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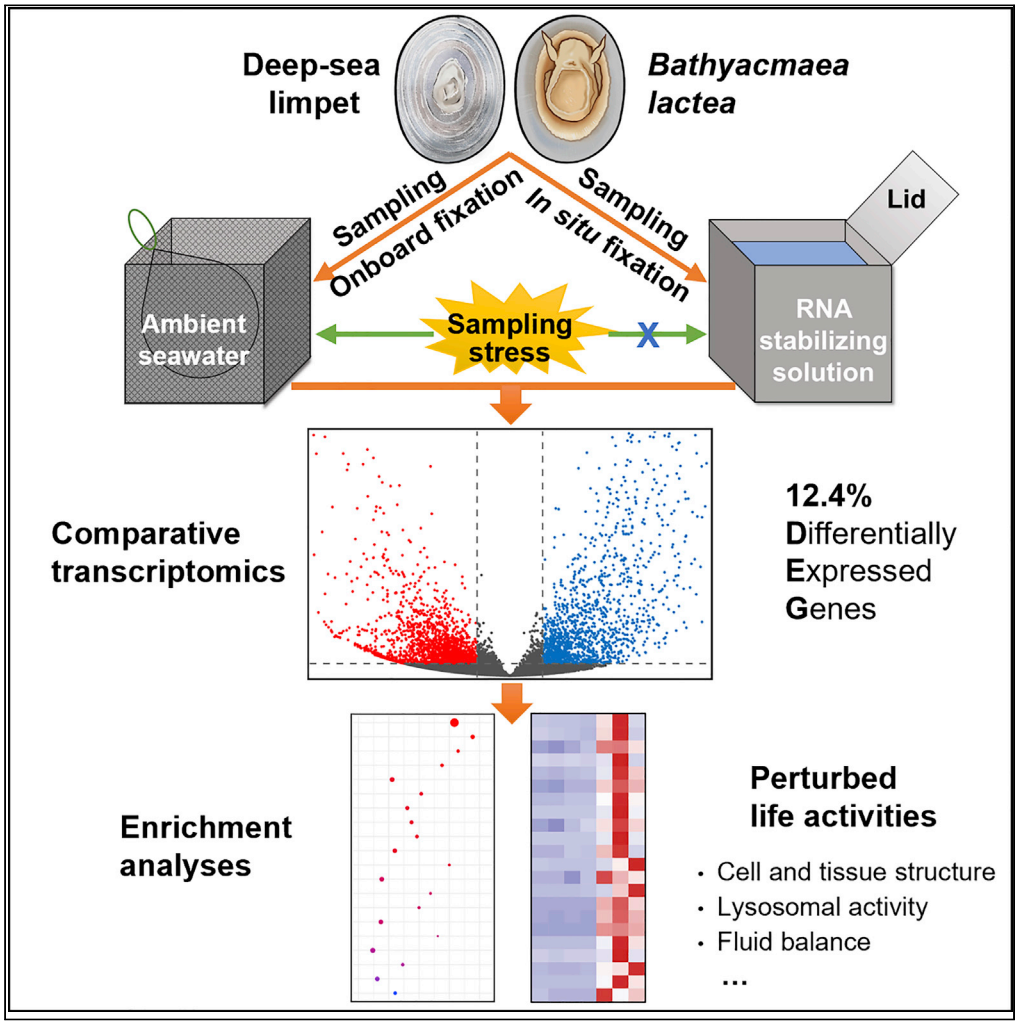

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Highlights

Deep-sea sampling without RNA *in situ* fixation influenced the gene expression greatly

Concomitant sampling stress perturbed various life activities of deep-sea limpets

Providing a high-quality *in situ* transcriptome of limpet *Bathyacmaea lactea*

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Comparative transcriptomic analysis of *in situ* and onboard fixed deep-sea limpets reveals sample preparation-related differences

Guoyong Yan,[1,2,4] Yi Lan,[1,2,4] Jin Sun,[3] Ting Xu,[1,2] Tong Wei,[1,2] and Pei-Yuan Qian[1,2,5,*]

SUMMARY

Precise gene expression reflects the molecular response of deep-sea organisms to their harsh living environments. However, changes in environmental factors during lifting samples from the deep sea to a research vessel can also affect gene expression. By using the transcriptomic approach, we compared the gene expression profiles of the onboard fixed with the *in situ* fixed samples of the deep-sea limpet *Bathyacmaea lactea*. Our results revealed that the concomitant stress during conventional deep-sea sampling without RNA *in situ* fixation greatly influenced the gene expression. Various biological activities, such as cell and tissue structure, lysosomal activity, fluid balance, and unsaturated fatty acid metabolism, were perturbed, suggesting that the sampling stress has exerted systemic impacts on the life of the limpets. These findings clearly illustrate that deep-sea samples without RNA *in situ* fixation can easily lead to biased results in gene expression analysis, which requires to be appropriately addressed in future studies.

INTRODUCTION

Approximately 88.3% of the ocean is deeper than 1000 m, commonly known as the deep sea (Weatherall et al., 2015), which is characterized by high hydrostatic pressure, low temperature (except for the hydrothermal vents), deficient food supply, and total darkness, and has therefore been considered hostile to life (Rex and Etter, 2010). However, both microorganisms and macroorganisms have been discovered in the deep sea, which is actually the largest habitat on Earth (Ramirez-Llodra et al., 2010). The deep sea supports various ecosystems with high biodiversity and biomass, such as hydrothermal vents, cold seeps, and whale falls (Kiel, 2016). Deep-sea organisms must have evolved various adaptive strategies to survive and thrive under such harsh environmental conditions. High-quality samples and free access to their genetic information are necessary to obtain a comprehensive and in-depth knowledge of the molecular mechanisms underlying deep-sea adaptation. Nevertheless, deep-sea sampling has always been quite technically challenging because of the great depth and far distance from land.

