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RESEARCH ARTICLE

# Membrane lipid metabolism, heat shock response and energy costs mediate the interaction between acclimatization and heat-hardening response in the razor clam *Sinonovacula constricta*

**Wenyi Zhang[1,2] and Yunwei Dong[3,4,*]**

## ABSTRACT

Thermal plasticity on different time scales, including acclimation/acclimatization and heat-hardening response – a rapid adjustment for thermal tolerance after non-lethal thermal stress, can interact to improve the resilience of organisms to thermal stress. However, little is known about physiological mechanisms mediating this interaction. To investigate the underpinnings of heat-hardening responses after acclimatization in warm seasons, we measured thermal tolerance plasticity, and compared transcriptomic and metabolomic changes after heat hardening at 33 or 37°C followed by recovery of 3 or 24 h in an intertidal bivalve *Sinonovacula constricta*. Clams showed explicit heat-hardening responses after acclimatization in a warm season. The higher inducing temperature (37°C) caused less effective heat-hardening effects than the inducing temperature that was closer to the seasonal maximum temperature (33°C). Metabolomic analysis highlighted the elevated content of glycerophospholipids in all heat-hardened clams, which may help to maintain the structure and function of the membrane. Heat shock proteins (HSPs) tended to be upregulated after heat hardening at 37°C but not at 33°C, indicating that there was no complete dependency of heat-hardening effects on upregulated HSPs. Enhanced energy metabolism and decreased energy reserves were observed after heat hardening at 37°C, suggesting more energy costs during exposure to a higher inducing temperature, which may restrict heat-hardening effects. These results highlight the mediating role of membrane lipid metabolism, heat shock responses and energy costs in the interaction between heat-hardening response and seasonal acclimatization, and contribute to the mechanistic understanding of evolutionary change and thermal plasticity during global climate change.

**KEY WORDS: Climate change, Heat shock protein, Intertidal bivalve, Membrane lipids, Thermal plasticity**

## INTRODUCTION

Ongoing global climate change, including increasing average temperature and more frequent extreme hot weather, is impacting species' survival and biogeographic distribution (IPCC, 2014). Thermal plasticity, such as long-term acclimation/acclimatization and short-term heat-hardening response, is important for organisms to reduce the overheating risk and delay extinction in the face of global warming (Deery et al., 2021). Acclimation or acclimatization refers to the slow adjustment of thermal tolerance during exposure to a particular temperature in the laboratory or to temperature variation in the field, and the heat-hardening response provides a thermal buffer for organisms after exposure to non-lethal thermal stress (Bowler, 2005). Understanding how thermal plasticity affects thermal tolerance and therein molecular mechanisms is an important step in developing a mechanistic basis for adaptive and evolutionary responses of organisms to the warming Earth. Acclimation/acclimatization and the short-term heat-hardening response can interact on multiple scales to improve the thermal tolerance of organisms in warm conditions. For example, the tropical populations of *Drosophila melanogaster* showed a greater heat-hardening response (Sgrò et al., 2010). Empirical evidence suggests that the magnitude of the heat-hardening response depends on the predictability of temperature variation and maximum temperature during long-term acclimatization (Phillips et al., 2016; Zhang et al., 2021). To further understand how acclimation/acclimatization and the heat-hardening response work together to improve thermal tolerance, we need to investigate the physiological and molecular mechanisms mediating these interactive processes.

It is unclear which physiological functions are activated by heat-hardening treatment to improve the thermal tolerance of organisms and the role of heat shock proteins (HSPs) in the interaction between acclimation/acclimatization and the heat-hardening response. The mechanisms underpinning heat hardening are not completely defined. HSPs, which can help to stabilize structures and maintain the functions of the cellular proteome, are considered as the key molecular players in the heat-hardening response (Evgen'Ev et al., 2014; Bilyk et al., 2012). For example, a positive correlation between HSP70 protein level and thermal tolerance following heat-hardening treatment was observed in an arthropod *Orhesella cincta* (Bahrndorff et al., 2009). However, similar results were not observed in the fly *D. melanogaster* (Jensen et al., 2010). After exposure to maximum critical temperature, upregulation of inducible HSP27 and HSP70 was also absent in the salamander *Eurycea bislineata* (Easton et al., 1987). These results argue against a necessary role of HSPs in the heat-hardening response and indicate that there are other mechanisms promoting this rapid thermal plasticity regardless of whether the HSP response is induced. Acclimation/acclimatization to warmer thermal environments can raise basal levels of HSPs in organisms to match the thermal stress experienced in the previous thermal history (Dietz and Somero, 1992; Maloyan et al., 1999). Therefore, in the interaction between acclimation/acclimatization and the

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**Received 16 June 2021; Accepted 6 September 2021**

heat-hardening response, the heat-hardening response induced by the thermal stress which can be predicted by the recent thermal history of the organism may depend on additional mechanisms and not completely on the induction of HSPs. However, the expression of HSPs can increase with elevated thermal stress and can indeed improve the thermal tolerance of most species (Solomon et al., 1991; Sørensen et al., 2001). In this way, greater thermal stress is expected to induce a more effective heat hardening because stronger thermal stress can result in higher levels of HSPs that can improve thermal tolerance. However, the response of HSPs carries huge energy costs (Krebs and Feder, 1998; Viant et al., 2003), and the adverse effects of energy consumption may affect the response of organisms to subsequent thermal stress, therefore altering heat-hardening effects. This may be predicted as a physiological constraint for the heat-hardening response.

The intertidal zone is an ideal system to explore the adaptive and evolutionary strategy of ectotherms in the context of global climate change because the temperature there fluctuates drastically in the daily or seasonal cycles (Brahim et al., 2019; Dong et al., 2017). Species living in the intertidal zone are vulnerable to high temperature and climate change because they live close to their upper thermal limits (Dong et al., 2017; Somero, 2010; Wang et al., 2020). The heat-hardening response can be observed in various species from the intertidal zone (Dong et al., 2010; Dunphy et al., 2018; Zhang et al., 2021). In our previous study, we observed an interaction between seasonal acclimatization and the short-term heat-hardening responses in which the stronger heat-hardening response worked synchronously with acclimatization to warmer seasons, and an initial thermal stress resembling the seasonal maximum temperature induced stronger heat-hardening effects, in the intertidal bivalve *Sinonovacula constricta* (Zhang et al., 2021). Therefore, intertidal species are ideal for further revealing the physiological mechanisms underpinning the interactive process between long-term and short-term thermal plasticity.

In the present study, to reveal the mechanisms mediating the interactive process between acclimation/acclimatization and the short-term heat-hardening response, we considered whether, after acclimatization in warm seasons, there are other mechanisms supporting the heat-hardening response without extra induction of HSPs, and whether activated HSPs correspond with a more effective heat-hardening response. We measured the heat-hardening responses of the razor clam *S. constricta* collected in June 2020 and undertook transcriptional and metabolomic analysis in clams that experienced different initial heat stresses followed by different recovery periods to identify key physiological functions or metabolite families involved in the heat-hardening response and the interaction between seasonal acclimatization and short-term heat hardening.

