreover, the metabolic responses related to energy metabolism and cellular stress were highly induced in the emergent rock microhabitat during the night, implying divergent metabolomic responses to different microclimates.

Diurnal metabolic responses coordinated with diel environmental changes

Various metabolic pathways interacted with each other and coordinated with diurnal environmental changes.[9,56] Purine metabolism constitutes a key pathway for organisms involved in the production of DNA and RNA and regulating various metabolic pathways via cofactors.[57] As a strong feature of circadian rhythms in organisms,[58] in the present study, the enhanced purine metabolism during night might be because of physiological needs for DNA repair or RNA synthesis.

It is crucial for organisms to cope with environmental stress by upregulating metabolism and increasing energy supply to meet the growing demand for energy in stressful environments.[6,7] The TCA cycle is one of the iconic pathways in energy metabolism that contributes to the generation of ATP.[59] In the present study, the relative concentrations of citrate, isocitrate, 2-oxoglutarate, and malate, involved in the TCA cycle, were induced significantly during night, indicating enhanced energy production.

Moreover, coenzyme A (CoA) is predicted to be involved in the regulation of the TCA cycle, and energy metabolism.[60] For synthesizing CoA, pantetheine is a preferred substance component.[61] In the present study, the level of pantetheine was maintained at a low level during the night, indicating the need for CoA synthesis for coping with enhanced energy consumption.

Our results also implied that accumulating catecholamine neurotransmitters may be important for *C. toreuma* confronting environmental stress. We noted that levels of phenylalanine and adrenaline increased during low tides, especially at night. Phenylalanine is a key metabolite in neurotransmitter regulation, because it is transformed into tyrosine and, subsequently, into catecholamine neurotransmitters (e.g., dopamine, norepinephrine, and adrenaline).[62] Adrenaline has long been known to play an active role in the nervous systems and has active effects on the cardiac performance of limpets.[63] An increase in cardiac rate is important for organisms to satisfy the demands of aerobic metabolism under stress.[64,65] In the present study, elevated phenylalanine and adrenaline, which are closely associated with nervous regulation, could lead to increased cardiac rate and thus promote oxygen delivery to tissues when limpets experienced severe cold stress during night.

Extreme osmotic stress placed on cells was highly injurious in the face of cold stress.[66] Osmolytes are important for preventing cellular dehydration owing to extracellular ice formation and modulating freeze plasticity in intertidal invertebrates.[10,47] For instance, the accumulations of osmolytes (e.g., taurine, glycine, TMAO, betaine, and alanine) in *Mytilus trossulus* were shown to be consistent with increased freezing tolerance.[10,48] In addition to their role in establishing osmotic balance, the favorable effects of these osmolytes on protein and membrane stability could also be important in assisting organisms to resist extreme temperatures.[48] As previous studies described, important osmolytes in intertidal invertebrates include amino acids and sugars.[67–69] In the present study, sugars (G6P and F6P) and amino acids (threonine, serine and methionine) were accumulated during the night, which could potentially maintain cellular homeostasis during diurnal environmental changes.

Another consequence for organisms exposed to low temperatures is cellular oxidative stress.[70] The overproduction of reactive oxygen species (ROS) can damage lipids, proteins, and DNA.[70,71] Vitamin B6, including pyridoxamine, pyridoxal and pyridoxine, is served as an important cofactor for numerous enzymes and is involved in the regulation of cellular stress responses.[72] In the present study, the level of pyridoxamine increased during low tides and was maintained at a relatively high level during the night. As the primary catabolic product of vitamin B6, 4-pyridoxic acid concentration was maintained at a high level during the night. These results indicated that the enhancement of vitamin B6 metabolism could scavenge ROS, and protect limpets from oxidative damage during diurnal environmental changes.