Owing to the rapid development of deep-sea sampling tools and techniques during the past decades (Clark et al., 2016), obtaining samples from deep-sea ecosystems has become easier. Furthermore, many studies have been conducted to obtain the genomic information of deep-sea organisms and explore the deep-sea adaptation mechanisms through genome, transcriptome, and proteome sequencing and analyses (Lan et al., 2018; Sun et al., 2017; Wang et al., 2019a; Yang et al., 2020). However, most of these studies were based on deep-sea samples obtained by conventional deep-sea sampling method, that is, the samples were fixed on the board of a research vessel after retrieval from the seafloor. For organisms that have adapted well to the deep sea, the harsh local environment has been optimum for them. Sampling these organisms from the seafloor to the surface might cause inevitable stress because of the changes in the ambient environment. Hence, conventionally obtained faunal samples can hardly reflect their natural physiological and biochemical status as they were inhabiting the deep sea. It is worth mentioning that some researchers realized this issue and tried to obtain *in situ* fixed deep-sea samples and advocated for the utilization of *in situ* fixed deep-sea samples in their studies (Chen et al., 2021; Gao et al., 2019; Mat et al., 2020; Motoki et al., 2020; Sanders et al., 2013; Sun et al., 2020; Wang et al., 2019b; Watsuji et al., 2014; Wei et al., 2020). Nevertheless, studies that explicitly compare the biological difference between *in situ* fixed and conventional sampled deep-sea fauna are still lacking.

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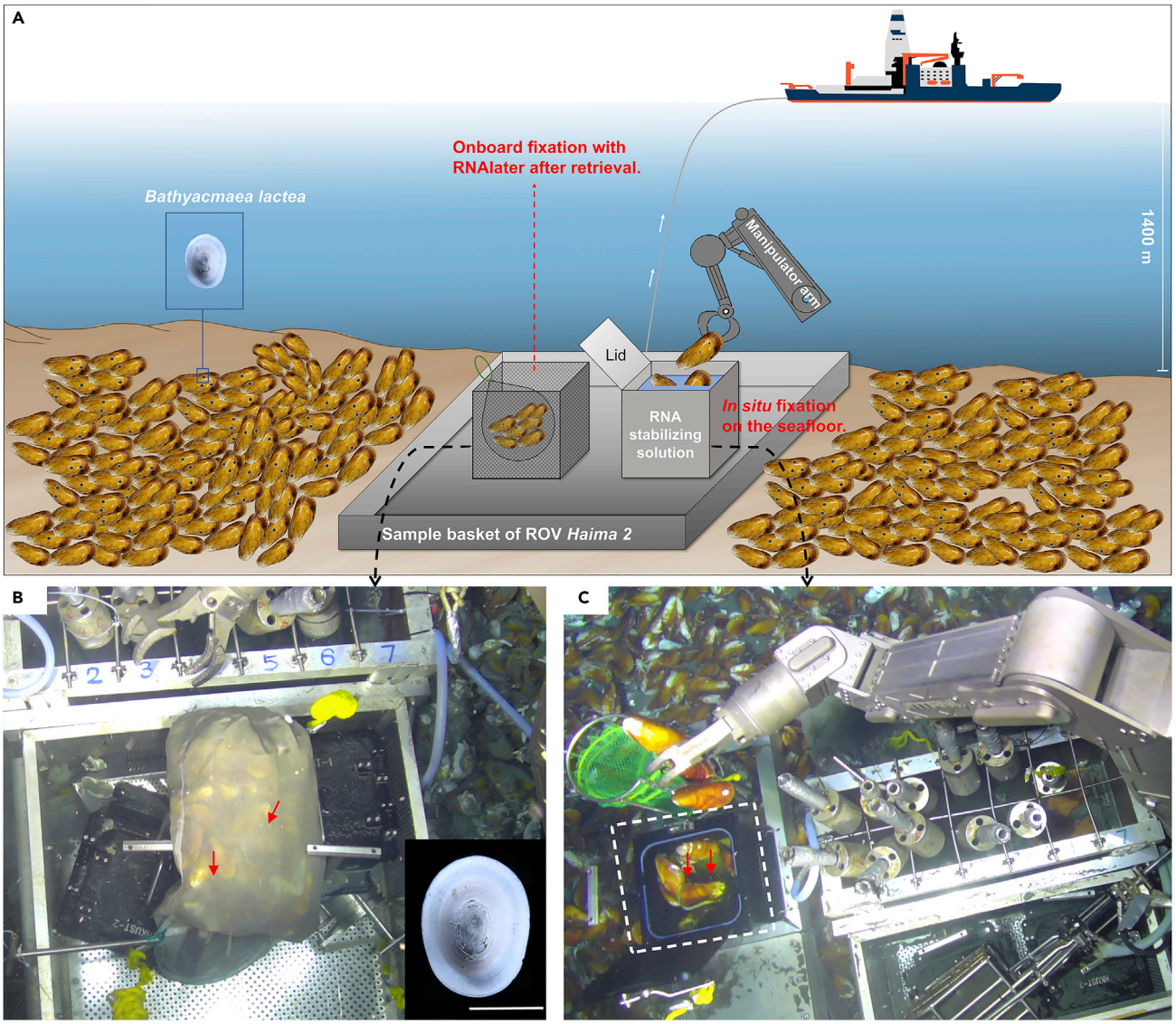

Figure 1. Pictures showing the two sampling methods applied in this study

(A) Schematic of the two sampling methods.

(B) An on-site photograph of the tuck net filled with the deep-sea limpet *Bathyacmaea lactea* attached to the mussel shells (indicated with red arrows). Inset: dorsal view of a *B. lactea* specimen. Scale bar, 0.5 cm.

(C) An on-site photograph of the *in situ* fixation. *Bathyacmaea lactea* attached on mussel shells (indicated with red arrows) were fallen off and *in situ* fixed in the sampling chamber (indicated with a white dotted box) fully filled with in-house RNA stabilizing solution.

The cold seep is a typical chemosynthetic ecosystem in the deep sea, where reduced sulfur compounds and methane emanate from the seafloor to the water (Feng et al., 2018; Levin, 2005). In addition to the general deep-sea environmental stressors mentioned earlier, the organisms inhabiting cold seeps also need to cope with extra stressors, such as low oxygen and potentially harmful substances in the seepage fluid (McMullin et al., 2000). The deep-sea patellogastropod limpet *Bathyacmaea lactea* is one of the dominant macrobenthos in the Haima cold seep in the South China Sea (Liu et al., 2020), and no evidence of harboring endosymbionts in *B. lactea* has been reported until now. Given its small size and open-shell structure, *B. lactea* is relatively easier to be *in situ* fixed during deep-sea sampling than the others because it does not need to be cracked. In this study, two methods were used for sampling this limpet species (Figure 1): the conventional onboard fixation method in which samples are fixed on board by RNA stabilizing solution after retrieval and the *in situ* fixation method in which the samples are fixed on the seafloor