## MATERIALS AND METHODS

### Clam collection, heat-hardening response and heart rate measurement

Razor clams, *Sinonovacula constricta* (Lamarck 1818) (mean±s.d. mass 10.02±2.59 g, $n$=336), were obtained from a natural mudflat in Xiamen, Fujian Province, China (24.6°N, 118.3°E) in June 2020 and were transported to the laboratory in Xiamen University. Our previous study showed that, in May and June 2019, the maximum operative temperature of clams in the mudflat can reach 32°C, and operative temperature was approximately 25 or 26°C in most cases (Zhang et al., 2021). Before the experiments, clams were given a short acclimation of 3–6 days in aerated natural seawater at 25°C, pH 8.4, and salinity of 28 psu.

After the short acclimation, 120 clams were randomly selected to measure the heat-hardening response. This was done by determining cardiac performance after initial heat stress and subsequent recovery periods as described in our previous study (Zhang et al., 2021). In that study, the initial heat stress at 33°C, which was closer to the seasonal maximum operative temperature, induced a more effective heat-hardening response than those at 35 and 37°C in *S. constricta* collected in June, especially after a 12 and 24 h recovery period (Zhang et al., 2021). Therefore, 33 and 37°C were used as inducing temperatures for the heat-hardening response in the present study. Clams were heated in the mud (obtained from the sampling mudflat, pre-sifted through a 40 mesh (hole size 0.425 mm) and with ∼40% water content) at a heating rate of 0.1°C $min^{-1}$ using a controllable water bath (TFX200, Grant). The temperature in the mud was monitored using a temperature sensor (54-II, Fluke). The target temperatures (33 and 37°C) were maintained for 1 min and then the clams were returned to the acclimation condition immediately for recovery. For each inducing temperature, 48 clams were heat hardened and, after 3 and 24 h of recovery, 24 individuals were used for cardiac performance measurements. A control group containing 24 clams without heat hardening was also created. Cardiac performance was measured using the method described by Dong and Williams (2011). The infrared heart beat signal of clams was amplified (AMP03, Newshift), filtered, smoothed, and recorded with a PowerLab AD converter (16/30, ADInstruments). The countable heart beat data were analyzed with LabChart (v.7.2). The Arrhenius break temperature (ABT), the temperature at which heart rate drops rapidly, having first increased with rising temperature, was used to indicate the sublethal heat tolerance, and flatline temperature (FLT), where the heart beat ceased, indicated the lethal heat tolerance (Zhang et al., 2020). ABT values were calculated by segmented linear regression using Origin software (OriginLab Corp.).

### Transcriptomic and metabolomic analysis of heat hardening

The heat hardening and recovery treatments were conducted on another 150 clams to collect samples for transcriptomic and metabolomic analysis. Each group [one control group and four heat-hardened groups (two inducing temperatures×two recovery periods)] contained 30 clams. After the recovery period, the foot muscle tissue of each clam was isolated and frozen in liquid nitrogen. Because of the small size of *S. constricta* muscle tissue, 5 pooled samples that each contained 6 individual samples were used for subsequent sequencing and analysis.

Transcriptomic analysis was conducted as described in our previous study (Zhang et al., 2020). Total RNA was extracted from pooled samples and used for library preparation. RNA purity, concentration and integrity were determined with agarose gel electrophoresis, a NanoDrop spectrophotometer (2000, Thermal Fisher Scientific) and a bioanalyzer (2100, Agilent), respectively. Subsequently, isolation of mRNA, fragmentation, synthesis of first and second strand cDNA, purification of cDNA fragments of preferentially 240 bp, and PCR amplification were conducted in turn. The library was paired-end sequenced on the Illumina HiSeq 2000 platform. Quality control was conducted to obtain clean reads, which were then *de novo* assembled using Trinity (v.2.5.1). Databases including the non-redundant protein database (NR), Swiss-Pro protein database, clusters of proteins database (COG/KOG/eggNG), gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) were employed for gene function annotation and the threshold value was set as an E value $<e^{-5}$. Gene expression was assessed as fragments per kilobase of exon million

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(FPKM). Differentially expressed genes (DEGs) were screened out from the following paired comparisons: each heat-hardened group versus control group, heat-hardened groups with the same inducing temperature followed by 3 h of recovery versus 24 h of recovery, and heat-hardened groups with the inducing temperature at 33°C versus 37°C followed by the same recovery period. The threshold value for DEGs was fold-change (FC)>2 and false discovery rate (FDR)<0.05. TopGO R package (v.2.28.0) and KOBAS software were used for GO and KEGG enrichments, respectively, to obtain information on DEGs (http://bioconductor.org/packages/topGO/; Mao et al., 2005). Significant GO enrichment was determined based on $P$<0.05 and significant value>expected value. Significant KEGG enrichment was determined based on an adjusted $P$-value ($P_{adj}$)<0.05.

Metabolites were extracted from pooled samples in methanol and internal metabolites were added. Proteins were precipitated via ultrasound treatment and incubation at −20°C. Samples were centrifuged and the supernatant was used for UHPLC-QTOF-MC analysis. LC-MC analysis was conducted on a UHPLC system (Agilent Technologies) using a UPLC BEH amide column (Waters) connected to a TripleTOF 5600 platform (AB Sciex). The injection volume was 3 µl and the MS data of full scan survey were evaluated using the acquisition software (AB Sciex). Electrospray ionization (ESI) source conditions were an ion spray voltage of 5 kV ($ESI^+$) and −4 kV ($ESI^-$), gas 1 and 2 of 60 psi (where 1 psi≈6.89 kPa), curtain gas of 35 psi and turbo spray temperature of 650°C. The results including retention time, mass-to-charge ratio values and peak intensity were used for metabolite identification. Differential metabolites (DMs) were screened out from the same paired comparisons that were used in the transcriptomic analysis. The orthogonal projections to latent structures-discriminant analysis (OPLS-DA) model was used to filter variables and obtain variable importance in the projection (VIP) values. The threshold value for DMs was FC>2, $P$<0.05 and VIP>1. The KEGG database and the human metabolome database (HMBD) were used for DM annotation. KEGG enrichments were conducted on the MetaboAnalyst platform (https://www.metaboanalyst.ca/) with all DMs obtained from $ESI^+$ and $ESI^-$ platforms.