Divergent metabolic responses between microhabitats

Small-scale variations in environmental conditions are an important determinant of physiological performance, population persistence, and distribution.[17,20,73] Living in different microhabitats, organisms experience different magnitudes of environmental stress.[19,23] Shaded microhabitats, for instance, are often cooler than sun-exposed microhabitats in summer, and organisms in the shaded microhabitats are predicted to survive the hot summer.[17,73] In winter, intertidal molluscs were frequently exposed to freezing, and exhibited intermediary levels of cold hardiness and limited survival to the freezing of their tissues.[74] For instance, the limpet, *Acmaea digitalis* (now *Lottia digitalis*), would be frozen at temperatures between −10 and −12°C and few individuals survived more than 24 h.[75] Among microhabitats, tidal pool microhabitats are often considered as refuges that can provide shelter from winds and air exposure and have served as nurseries for limpets.[38] On the other hand, emergent rock microhabitats are often considered to be stressful, in which organisms exposed to air during low tides were sometimes frozen.[10,76]

In the present study, the limpets were not found to be frozen during the sampling period, which might be attributed to the effects of freeze avoidance.[77] The environmental temperature of emergent rock microhabitats was lower than that of tidal pools, especially at night. Responding to the harsh microclimate, the limpets in the emergent rock microhabitat deployed higher intensity of cellular stress response. As mentioned above, the cellular stress responses are activated in response to macromolecular damage and play central roles in the regulating repair of stress-induced cellular damages at the expense of higher energy costs.[70,78] Energy balance plays a critical role in setting limits for the survival of organisms under stressful conditions and is a fundamental requirement of stress adaptation and tolerance.[8] In the present

study, the severer cold stress in emergent rock microhabitats could cause higher levels of cellular stress response in limpets and thus more energy was required. Comparatively, the intensity of the cellular stress response of limpets living in thermally mild tidal pool microhabitats was lower, which would allow increased amounts of energy to be directed to growth, storage or reproduction.[8]

In addition, the results of PERMANOVA analysis showed the independent effects of day/night and microhabitats on the metabolic responses of limpets to winter cold stress. Moreover, the PERMANOVA analysis indicated the important effects of the diurnal cycle and microhabitats on the metabolic responses of limpets to winter cold stress. Specifically, the diurnal cycle of metabolomic responses reflected the coordinated changes in physiological performance and affected the survival of organisms in the changing environment.[14,15] The divergent diurnal metabolic responses between microhabitats were consistent with the environmental stresses of rock and tidal pool microhabitats, indicating the importance of metabolic adaptation to small-scale environmental variations in winter.

In conclusion, the present study revealed diurnal metabolomic responses of the intertidal limpet, *C. toreuma*, to winter cold stress and the divergent metabolic adaptive responses between rock and tidal pool microhabitats. A series of metabolic responses, including increases in purine metabolism and energy metabolism, and accumulation of osmolytes and metabolites protecting organisms from cellular stress, were induced during the night. In the more stressful emergent rock microhabitats, metabolic responses which are associated with cellular stress response and energy metabolism were highly induced. These results emphasized the existence of diurnal metabolic adaptation in the intertidal molluscs and differential metabolic responses to divergent microclimates in the face of low temperatures.

Limitations of the study

Although the present study provides insight into the diurnal patterns of metabolic adaptation of intertidal molluscs under low temperatures in nature and emphasizes the different metabolic patterns between different microhabitats, several limitations of the study remain to be addressed. First, metabolomic responses under prolonged periods with multiple tidal cycles can potentially reveal more refined metabolic response strategies to environmental stresses. Second, assessing the effects of multiple stressors (e.g., air exposure, salinity, pH, dissolved oxygen, etc.) on the physiological performances of marine organisms will further contribute to revealing the divergent physiological responses among microhabitats. Finally, the behavioral strategies of cold tolerance in intertidal invertebrates inhabiting different microhabitats are worth to be investigated. These analyses will be important to understand how species survive cold temperatures in winter and the ecological impacts of various microhabitats.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106128.