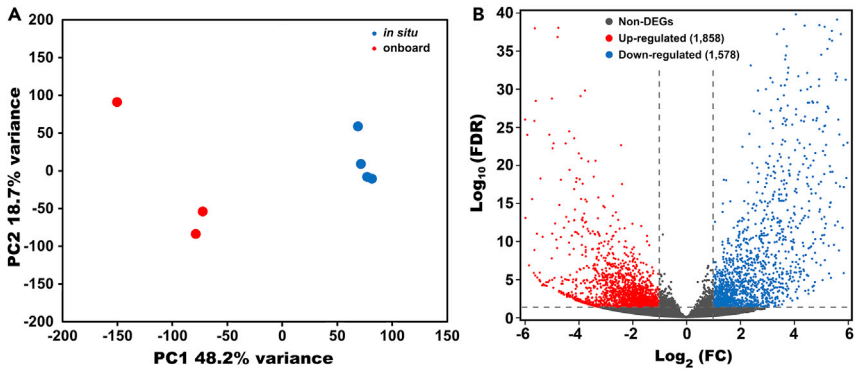

Figure 2. Comparative transcriptomic analysis of the *in situ* and onboard fixed groups

(A) Principal component analysis (PCA) result based on gene expression levels of four *in situ* and three onboard fixed individuals. Significance of PCA was examined by the PERMANOVA analysis on Bray-Curtis dissimilarities (p value = 0.027).

(B) A volcano plot showing the relationship between false discovery rate (FDR) and fold change (FC). Red spots indicate upregulated transcripts in the onboard fixed group, and blue spots indicate downregulated transcripts in the onboard fixed group.

before suffering sampling stress. Further comparative transcriptomic analysis was performed on the limpet samples fixed with different methods to investigate the influences of deep-sea sampling stress on their gene expression at the transcriptional level.

RESULTS AND DISCUSSION

Transcriptome sequencing, assembly, and annotation

A total of 27,674 nonredundant transcripts with predicted open-reading frames were obtained by using 648,458,172 reads from seven individuals of *B. lactea* for transcriptome assembly, with an N50 value of 3610 bp (Table S1). The Benchmarking Universal Single-Copy Orthologs (BUSCO) assessment results show 98% completeness (single copy: 84.1% and duplicated: 13.9%) for the predicted transcripts. The functional annotation of the transcripts indicated that 23,327 transcripts had hits to the NCBI nonredundant (NR) database; 17,160 to the EggNOG database; 16,462 to Gene Ontology (GO) items; and 8424 to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure S1). To the best of our knowledge, this is the first report on the *in situ* transcriptome of *B. lactea*.

Differential gene expression analysis between the *in situ* and onboard fixed samples

The expression level of each transcript was quantified in seven individuals. The principal component analysis (PCA) result shows a clear separation between the *in situ* fixed and onboard fixed groups along PC1, explaining the 48.2% variance (Figure 2A). Although variability also exists among replicates of the same group, we consider it acceptable, as these samples grew in a volatile wild environment that might result in individual differences. Moreover, replicates among the onboard fixed group showed more variability than that of *in situ* fixed group (Figure 2A), possibly because of having suffered the sampling stress.

Differential gene expression analysis was performed between the two groups to investigate the impacts of sampling stress during retrieval from the seafloor on the gene expression of cold seep-adapted limpets. A total of 3,436 (12.4%) differentially expressed genes (DEGs) were identified, with 1,858 upregulated and 1,578 downregulated in the onboard fixed limpets compared with *in situ* fixed ones (Figure 2B). This result indicated that sampling stress indeed led to dramatic changes in the gene expression of the deep-sea limpets during the ~80 min sampling process. During the conventional deep-sea sampling process, the samples were exposed to the ambient environment during retrieval from the seafloor to the surface. As a result, the samples suffered from decreased hydrostatic pressure, increased water temperature, increased dissolved oxygen, and decreased salinity (Figure S2). Other environmental parameters, such as hydrogen sulfide, metal ion, and pH, might also have changed. In a recent study conducted under laboratory conditions, few (< 0.1%) DEGs were identified in the shallow-water sea cucumber *Apostichopus japonicus* after the hydrostatic pressure changed in the first hour (Chen et al., 2020), and in another study conducted in a cold seep, approximately 40 min of decompression from the 1,119 m depth resulted in 337 DEGs in the gill

Table 1. GO enrichment analysis of the upregulated transcripts in the onboard fixed group

GO ID	GO description	Category	FDR
GO:0005198	Structural molecule activity	Molecular Function	7.86E-7
GO:0003735	Structural constituent of ribosome	Molecular Function	7.53E-4
GO:0022857	Transmembrane transporter activity	Molecular Function	7.69E-4
GO:0005215	Transporter activity	Molecular Function	7.69E-4
GO:0005840	Ribosome	Cellular Component	7.69E-4
GO:0032501	Multicellular organismal process	Biological Process	4.96E-3
GO:0006518	Peptide metabolic process	Biological Process	6.07E-3
GO:0006412	Translation	Biological Process	6.07E-3
GO:1901566	Organonitrogen compound biosynthetic process	Biological Process	6.07E-3
GO:0034645	Cellular macromolecule biosynthetic process	Biological Process	6.07E-3
GO:1901576	Organic substance biosynthetic process	Biological Process	6.07E-3
GO:0044249	Cellular biosynthetic process	Biological Process	6.07E-3
GO:0043043	Peptide biosynthetic process	Biological Process	6.07E-3
GO:0043604	Amide biosynthetic process	Biological Process	6.07E-3
GO:0043603	Cellular amide metabolic process	Biological Process	6.07E-3
GO:0009059	Macromolecule biosynthetic process	Biological Process	6.07E-3
GO:0044271	Cellular nitrogen compound biosynthetic process	Biological Process	6.07E-3
GO:0043228	Nonmembrane-bounded organelle	Cellular Component	9.89E-3
GO:0043232	Intracellular nonmembrane-bounded organelle	Cellular Component	9.89E-3
GO:0005576	Extracellular region	Cellular Component	9.89E-3
GO:0050877	Nervous system process	Biological Process	1.73E-2
GO:0003008	System process	Biological Process	1.98E-2
GO:0005886	Plasma membrane	Cellular Component	4.58E-2
GO:0016020	Membrane	Cellular Component	4.58E-2

of the deep-sea mussel *Gigantidas platifrons* (formerly "*Bathymodiolus*" *platifrons*) (Chen et al., 2021). Therefore, we speculate that the large number of DEGs identified in this study might be induced by combined factors rather than a single factor during a sampling process of only ~80 min.