### Real-time PCR and measurement of glycogen

To validate the results from omics analysis, 60 clams were heated to 33 or 37°C in the mud followed by recovery. After 3, 6, 12, 18 and 24 h of recovery, for each inducing temperature, 6 individuals were sampled and foot muscle was isolated. The clams without heat-hardening treatment were used for the control group. Total RNA was isolated from about 0.1 g muscle tissue using TRIzol (ThermoFisher Scientific) with an RNA isolation kit (Qiagen) and reverse transcribed into cDNA (Takara). The expression level of five HSPs (HSP19.5, HSP23, HSP60, HSP70 and HSP90) and two

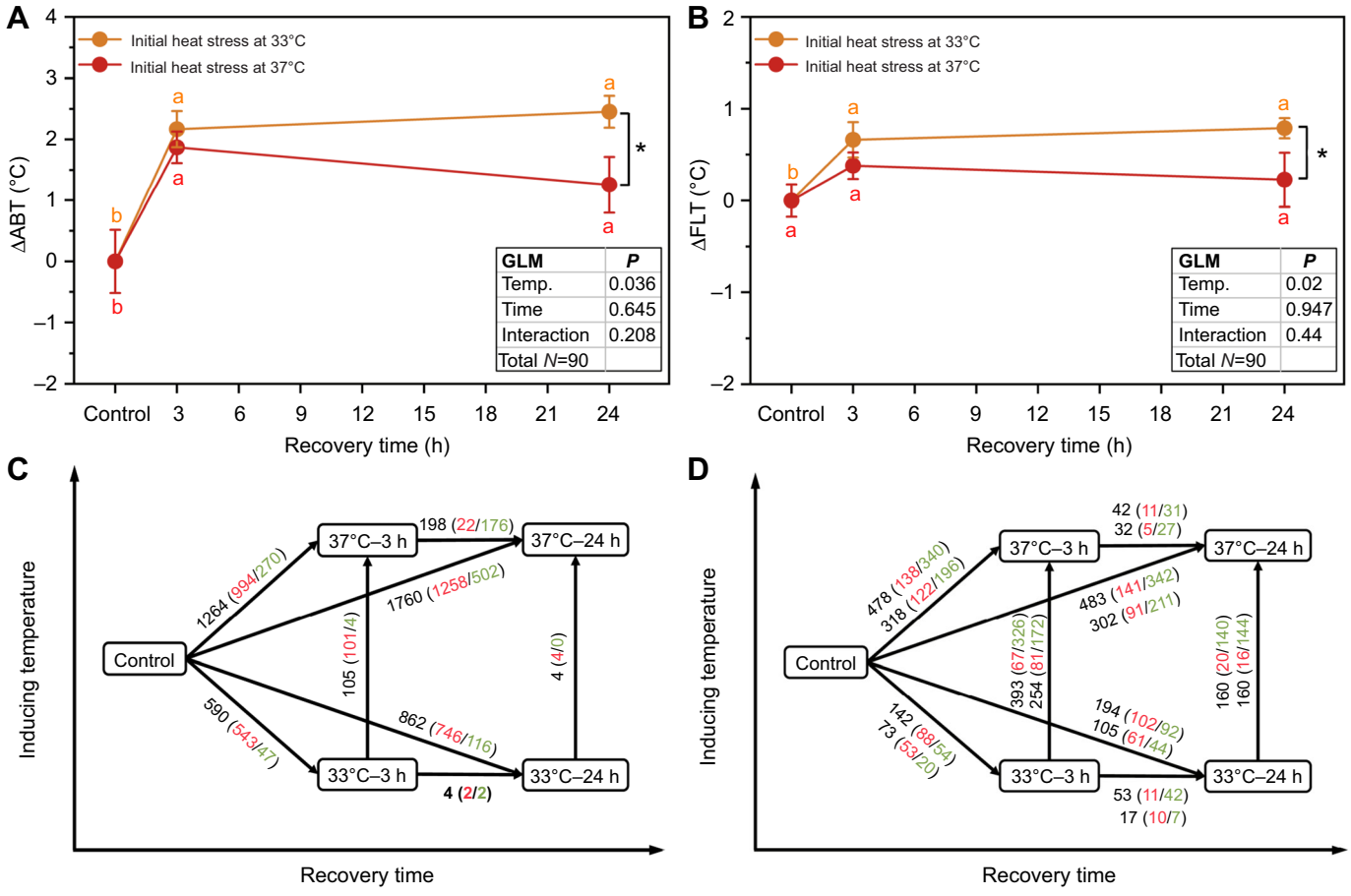


**Fig. 1. The heat-hardening response and number of differentially expressed genes and differential metabolites in the clam *Sinonovacula constricta* after heat hardening at 33 or 37°C with 3 or 24 h of recovery.** (A,B) Change in Arrhenius break temperature (ΔABT; A) and flatline temperature (ΔFLT; B). Letters of the same color indicate the effects of the same inducing temperature with different recovery periods on the metrics. Asterisks indicate a significant difference caused by different inducing temperatures after the same recovery period. Significance was set at $P$<0.05. (C,D) The number of differentially expressed genes (DEGs; C) and differential metabolites (DMs; D). Red and green numbers indicate, respectively, the number of upregulated and downregulated DEGs or DMs. In D, the two lines of numbers show the results from $ESI^+$ (top) and $ESI^-$ (bottom) platforms of LS-MC analysis.

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metabolic enzyme genes (endoglycanase E-4 and trehalase), which were screened out from transcriptomic analysis were assessed via real-time PCR (qPCR) with β-actin employed as the control gene. qPCR was conducted on a StepOne Plus Real-time PCR platform (Applied Biosystems) with the following conditions: 10 µl SYBR Green PCR Master Mix (Applied Biosystems), 8 µl Milli-Q $H_2O$, 0.25 µl forward and reverse primers, and 1.5 µl cDNA template. The primers are shown in Table S1 and relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Outliers beyond the mean±3 s.d. were excluded.

In these samples (two inducing temperatures×5 recovery periods), changes in the content of glycogen were assessed because of its important role in supplying energy when bivalves encounter environmental stress (Hummel et al., 1989). The content of glycogen was determined using a reagent kit (Nanjing Jiancheng Bioengineering Institute) according to the anthrone method, in which the absorption value was measured at 620 nm after the tissues were digested in potassium hydroxide and reacted with anthrone (Roe and Dailey, 1966).

### Statistical analysis

The change in ABT and FLT (ΔABT and ΔFLT) was calculated as all ABT and FLT values minus the average values in the control group. Because the homogeneity of variance was not appropriate for all ΔABT, ΔFLT, qPCR and glycogen measurement data, a generalized linear model followed by a least significant difference *post hoc* test was employed to explore the effects of inducing temperature, recovery period and their interaction on these metrics. The significance was set as $P<0.05$. Statistical analysis was conducted using SPSS software (v.17.0, IBM).

## RESULTS

Exposure to both 33 and 37°C induced a heat-hardening response that increased the ABT of *S. constricta* collected in June 2020, but 37°C did not significantly increase the FLT of *S. constricta* (Fig. 1A,B). Initial heat stress at 33°C induced a relatively stronger heat-hardening response than that at 37°C, resulting in higher ΔABT and ΔFLT values (Fig. 1A,B). After 24 h of recovery, the clams treated at 37°C showed significantly lower ΔABT and ΔFLT than those treated at 33°C (Fig. 1A). Heat-hardening treatment resulted in plenty of DEGs and DMs in *S. constricta* (Fig. 1C,D). The effects caused by different inducing temperatures were mainly reflected in the DEGs after 3 h of recovery, in which 101 DEGs showed higher expression with heat hardening at 37°C, as well as in the DMs after 3 h and 24 h of recovery, in which more metabolites showed lower content after heat hardening at 37°C (Fig. 1C,D). In clams treated at 37°C, 176 DEGs showed downregulated expression after 24 h of recovery when compared with those after 3 h of recovery (Fig. 1C), and only 4 DEGs were observed between the clams treated at 33°C followed by different recovery periods (Fig. 1C).