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AUTHOR CONTRIBUTIONS

Y-W.D., Y-X.S., and L-S.H. designed research; Y-X.S. performed research; Y-X.S. and Y-W.D. analyzed data; Y-X.S. and L-S.H. created all figures; Y-X.S., L-S.H., and Y-W.D. wrote and revised the paper. All authors contributed critically to the drafts and gave final approval for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Metabolomic data	This study	https://doi.org/10.5061/dryad.79cnp5hzk
Original code	This study	https://github.com/sunyongxu/ISCIENCE-D-22-03956R1-.git
Software and algorithms		
R software	https://www.r-project.org	V 4.2.1
SmartView	Fluke Corporation	V 4.4
Other		
LC-MS	ThermoFisher Scientific	N/A
Vanquish UHPLC system	ThermoFisher Scientific	N/A
Orbitrap Q Exactive™ HF mass spectrometer	ThermoFisher Scientific	N/A
Fluke Infrared camera	Fluke Corporation	TiX660

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yun-Wei Dong (dongyw@ouc.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The metabolomic data have been deposited at the Dryad Digital Repository and are publicly available as of the date of publication. The accession number is listed in the key resources table.
- All original code has been deposited at GitHub and is publicly available as of the date of publication. The DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study was based on wild limpet *C. toreuma*, and no experimental models were used.

METHOD DETAILS

In situ operative temperature measurement

Biomimetic loggers (HOBO, MX2201) with a height of 1.5 cm and a diameter of 3.0 cm were used to monitor the operative temperature. Six biomimetic loggers were deployed at a field site using A-788 Z-Spar Splash Compound (Kopper's Co.,USA) in Qingdao, Shandong, China (36.03°N, 120.23°E).[79] The temperature recorded by loggers was similar to the operative body temperature of limpets based on the preliminary experiment in winter. Loggers were positioned in different microhabitats: 1) Loggers were deployed on the bottom of the tidal pools where the limpets usually occupy; and 2) Loggers were deployed on emergent rock surfaces near to limpets. From November 2021 to February 2022, three loggers were deployed in each microhabitat. The measuring accuracy was set as 0.5°C, and the interval was 20 min for long-term monitoring from November 2021 to February 2022 and 10 min for short-term monitoring to get a more specific temperature dataset during sampling periods.[17,80,81]

To characterize the different thermal properties between tidal pool and emergent rock microhabitats in visual, infrared thermal imaging was applied by using a Fluke TiX660 Infrared camera (Fluke Corporation, USA) on December 24th, 2021 following the methods as described in Lathlean and Seuront.[82] Thermal images were analyzed using the software SmartView V 4.4 (Fluke Corporation, USA). For each thermal imaging, points were drawn to determine the temperature of microhabitats.

Animal collection

On December 12th, 2021, individuals of *C. toreuma* were randomly collected at emergent rock and tidal pool microhabitats in Qingdao, Shandong, China (36.03°N, 120.23°E). In Qingdao, these limpets experience semi-diurnal tidal regimes and exposure to air during day and night, respectively (Figure 1C). Eight individuals (n = 8) were collected at both microhabitats after the tide had receded (just emerged, D0, N0), when the tide faded to the lowest (emerged for 3 hours, D3, N3), and just before submergence by the returning tide (emerged for 5 hours, D5, N5), respectively. After collection, animals were frozen in liquid nitrogen and stored at −80°C for subsequent use in metabolomic profiling.

Metabolomic profiling

Living on the shore, limpets keep attached with their foot to the substrate and thus foot tissue is one of the tissues to be first impacted by cold temperatures.[83] In addition, foot tissue was large enough in meeting the quantity requirement in metabolomic analysis. Thus, foot tissues (80 mg) were individually frozen with liquid nitrogen and the homogenate was resuspended with prechilled 80% methanol by vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15,000 g, 4°C for 20 min. The supernatant was diluted to a final concentration containing 53% methanol by LC-MS/MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 15,000 g, 4°C for 20 min. Finally, the supernatant was injected into the LC-MS/MS system for analysis. UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q Exactive™ HF mass spectrometer (ThermoFisher, Germany) in Novogene Co., Ltd. (Beijing, China). Samples were injected onto a Hypersil Gold column (100 × 2.1 mm, 1.9 μm) using a 17-min linear gradient at a flow rate of 0.2 mL min^{-1}. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% eluent B, 1.5 min; 2–85% eluent B, 3 min; 85–100% eluent B, 10 min; 100-2% eluent B, 10.1 min; 2% eluent B, 12 min. Q Exactive™ HF mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.5 kV, capillary temperature of 320°C, sheath gas flow rate of 35 psi, and aux gas flow rate of 10 L/min, S-lens RF level of 60, Aux gas heater temperature of 350°C.