Effect of the sampling stress caused differential gene expression on the deep-sea limpets

GO and KEGG enrichment analyses were performed on the identified DEGs to discover the biological activities and metabolic pathways influenced by the DEGs induced by sampling stress. GO enrichment analysis of the upregulated DEGs in the onboard fixed group identified 24 significantly enriched GO terms, including structural molecule activity (GO:0005198), transmembrane transporter activity (GO:0022857), ribosome (GO:0005840), translation (GO:0006412), cellular biosynthetic process (GO:0044249), and plasma membrane (GO:0005886) (Table 1). GO enrichment analysis of the downregulated DEGs in the onboard fixed group resulted in 27 GO terms, including catalytic activity (GO:0003824), hydrolase activity (GO:0016787), peptidase activity (GO:0008233), small molecule metabolic process (GO:0044281), lipid metabolic process (GO:0006629), and lysosome (GO:0005764) (Table 2). KEGG enrichment analysis of all the DEGs identified 14 significantly enriched pathways, which were involved in transport and catabolism (lysosome [ko04142]); glycan biosynthesis and metabolism (glycosaminoglycan degradation [ko00531], other glycan degradation [ko00511]); carbohydrate metabolism (pentose and glucuronate interconversions [ko00040], amino sugar and nucleotide sugar metabolism [ko00520]); amino acid metabolism (histidine metabolism [ko00340], glycine, serine, and threonine metabolism [ko00260]); digestive system (protein digestion and absorption [ko04974]); cell growth and death (ferroptosis [ko04216]); lipid metabolism (biosynthesis of unsaturated fatty acids [ko01040]); endocrine system (PPAR signaling pathway [ko03320], renin-angiotensin system [ko04614]); and metabolism of cofactors and vitamins (one carbon pool by folate [ko00670], retinol metabolism [ko00830]) (Figure 3). Many affected biological functions

Table 2. GO enrichment analysis of downregulated transcripts in the onboard fixed group

GO ID	GO description	Category	FDR
GO:0003824	Catalytic activity	Molecular Function	1.47E-34
GO:0016798	Hydrolase activity, acting on glycosyl bonds	Molecular Function	5.55E-24
GO:0016491	Oxidoreductase activity	Molecular Function	4.48E-23
GO:0016787	Hydrolase activity	Molecular Function	2.04E-21
GO:0005975	Carbohydrate metabolic process	Biological Process	6.83E-15
GO:0008233	Peptidase activity	Molecular Function	1.23E-12
GO:0140096	Catalytic activity, acting on a protein	Molecular Function	1.23E-12
GO:0044281	Small molecule metabolic process	Biological Process	1.13E-11
GO:0006082	Organic acid metabolic process	Biological Process	5.24E-10
GO:0006520	Cellular amino acid metabolic process	Biological Process	5.24E-10
GO:0043436	Oxoacid metabolic process	Biological Process	5.24E-10
GO:0005773	Vacuole	Cellular Component	5.24E-10
GO:0019752	Carboxylic acid metabolic process	Biological Process	5.24E-10
GO:0005576	Extracellular region	Cellular Component	1.60E-9
GO:0022857	Transmembrane transporter activity	Molecular Function	9.62E-9
GO:0005215	Transporter activity	Molecular Function	9.62E-9
GO:0006629	Lipid metabolic process	Biological Process	2.85E-8
GO:0005764	Lysosome	Cellular Component	1.45E-7
GO:0000323	Lytic vacuole	Cellular Component	1.45E-7
GO:0016810	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	Molecular Function	4.89E-5
GO:0009056	Catabolic process	Biological Process	5.04E-5
GO:0006790	Sulfur compound metabolic process	Biological Process	1.24E-4
GO:0005615	Extracellular space	Cellular Component	6.00E-4
GO:0016829	Lyase activity	Molecular Function	6.23E-3
GO:0071704	Organic substance metabolic process	Biological Process	1.26E-2
GO:0044238	Primary metabolic process	Biological Process	1.26E-2
GO:0043167	Ion binding	Molecular Function	1.26E-2

and metabolic pathways were identified, suggesting that the sampling stress during retrieval from the seafloor exerted a systemic influence on the vital activity of the sampled deep-sea limpets.

Cell and tissue structure

GO enrichment analysis showed that cell structure maintenance, transmembrane transport, and biosynthetic process-related activities were upregulated (Table 1), whereas catabolism- and metabolic-process-related activities were downregulated potentially because of the sampling stress (Table 2). Collagen is the main structural protein in the extracellular matrix of diverse connective tissues, playing important roles in tissue morphogenesis and the maintenance of tissue structural integrity (Gelse et al., 2003). Previous studies uncovered that some collagens are positively selected in the deep-sea fish *Aldrovandia affinis* (Lan et al., 2018) and the deep-sea alvinocaridid shrimp *Shinkaicaris leurokolos* (Zhu et al., 2020), suggesting their potential roles in deep-sea adaptation. Herein, 86 collagen-encoding transcripts were identified, with 19 upregulated and 4 downregulated in the onboard fixed group (Table S2). The alteration of the expression levels of such a large number of collagen-encoding transcripts suggests that sampling stress, especially the pressure and temperature variations, will likely have an impact on the cell and tissue structure.

Lysosomal activity and fluid balance

KEGG enrichment analysis indicated that lysosome (ko04142) was the most affected pathway (Figure 3), implying that lysosomal activity was greatly altered in the cell of the limpets that suffered from sampling