### Physiological mechanisms supporting heat-hardening responses based on acclimatization

Most biological process categories that were enriched with DEGs and shared by all heat-hardened groups were related to general metabolic processes or gene expression, and no pathway enriched with DEGs was shared by all these groups (Table S2). In particular, in clams with an initial heat stress at 33°C with 3 h of recovery, no pathway was enriched with DEGs (Table S2).

Heat-hardening treatment altered the content of various membrane lipids in *S. constricta*, including glycerophosphocholines (PCs),

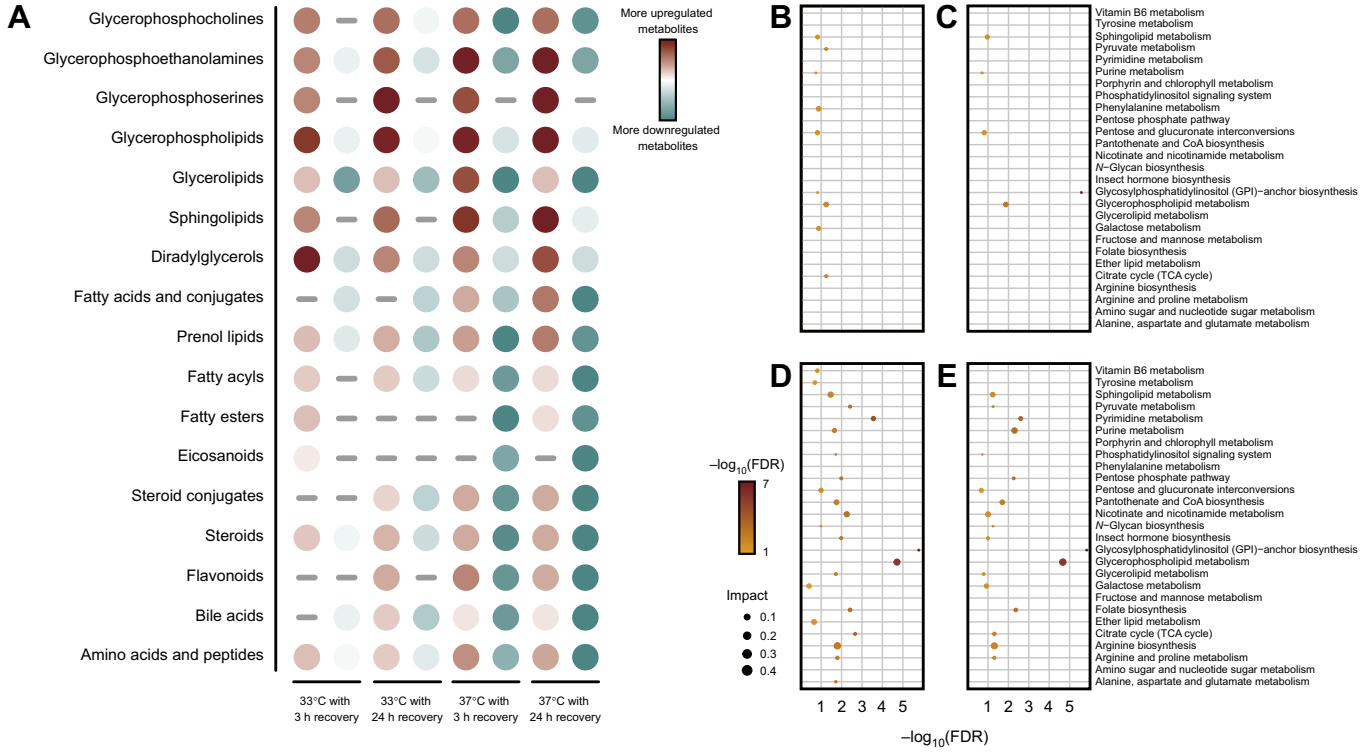


**Fig. 2. Results of metabolomic analysis in *S. constricta* after heat hardening at 33 or 37°C with 3 or 24 h of recovery.** (A) Relative number of DMs enriched in classifications related to membrane glycerophospholipids or energy reserves in heat-hardened clams when compared with clams from the control groups. Relative numbers were standardized based on the highest number in each classification; the gray line indicates no different metabolites. (B–E) Pathways enriched with DMs in heat-hardened clams when compared with clams from the control groups: 33°C with 3 h of recovery (B), 33°C with 24 h of recovery (C), 37°C with 3 h of recovery (D) and 37°C with 24 h of recovery (E). Each group contained 5 pooled samples. FDR, false discovery rate.

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glycerophosphoethanolamines (PEs), glycerophosphoserines (PSs) and sphingolipids (Fig. 2A; Table S3). These DMs were enriched in sphingolipid metabolism, glycerophospholipid metabolism or glycerolipid metabolism pathways (Fig. 2). Clustering analysis showed that, in general, the content of many PCs, PEs or PSs increased after heat-hardening treatment and the content of lysoPEs and lysoPCs, which are the degradation product of PEs or PCs, decreased (Fig. 3A,B). Additionally, we found the effects of initial heat stress on the content of membrane lipids can last for 24 h in *S. constricta* (Fig. 3).

### Mechanisms underlying effect of different inducing temperatures on heat-hardening response

After 3 h of recovery, clams that were treated with different inducing temperatures showed enrichment of DEGs involved in cellular macromolecule metabolic processes, starch and sucrose metabolism and protein processing in endoplasmic reticulum pathways (Fig. 4A,C). These DEGs included various HSPs and metabolism-related genes, such as endoglycanase E-4 and trehalase. DEGs were also identified via GO enrichment for the DEGs that decreased expression with extended recovery time in clams treated with 37°C (Fig. 4B,D). In general, HSPs showed higher expression levels in clams treated at 37°C followed by 3 h of recovery (Fig. 5A). qPCR further showed that, after 3 h of recovery, HSPs had significantly higher expression levels in clams treated with an initial heat stress at 37°C than in those treated at 33°C (Fig. 5B–F). Initial heat stress at 33°C caused little fluctuation in the expression of HSPs (Fig. 5B–F).

In *S. constricta* exposed to 37°C, the content of many metabolites related to energy reserves, especially lipids and peptides, decreased after 3 and 24 h of recovery (Fig. 2A). Compared with clams treated at 33°C, many metabolites, most of which were steroids, prenol lipids, peptides or fatty acyls, showed a lower content in clams that experienced initial heat stress at 37°C followed by 3 and 24 h of recovery (Fig. 6; Table S3). Initial heat stress at 37°C resulted in significantly higher expression levels of the metabolism-related genes endoglycanase E-4 and trehalase in *S. constricta* after 3 or 6 h of recovery (Fig. 7A,B). Similarly, initial heat stress at 37°C led to a higher glycogen content in clams after 3 and 6 h of recovery than with 33°C treatment (Fig. 7C). Effects of different initial heat stress