Microhabitat thermal variation analysis

To characterize the thermal variation between tidal pool and emergent rock microhabitats, the daily thermal variation was calculated by the difference between the daily maximum temperature and the daily minimum temperature from November 2021 to February 2022. In addition, the minimum temperature during the sampling period was calculated to characterize the variation of cold stress between tidal pool and emergent rock microhabitats. Differences in the daily thermal variation and the minimum temperature between microhabitats were analyzed using t-test in R V 4.2.1. The numerical results were presented as mean ± SEM.

Metabolomic analysis

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, ThermoFisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 12 seconds; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; minimum intensity, and adduct ions. After that, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/), mzVault, and MassList database to obtain the relative quantitative results.

The MetaboAnalyst 5.0 website (https://www.metaboanalyst.ca/) was used to perform statistical analysis (normalization, generalized logarithm transformation, auto-scaling, and cluster analysis) and pathway

analysis.[84] To obtain differentially expressed metabolites (DEMs), one-way ANOVA(adjusted $p < 0.05$) and partial least squares discriminate analysis (PLS-DA) (VIP score >1) were performed among different sampling times.

In order to clarify the variation of diurnal metabolomic response, the metabolic pathway analyses were performed in tidal pool and rock microhabitats, respectively. In the pathway analysis, the metabolites that varied significantly among sampling times (D0, D3, D5, N0, N3, and N5) were selected by the differentially expressed metabolites (DEMs), and then enriched in the KEGG pathway database (https://www.metaboanalyst.ca/). Thus, significantly enriched pathways indicated the important metabolic responses to diurnal environmental change. The negative log-transformed p values (-log *P*) were used to explain the degree of difference in metabolic pathways.

PERMANOVA analysis

To detect the effects of the diurnal cycle, microhabitats, and the interaction between diurnal cycle and microhabitats on the variations of metabolites, a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was performed using *vegan* package in R V 4.2.1.[85] To detect the differences in the relative concentrations of metabolites among sampling times and microhabitats, generalized linear models (GLM) with Gaussian models were performed in SPSS 22 (SPSS, Chicago, IL, USA).[86] For statistical analysis, tests for normality distribution and variance homogeneity were assessed with the Shapiro-Wilk test and Levene's test, respectively. Data were log-transformed for normality and homogeneity. The numerical results were presented as mean $\pm$ SEM (n = 8).

Redundancy analysis

The influence of environmental variables on the relative concentrations of metabolites was analyzed using the redundancy analysis (RDA). During the RDA, the importance of each variable (diurnal cycle, microhabitats, exposure time, and temperature) was assessed by hierarchical partitioning using the *rdacca.hp* package in R V 4.2.1.[87] The environmental temperatures were calculated by the hourly mean values of the temperature recorded during the sampling periods (December 12th, 2021) at 12:00 (D0), 15:00 (D3), 17:00 (D5) in the day and at 0:00 (N0), 3:00 (N3), 5:00 (N5) at night, respectively.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis for thermal variation between microhabitats was performed in R software V 4.2.1. The one-way ANOVA(adjusted $p < 0.05$) and partial least squares discriminate analysis (PLS-DA) (VIP score >1) were used to select differentially expressed metabolites (DEMs). Statistical analysis for the relative concentrations of metabolites among sampling times and microhabitats was performed using GLM model in SPSS 22. The PERMANOVA and RDA analyses were performed using *vegan* and *rdacca.hp* package in R V 4.2.1, respectively. Normality of the data was tested using the Shapiro-Wilk test. The homogeneity of variance was tested using Levene's test. If one of the two conditions was not validated ($p < 0.05$), a log transformation of the data was performed before further analysis. Description of the specific statistical tests used are stated in the method details and figure legends.