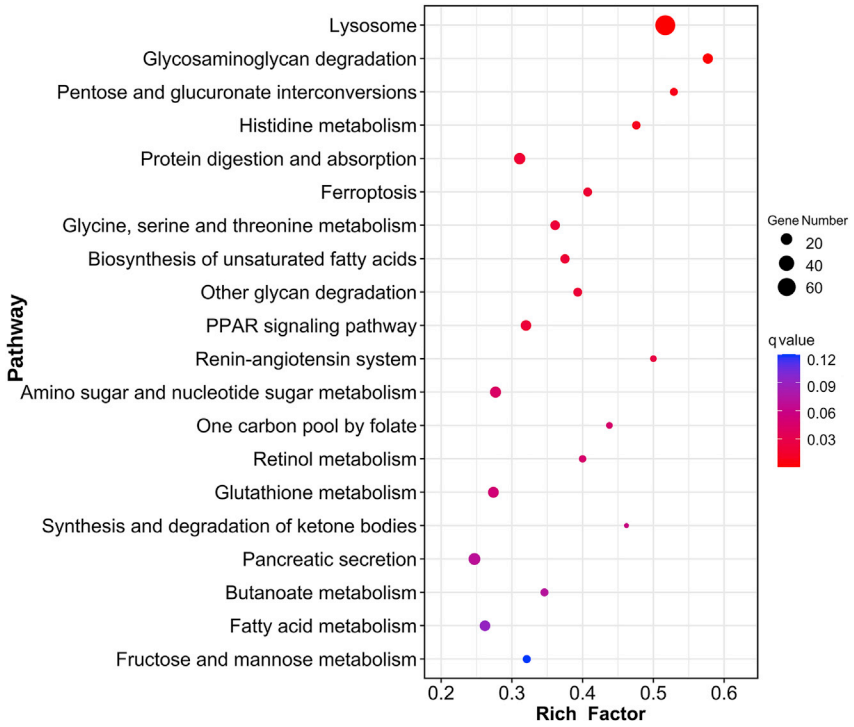

Figure 3. A bubble diagram showing the enriched pathways obtained in the KEGG enrichment analysis of all the differentially expressed genes between the *in situ* and onboard fixed groups

stress. Lysosome (ko04142) was also significantly enriched in the enrichment analysis of the downregulated DEGs (Figure S3A), suggesting that the lysosomal activity might be downregulated in onboard fixed limpets. In addition, the renin-angiotensin system (ko04614) pathway, which is mainly engaged in osmoregulation, was also significantly enriched (Figure 3), suggesting that the sampling stress might perturb the fluid balance of the deep-sea limpets (Salzet et al., 2001), which would be caused by decompression-associated osmotic pressure change or by dehydration when the remotely operated underwater vehicle (ROV) was retrieved on board (Nobata et al., 2013; Yancey et al., 2014).

Unsaturated fatty acid metabolism

High hydrostatic pressure and low temperature are the key limiting factors in the colonization of deep-sea organisms (Brown and Thatje, 2014). Increased hydrostatic pressure and decreased temperature can reduce the fluidity of biological membranes, which are mainly composed of lipid bilayers and various proteins, leading to their disfunction (Balny et al., 2002; Hazel, 1995; Kato et al., 2002; Marques et al., 2003). Many deep-sea organisms are known to rely on a large proportion of unsaturated fatty acids to cope with high hydrostatic pressure and low-temperature-caused rigidity of the membranes (Parzanini et al., 2018; Van Campenhout et al., 2016; Wang et al., 2019a). Deep-sea vent shrimps exposed to atmospheric pressure exhibit a lower level of unsaturated fatty acids than those kept under natural high pressure (Shillito et al., 2020). When the shallow-water amphipod *Eogammarus possjeticus* was exposed to high pressure, the expression of fatty acid desaturase and the elongation of the very long-chain fatty acids protein (ELOVL) involved in the production of unsaturated fatty acids increased (Chen et al., 2019b). As the biosynthesis of unsaturated fatty acids (ko01040) pathway regulating the production of unsaturated fatty acids was significantly enriched from all the DEGs (Figure 3), and the fatty acid metabolism (ko01212) pathway was significantly enriched from the downregulated DEGs as well (Figure S3A), the expression of fatty acid desaturase and fatty acid elongation protein was investigated. Four fatty acid desaturase-encoding transcripts and three fatty acid elongation protein-encoding transcripts were downregulated in the onboard fixed group (Figure 4A, Table S3), indicating that the biosynthesis of unsaturated fatty acids in the deep-sea limpets decreased during retrieval, which might have been caused by the decreased hydrostatic pressure along with the increased ambient temperature during the sampling process.

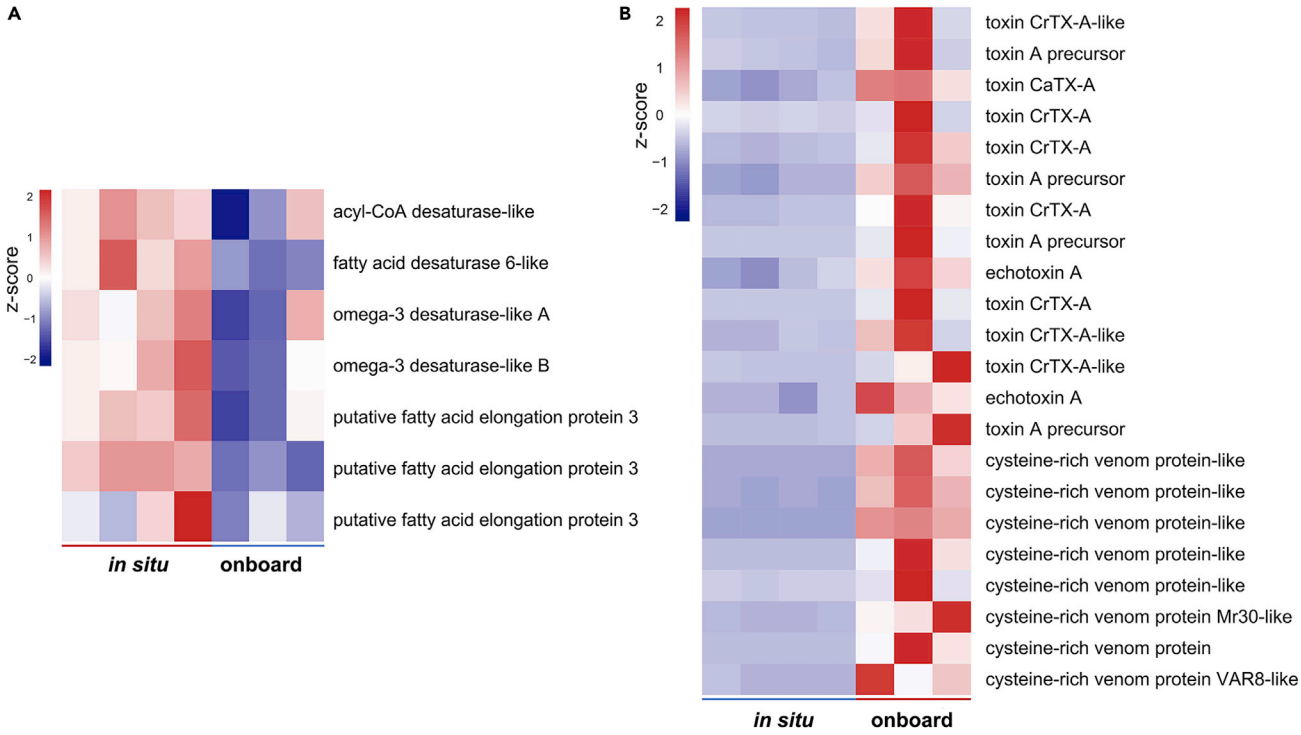

Figure 4. Heatmaps showing the expression pattern of selected transripts

(A) Downregulated fatty acid desaturase- and fatty acid elongation protein-encoding transcripts. (B) Upregulated toxin and cysteine-rich venom protein-encoding transcripts.