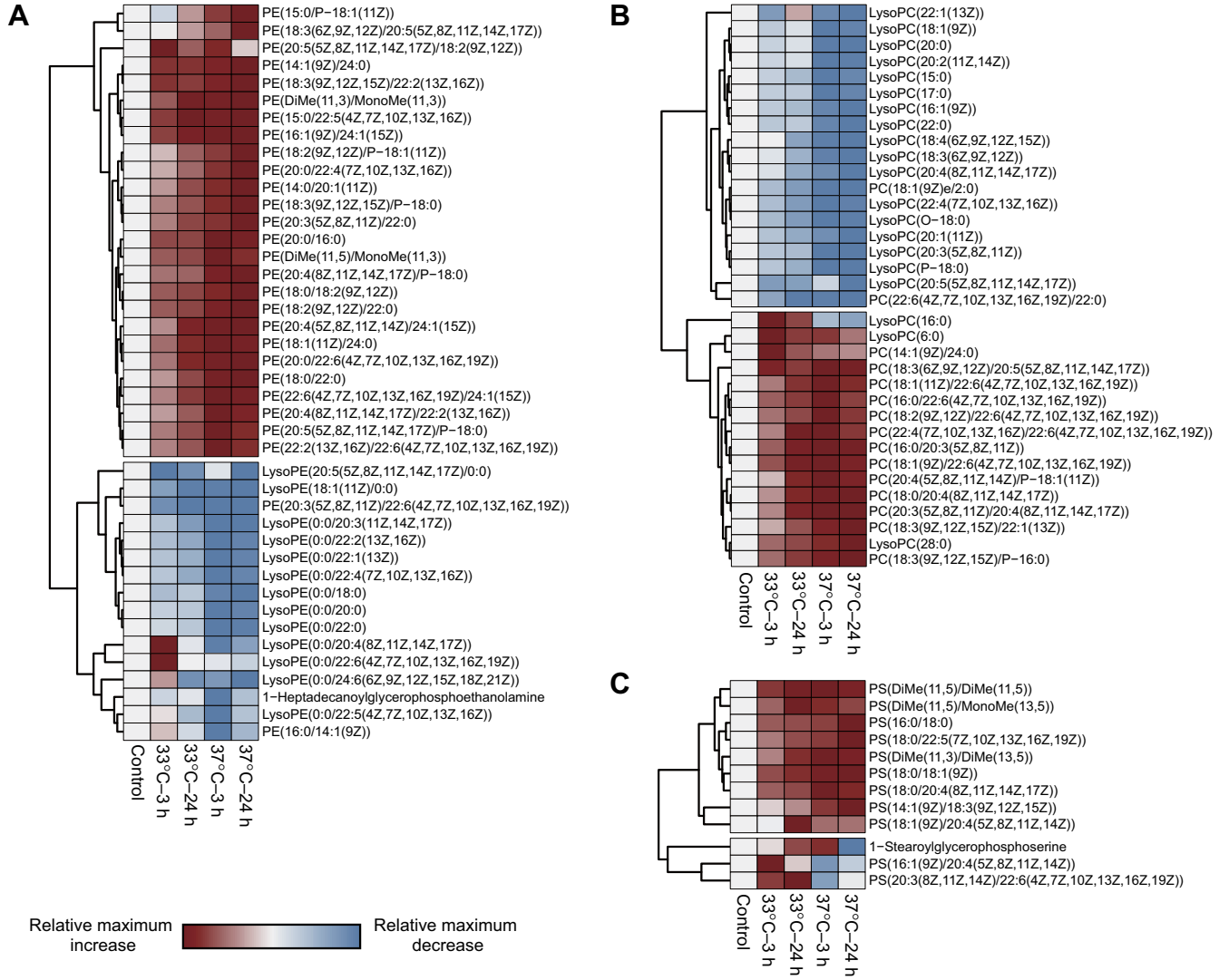


**Fig. 3. Clustering analysis of changes in membrane lipids in *S. constricta* subjected to heat hardening.** (A) Changes in glycerophosphoethanolamines (PE). (B) Changes in glycerophosphocholines (PC). (C) Changes in glycerophosphoserines (PS). Lipid content was standardized based on the control group. Each group contained 5 pooled samples.

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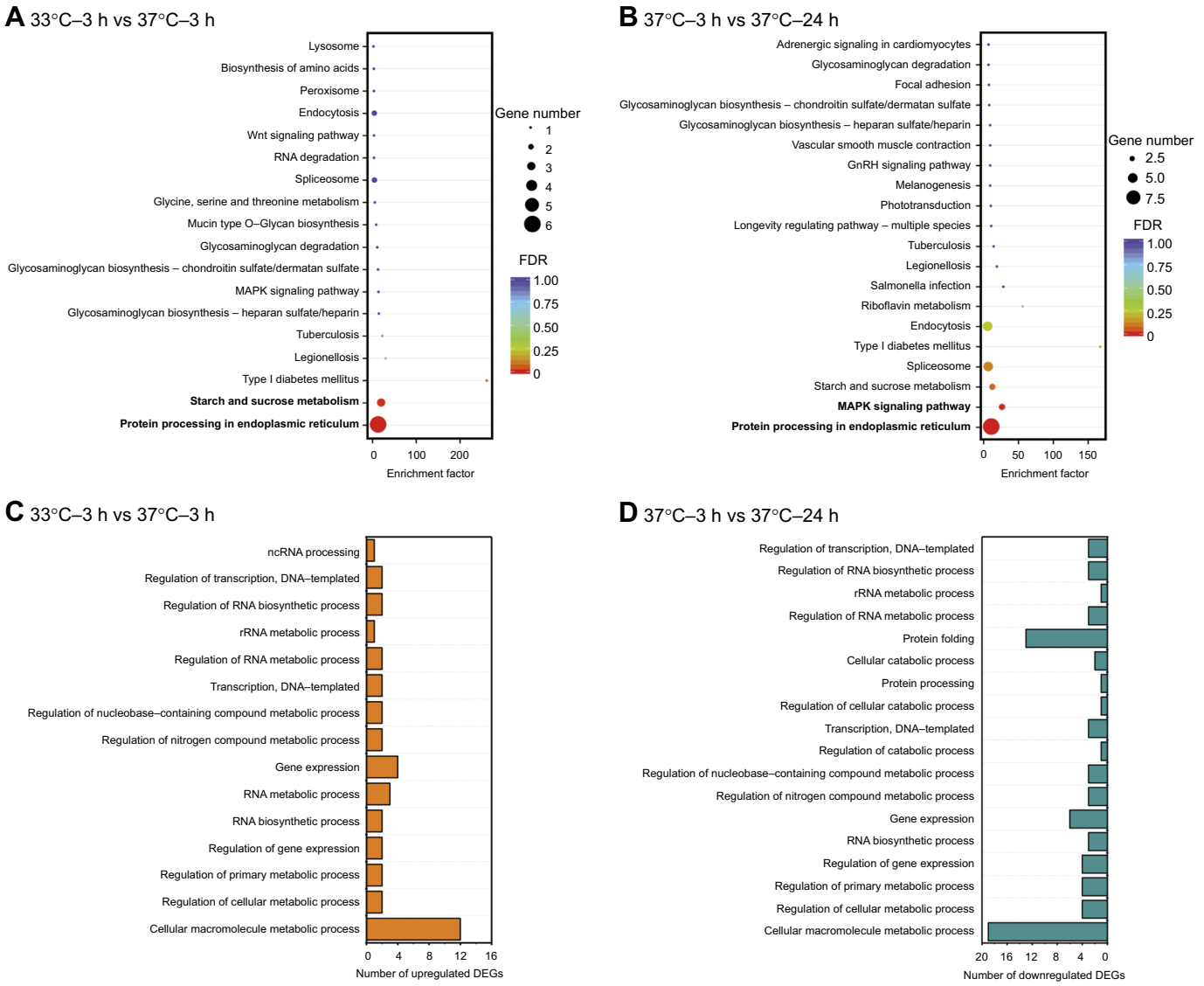