Chemical defense reactions

Many marine animals, such as squids release secretions in defense against predator attacks (Wood et al., 2010), and sea anemones maintain their venom quality and quantity when suffering from environmental stressors (Hoepner et al., 2019). Among all the DEGs identified between the onboard and *in situ* fixed groups, 14 toxin-encoding transcripts and eight cysteine-rich venom protein-encoding transcripts were significantly upregulated during the retrieval process of deep-sea limpets from the seafloor (Figure 4B, Table S4). We speculated that sampling stress might also trigger their chemical defense reactions, resulting in the increased expression of toxin- and cysteine-rich venom protein-encoding transcripts. These reactions might be mediated by pathways involved in neural signal transduction, such as neuroactive ligand-receptor interaction (ko04080) and calcium signaling pathway (ko04020), which were significantly enriched from the upregulated DEGs (Figure S3B).

Insights into deep-sea adaptation through the *in situ* fixed limpet transcriptome

The highly expressed transcripts of a transcriptome are likely to play decisive roles. The top 10% most abundant transcripts (top 2,767 transcripts ranked by transcripts per million [TPM] values) of the *in situ* fixed *B. lactea* transcriptome were applied to the KEGG enrichment analysis to investigate the pathways involved in their environmental adaptation to the seep habitat. Results showed that 19 pathways were significantly enriched, several of which have been reported to play potential roles in deep-sea adaptation, such as lysosome (ko04142), metabolism of xenobiotics by cytochrome P450 (ko00980), glutathione metabolism (ko00480), and fatty acid elongation (ko00062) (Figure 5A).

Lysosome (ko04142) is the most significantly enriched pathway in the KEGG enrichment analysis of the top 10% most abundant transcripts in the *in situ* fixed *B. lactea* transcriptome (Figure 5A). A total of 64 transcripts mapped to lysosome (ko04142), including lysosomal acid hydrolases, lysosomal membrane proteins, other lysosomal enzymes and activators, mannose-6-phosphate receptor, clathrins, adaptor protein complex 3 (AP-3), and V- ATPase, were highly expressed (Figure 5B and Table S5), suggesting high lysosomal activity in the limpet living in the natural cold seep environment. High lysosomal activity

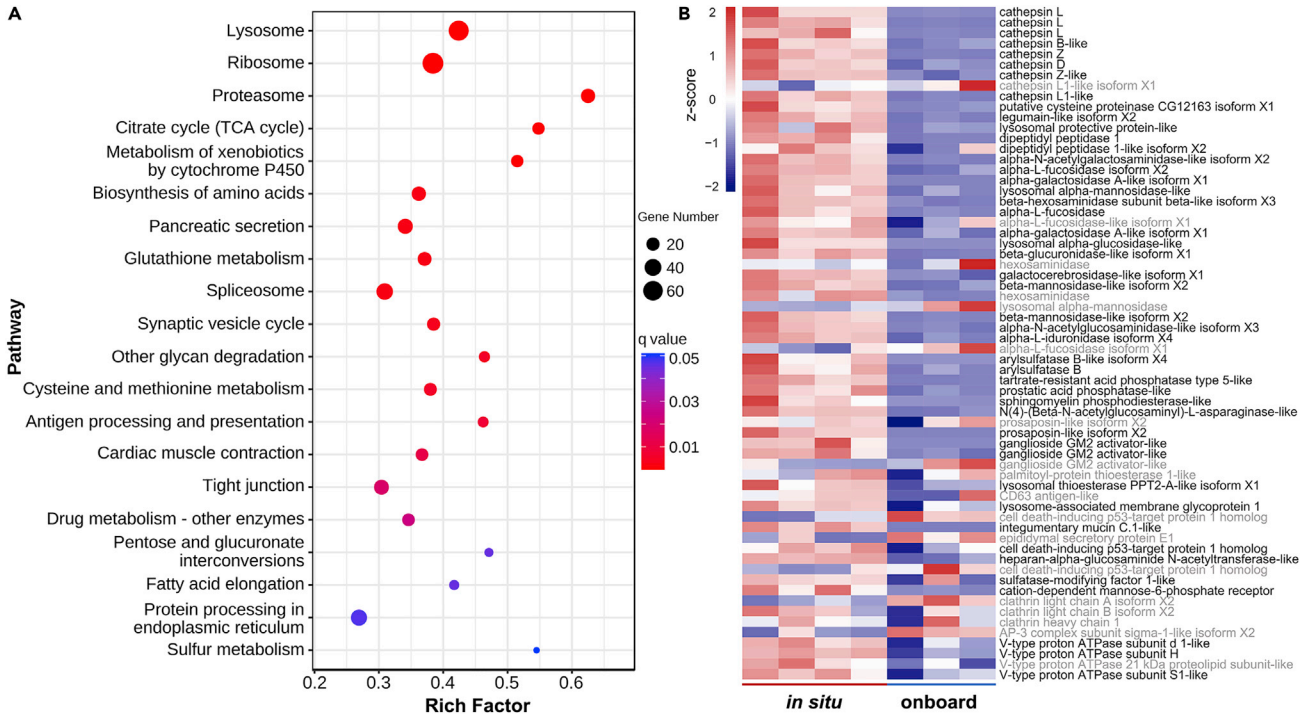

Figure 5. KEGG enrichment analysis of the top 10% most abundant transcripts in the *in situ* fixed limpet transcriptome

(A) Bubble diagram showing the enriched pathways of the top 10% most abundant transcripts.

(B) Heatmap showing the expression pattern of the top 10% highly expressed transcripts mapped to the lysosome (ko04142) pathway. Differentially expressed transcripts are in black and those not are in gray.

has been reported in the symbiotic organs of several cold-seep symbiotic species, including mussels (Yu et al., 2019; Zheng et al., 2017), clams (Ip et al., 2021; Lan et al., 2019), and tubeworms (Sun et al., 2021), and was deduced to digest the symbionts in the specialized bacteriocytes for nutrition and to control the symbiont populations as well. Nevertheless, the limpets of *Bathyacmaea* are considered to mainly graze the bacterial film on the substrates they attached to (i.e., mainly mussel shells) for nutrition (Chen et al., 2019a; Liu et al., 2020), implying that the high lysosomal activity detected in *B. lactea* might play different roles. However, 45 of the 63 highly expressed transcripts were significantly downregulated in the onboard fixed group (Figure 5B), indicating that the role of high lysosomal activity might be interrupted by sampling stress, which is consistent with the result that lysosome (ko04142) was the most significantly enriched pathway in the KEGG enrichment analysis of all the DEGs (Figure 2A) and downregulated DEGs (Figure S3A). Lysosomes are ubiquitous cellular organelles known as the waste disposal system involved in the degradation and recycling of the cellular waste derived from both extracellular and intracellular regions, and growing evidence shows that they are also involved in many other cellular processes, including secretion, metabolic signaling, plasma membrane repair, and response to environmental cues, playing vital roles in maintaining cellular and organismal homeostasis (Ballabio and Bonifacino, 2020; Settembre et al., 2013).

In cold seeps, stressful environmental conditions might increase the production of intracellular waste (e.g., damaged organelles, oxidized lipids, and misfolded proteins) and the encounter with the pathogens of local organisms. Therefore, the high lysosomal activity would be conducive to increasing the efficiency of cellular clearance and reusage of the breakdown products for imperative nutritional needs. We assume that the high lysosomal activity might be a normalcy in the cold-seep-adapted nonsymbiotic invertebrates, which is a universal strategy for maintaining cellular and organismal homeostasis under the harsh environmental conditions of seep areas. However, various stressors can influence the function of lysosomes. For example, chemical contaminant treatments significantly decrease the lysosomal stability in molluscan hepatopancreas (Shaw et al., 2019), and the acute thermal stress on the deep-sea sponge holobiont causes significant lysosomal destabilization (Strand et al., 2017). Deep-sea sampling stress may have perturbed the lysosomal stability of the deep-sea limpets and thus decreased the lysosomal activity.

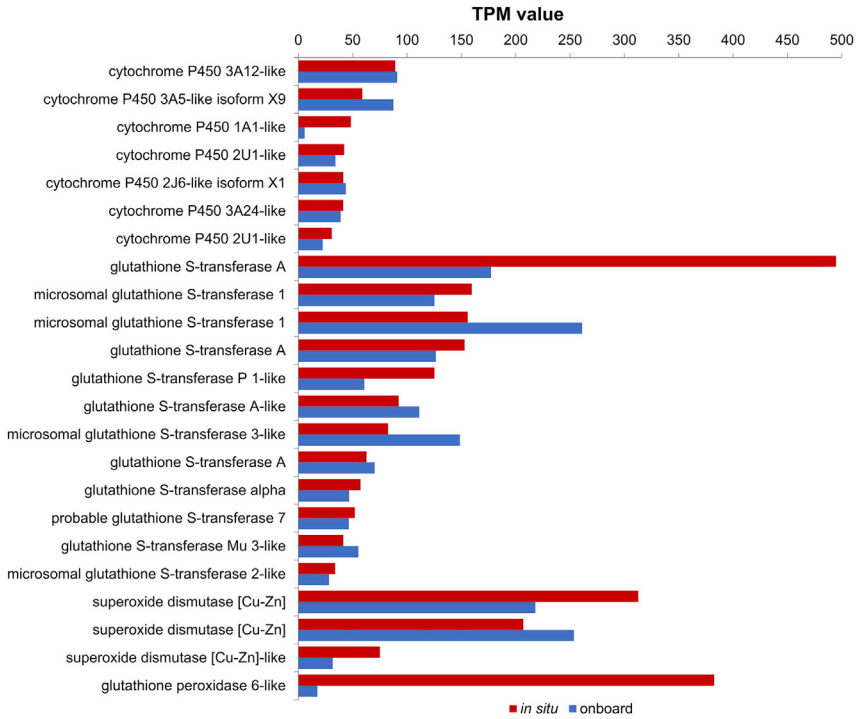

Figure 6. A histogram showing the expression levels of transcripts involved in xenobiotic detoxification of the *in situ* and onboard fixed groups

TPM: transcripts per million.

In the Haima cold seep, *B. lactea* usually attaches to the shells of the bathymodioline mussel *Gigantidas haimaensis* harboring methane-oxidizing endosymbionts, which makes use of methane emitted from the seafloor as the energy resource (Xu et al., 2019). Under such a condition, these limpets are also exposed to methane along with other toxic substances from their ambient environment and thus need to evolve suitable strategies for detoxification. Considering that the metabolism of xenobiotics by cytochrome P450 (ko00980) and glutathione metabolism (ko00480) pathways were significantly enriched from the top 10% most abundant transcripts, we investigated the expression of the mixed-function oxygenase (MFO) system components cytochrome P450 (CYP), conjugating enzyme glutathione S-transferase (GST), antioxidant enzymes superoxide dismutase (SOD), and glutathione peroxidase (GPX) (Lee, 1981; Ramos and Garcia, 2007; Xiao et al., 2020). Seven transcripts of CYP, twelve transcripts of GST, three transcripts of SOD, and one transcript of GPX ranked in the top 10% (Figure 6 and Table S6), indicating high MFO system and antioxidant enzyme activities in the limpet *B. lactea*, which might be responsible for the xenobiotic detoxification.

Conclusions

In this study, we successfully sampled the *in situ* fixed deep-sea limpet *B. lactea* and obtained its first *in situ* transcriptome. Comparative transcriptomic analysis of the *in situ* and onboard fixed samples revealed that the concomitant stress during conventional deep-sea sampling without *in situ* fixation affected their gene expression. Furthermore, sampling stress exerted systemic influences on the life of the sampled deep-sea limpets by perturbing the cell and tissue structure, lysosomal activity, fluid balance, and unsaturated fatty acid metabolism. These findings reveal that conventionally sampled samples from deep sea without RNA *in situ* fixation might lead to biased results in transcriptomic analyses and suggest that *in situ* fixed deep-sea samples are highly demanded for mRNA quantitative-analysis-based studies.

Limitations of the study

Due to the small size (0.5–0.8 cm) of *B. lactea* we collected, the whole body of each individual, rather than the dissected tissues, was used for transcriptome sequencing and downstream data analyses. This sampling strategy may bias the expression levels of some genes that actually exhibit opposite expression

patterns in different tissues. Nevertheless, we consider such impacts to be limited and would not influence the main findings of this work.

STAR★METHODS

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- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Gene expression analysis
  - Enrichment analyses

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104092.