**Fig. 4. KEGG and GO enrichment for DEGs in *S. constricta*.** (A) Pathways enriched with DEGs after 3 h of recovery for clams subjected to different initial heat stress. (B) Pathways enriched with DEGs for clams subjected to different recovery periods after heat hardening at 37°C. In A and B, pathways significantly enriched with DEGs are in bold. (C) Biological process categories enriched with upregulated DEGs after 3 h of recovery for clams subjected to different initial heat stress. (D) Biological process categories enriched with downregulated DEGs between clams subjected to different recovery periods after heat hardening at 37°C. Each group contained 5 pooled samples.

on the content of several key metabolites were observed. An inducing temperature of 37°C increased the content of nicotinamide adenine dinucleotide (NADH), α-ketoglutarate, citric acid, lactic acid and guanosine triphosphate (GTP) in *S. constricta* after 3 and 24 h of recovery, whereas an initial heat stress of 33°C did not affect the content of these metabolites (Fig. 7D–H).

## DISCUSSION

With more frequent stressful climate events being expected in the future, thermal physiological plasticity on multiple time scales is crucial for avoiding thermal stress although adverse effects of overheating may not be completely counteracted by plasticity (Deery et al., 2021; Gunderson et al., 2017; Gunderson and Stillman, 2015). Nonetheless, knowledge about the physiological mechanisms underpinning the interaction between thermal plasticity on different time scales, especially that regulating heat-hardening effects of organisms after seasonal acclimatization, is relatively scarce. In the present study, based on the phenomenon that *S. constricta* showed heat-hardening responses after acclimatization in summer and that the heat-hardening effect was stronger with a milder inducing temperature, our results suggested the role of membrane lipid metabolism in supporting the heat-hardening response of *S. constricta*, and further showed a potential constraint related to the induction of HSPs and energy costs on heat-hardening effects after acclimatization in the warm season.

An alteration in membrane lipid composition was observed in all heat-hardened razor clams. In particular, the duration of elevated content of PCs, PEs and PSs was in accord with that of the heat-hardening response in *S. constricta*. PCs, PEs and PSs are abundant phospholipids in biological membranes and are closely related to the regulation of cellular energy metabolism, the permeability of biological membranes and the activity of ATPases (Nemova et al., 2017; Van der Veen et al., 2017). The increase in phospholipid content, especially the relatively higher concentrations of PCs, is an

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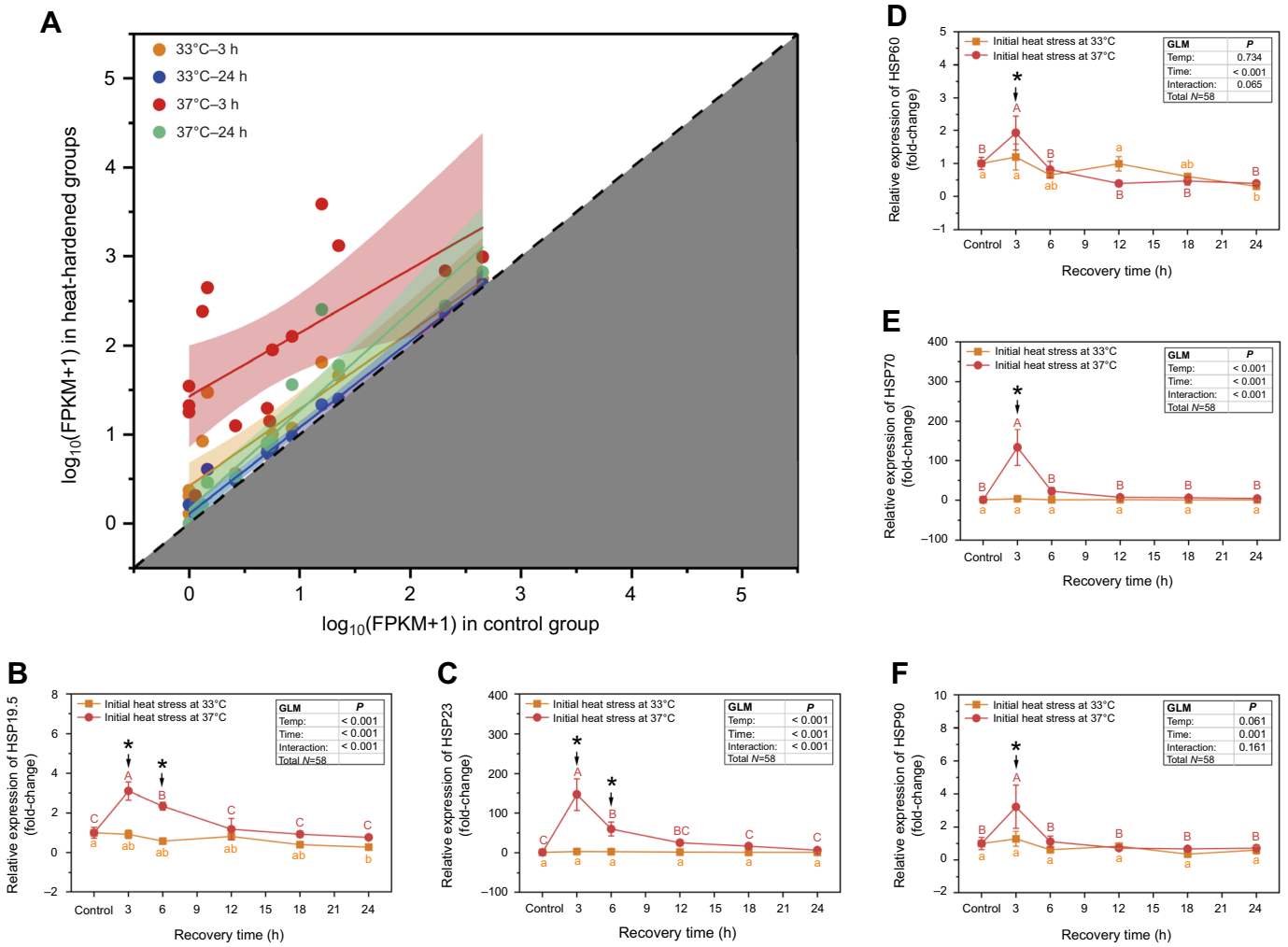


**Fig. 5. Changes in expression of heat shock proteins in heat-hardened *S. constricta*.** (A) Linear fitting and confidence interval for changes in expression of heat shock proteins (HSPs) from transcriptomic analysis. Each group contained 5 pooled samples. (B–F) Changes in expression of HSP19.5 (B), HSP23 (C), HSP60 (D), HSP70 (E) and HSP90 (F) with different recovery times in clams after heat-hardening treatment. Letters of the same color indicate the effects of the same inducing temperature with different recovery periods on the metrics. Asterisks indicate a significant difference caused by different inducing temperatures after the same recovery period. FPKM, fragments per kilobase of exon million.