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AUTHOR CONTRIBUTIONS

P-YQ and JS conceived this project. GY and TX collected the samples. GY and YL performed the bioinformatics analyses and drafted the manuscript. TW helped to prepare the figures. All authors contributed to the manuscript writing and approved it for submission and publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ammonium sulfate	Xilong Scientific	Cat#12600901
EDTA	Solarbio Life Sciences	Cat#E8040-100g
Sodium citrate	Xilong Scientific	Cat#10201601
TRIzol	Thermo Fisher Scientific	Cat#15596018
RNAlater	Thermo Fisher Scientific	Cat#AM7021
Deposited data		
Raw RNA-seq data of six limpet *Bathyacmaea lactea*	This study	NCBI under BioProject PRJNA765439
Software and algorithms		
Trimmomatic version 0.39	Bolger et al. (2014)	N/A
Trinity version 2.8.5	Grabherr et al. (2011)	N/A
Salmon version 1.2.1	Patro et al. (2017)	N/A
TransDecoder version 5.5.0	https://github.com/TransDecoder/TransDecoder/wiki	N/A
CD-HIT version 4.8.1	Fu et al. (2012)	N/A
BUSCO version 3.0.2	Waterhouse et al. (2018)	N/A
OmicsBox version 1.4.11	Biobam	N/A
RNA-seq 2G	Zhang et al., 2017	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pei-Yuan Qian (boqianpy@ust.hk).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability

- All raw sequencing data and the transcriptome assembly of *B. lactea* were deposited to NCBI under BioProject PRJNA765439.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study was based on wild deep-sea limpet *B. lactea*, and no experimental models were used.

METHOD DETAILS

Deep-sea sampling and fixation

Limpets of *B. lactea* were collected from a single colony in the Haima cold seep (~1400 m depth) in South China Sea by the ROV *Haima 2* onboard the R/V *Haiyangdizhi6* during the HYDZ6-202005 cruise in August 2020. CTD (Sea-Bird, Bellevue, WA, USA) data showed that the seawater temperature increased from 3.0°C to 30.7°C, the dissolved oxygen increased from 1.8 mg/L to 5.2 mg/L, and the salinity changed from 34.6 to 33.4 at the sampling site (Figure S2). Two methods were used for sample fixation. 1) Onboard fixation: *G. haimaensis* mussels attached

with limpets were wrapped up with a tuck net held by the ROV manipulator arm and placed into the sample basket of the ROV (Figure 1B). The tuck net was transferred into the laboratory on board after the ROV was retrieved, and the limpets were immediately fixed by RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and frozen with liquid nitrogen after 4°C overnight. The sampling process from the seafloor to the laboratory took approximately 80 min 2) *In situ* fixation: *G. haimaensis* mussels attached with limpet *B. lactea* were cracked slightly by the ROV manipulator arm before they were placed into the sampling chamber fully filled on board with ~12 L in-house RNA stabilizing solution to preserve their RNA *in situ* (Figure 1C). The chamber was sealed by closing the lid and returned to the sample basket of the ROV. After the ROV was retrieved on board, the sampling chamber was transferred to the lab on board. The *in situ* fixed limpets were transferred to the RNAlater (Thermo Fisher Scientific, USA) immediately and frozen with liquid nitrogen after 4°C overnight. All the limpets were stored at −80°C until usage. The in-house RNA stabilizing solution was prepared as previously described (Mat et al., 2020) with 700 g of ammonium sulfate, 40 mL of 0.5 M EDTA, 25 mL of 1 M sodium citrate, and 935 mL of distilled water; the pH was adjusted to 5.2, and the solution was stored in a 4°C cold room in the ship before the ROV dive.

RNA extraction and sequencing

Four individuals of *B. lactea* with *in situ* fixation and three with onboard fixation were used in this study. The whole body tissue of each individual was used for RNA extraction using TRIzol Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. The quality and quantity of the extracted RNA were measured by the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The mRNA of each individual was enriched by Oligo-dT probes and used for cDNA synthesis and eukaryotic library construction. All cDNA libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to yield paired-end reads with a length of 150 bp in Novogene (Beijing, China).

Transcriptome assembly and annotation

Adaptors and low-quality bases of the raw reads were trimmed by Trimmomatic version 0.39 (Bolger et al., 2014) with the following setting: "ILLUMINACLIP: TruSeq3-PE-2.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:36". The obtained clean reads of all the individuals were used for the *de novo* assembly of the transcriptome by Trinity version 2.8.5 (Grabherr et al., 2011). Salmon version 1.2.1 (Patro et al., 2017) was used to quantify the expression of each assembled transcript. The transcripts with a transcript per million value below 0.1 were removed. TransDecoder version 5.5.0 (https://github.com/TransDecoder/TransDecoder/wiki) was used to predict the open reading frame of the transcripts. Potential isoforms of the protein sequences were removed using CD-HIT version 4.8.1 (Fu et al., 2012) with c set to 0.95. The completeness of the assembled transcriptome was assessed using BUSCO version 3.0.2 to search against the metazoa_odb10 database (Waterhouse et al., 2018). The predicted protein sequences were used for functional annotation by searching their predicted protein sequences against the NCBI Non-Redundant (NR) databases using BLASTp version 2.10.0+ with an *E*-value cut-off of 1×10^{-5}, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via KEGG Automatic Annotation Server (KAAS), and the Gene Ontology (GO) via OmicsBox version 1.4.11 (BioBam, Valencia, Spain).

QUANTIFICATION AND STATISTICAL ANALYSIS

Gene expression analysis

Differential expression analysis was performed between four *in situ* and three onboard fixed limpets using DESeq2 (Love et al., 2014) implemented in RNA-seq 2G (Zhang et al., 2017) on the basis of the mapped reads count. Genes with a significant false discovery rate (FDR) value less than 0.05 and a fold change larger than two were identified as DEGs. The script "abundance_estimates_to_matrix.pl" implemented in Trinity (Grabherr et al., 2011) was used to generate a matrix of TMM-normalized expression values. This matrix was used for PCA by Past version 4.03 (Hammer et al., 2001). The significance of the comparison in PCA was further examined using PERMANOVA on a Bray-Curtis dissimilarity matrix (implemented in Past version 4.03) calculated for TMM-normalized expression values.

Enrichment analyses

The GO terms of the DE-Gs were enriched using the Fisher Exact Test implemented in OmicsBox 1.4.11 (BioBam). The KEGG pathways were enriched with the cumulative hypergeometric distribution method implemented in OmicShare online tool version 6.3.0 (http://www.omicshare.com/tools).