adaptive response to thermal stress to maintain the stability and function of biological membranes (Cooper et al., 2014; Qu and Ajuwon, 2018). Chen et al. (2021) reported that regulation of glycerophospholipid metabolism, in which the content of specific PEs increased, played an important role in thermal resistance of a thermal tolerant intertidal snail *Echinolittorina malaccana*. Our results further showed that the increase in phospholipid content may also occur during the subsequent recovery from thermal stress. This indicates that the effects of adaptative responses to thermal stress may not only cover the stress periods but can last for a relatively longer duration to provide further protection. After initial heat stress and recovery, the elevated levels of phospholipids in *S. constricta* may contribute to the heat-hardening response by helping the clams to prevent adverse effects of subsequent secondary heat exposure, such as phospholipid disorganization, and may be beneficial to energy supply and survival of the cell when the clams encounter a thermal stress again (Biederman et al., 2019; Wallaert and Babin, 1994). Interestingly, regulation of membrane lipid composition was widely reported as the key factor underpinning in the cold-hardening response, which is a rapid plastic response to improve the resistance of organisms to low temperature (Lee et al., 2006; Overgaard et al., 2010). These findings indicated that maintaining the stability and function of biological membranes is of great significance for the short-term thermal plasticity of organisms. Alterations in membrane lipid composition were also involved in long-term thermal acclimation of organisms (Hazel, 1979; Kraffe et al., 2007). Our results suggest that the mechanisms supporting short-term thermal plasticity may partly overlap with those underpinning long-term thermal plasticity.

After initial heat stress at 33°C and subsequent recovery, elevated expression of HSPs was not observed in clams that showed a significant heat-hardening response. This was novel and different from the findings of other studies, in which activated HSPs were observed in organisms after heat-hardening treatments (Bahrndorff et al., 2009; Gu et al., 2019). The mRNA level of HSP70 substantially increased and reached a peak after heat stress and following 2 h of recovery, and the HSP70 protein level showed a positive correlation with thermal tolerance after heat-hardening in the springtail *Orchesella cincta* (Bahrndorff et al., 2009). In the present study, *S. constricta* had experienced acclimatization to a warm season and the initial heat stress at 33°C may therefore be a relatively mild heat stress, which was close to the maximum

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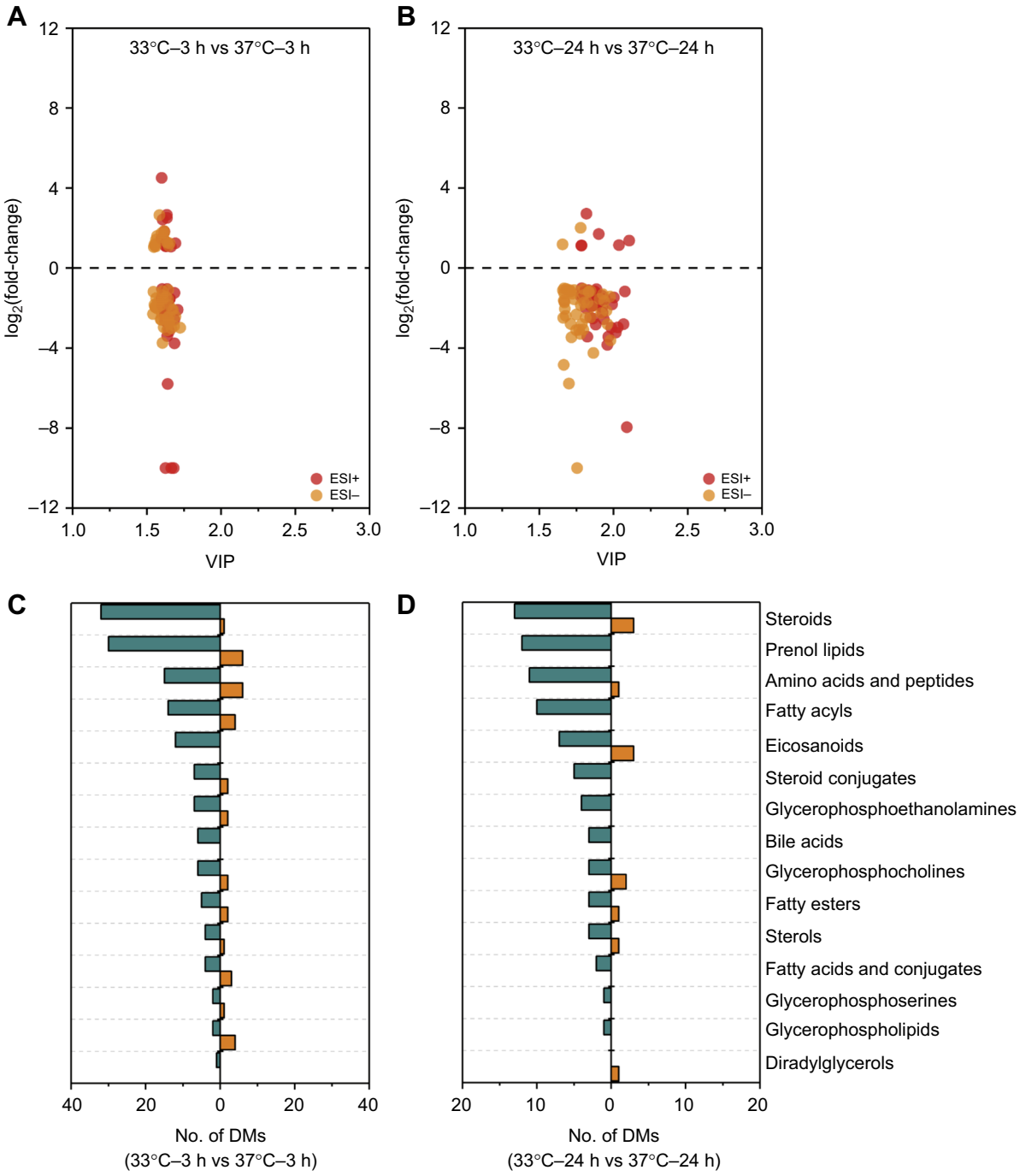


**Fig. 6. Differences in metabolites between *S. constricta* after heat hardening at different inducing temperatures.** (A,B) Relationship between variable importance in the projection (VIP) values and $\log_2$(fold-change) for DMs following treatment at two inducing temperatures after 3 h (A) and 24 h (B) of recovery. (C,D) Metabolite classifications for the most enriched DMs following treatment at two inducing temperatures after 3 h (C) and 24 h (D) of recovery. Green and orange bars indicate the number of downregulated and upregulated DMs, respectively, in clams that were heat hardened at 37°C when compared with that in clams that were heat hardened at 33°C. Each group contained 5 pooled samples.

operative temperature that the clams experienced in recent thermal history and may even have been encountered by the clams (Zhang et al., 2021). HSPs will be activated when temperature increases to levels substantially above normal to improve the heat tolerance of organisms; additionally, acclimation/acclimatization to a relatively warmer condition can raise the induction temperature of HSPs (Dietz and Somero, 1992; Roberts et al., 1997). For example, in the intertidal mussels of the *Mytilus* genus, the induction temperature of HSP70 was higher when the species were acclimated/acclimatized to warmer thermal environments (Buckley et al., 2001). Furthermore, acclimation/acclimatization to warm thermal environments can also lead to higher basal levels of HSPs in the tissues of organisms (Dietz and Somero, 1992). Therefore, after acclimatization in summer, 33°C was not a high enough temperature to induce a substantial activation of HSPs in *S. constricta*, and the basal levels of HSPs plus regulation of membrane lipid composition may jointly support the heat-hardening response of *S. constricta*.

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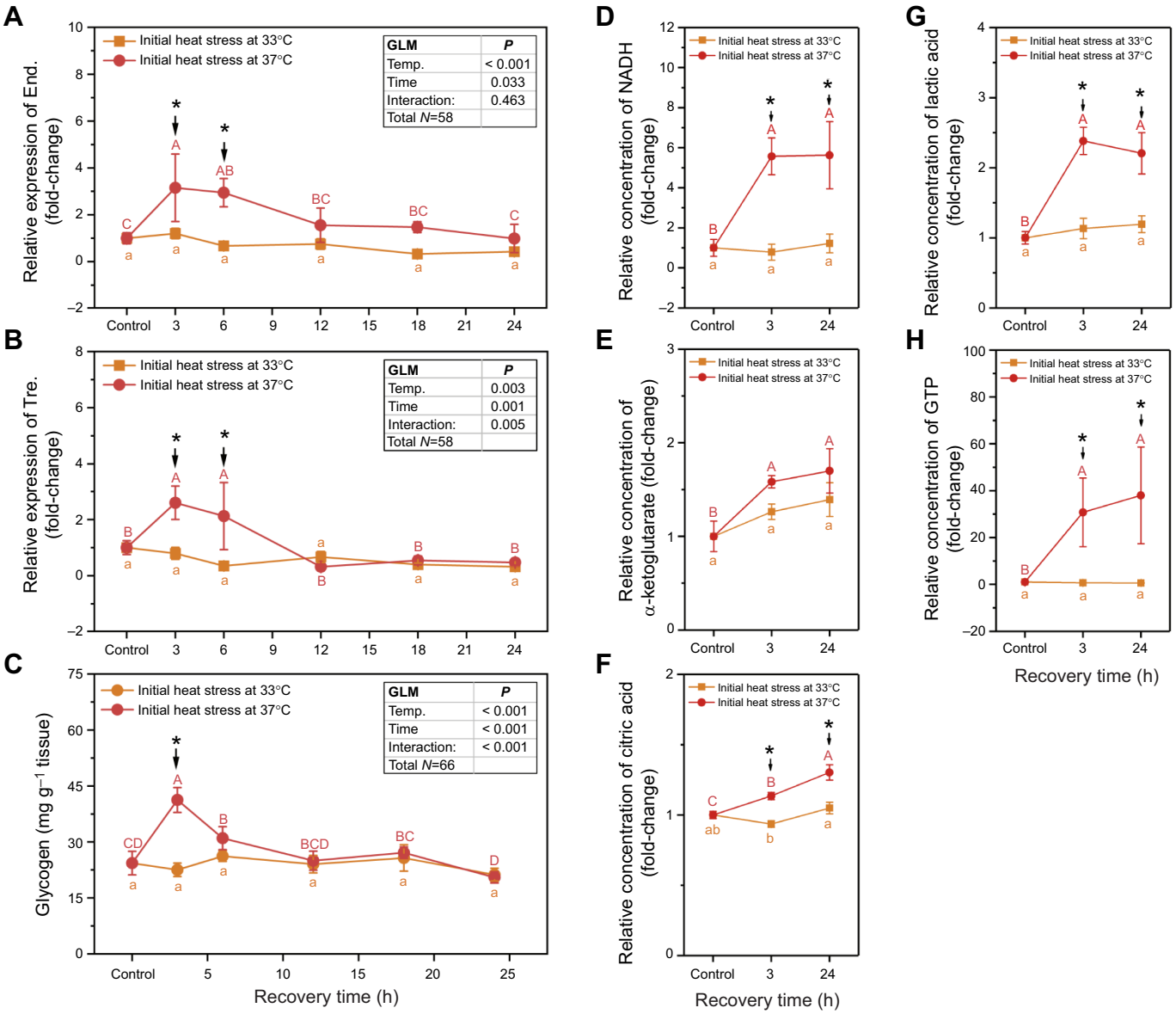


**Fig. 7. Changes in metrics related to energy metabolism in heat-hardened *S. constricta*.** (A,B) Changes in expression of endoglucanase E-4 (End.; A) and trehalase (Tre.; B) in clams after heat-hardening treatment and subsequent recovery. (C) Change in glycogen content in clams after heat-hardening treatment and subsequent recovery. (D–H) Change in NADH (D), α-ketoglutarate (E), citric acid (F), lactic acid (G) and GTP (H) content, which are related to the tricarboxylic acid cycle and glycogen metabolism, in clams after heat-hardening treatment and subsequent recovery. Letters of the same color indicate the effects of the same inducing temperature with different recovery periods on the metrics. Asterisks indicate a significant difference caused by different inducing temperatures after the same recovery period. In D–H, each group contained 5 pooled samples.

However, in clams that showed a less effective heat-hardening response after an initial heat stress at 37°C, elevated expression of HSPs was observed after 3 h of recovery. The expression pattern of HSPs in the clams that were heat hardened at 37°C was similar to the change of HSP70 reported in the springtail *O. cincta* after exposure to heat shock (Bahrndorff et al., 2009). Our results suggest that, although elevated expression of HSPs can be observed in the heat-hardening response, it may not always mean that heat-hardening effects will be enhanced by activation of HSPs. One possible cause of this phenomenon is the higher energy costs during initial heat stress at higher temperatures and subsequent recovery. Our results showed that the content of various metabolites, especially lipids, decreased in *S. constricta* treated at 37°C. These were similar to the results in *Drosophila* that thermal stress can lead to the depletion of energy reserves and this depletion cannot recover in a short recovery period (Klepsatel et al., 2016; Malmendal et al., 2006). The activated endoglycanase E-4 and trehalase and the increased content of glycogen after 3 h of recovery in *S. constricta* that were heat hardened at 37°C indicate an energy requirement here or in response to the previous heat stress (Shukla et al., 2015; Hummel et al., 1989; Sakamoto and Toyohara, 2009). Although these metabolic responses faded with longer recovery, an enhanced tricarboxylic acid cycle, which can be inferred from a higher content of NADH, α-ketoglutarate or citric acid, also suggests an enhanced metabolic process in the clams after heat hardening at 37°C (Chinopoulos, 2013; Li et al., 2017). This may result in decreased energy reserves or nutrients, which in turn affects the energy turnover when the clams encounter secondary thermal stress because sufficient energy supply matters in enduring thermal stress (Semsar-Kazerouni et al., 2020). The consumed energy in response to a heat stress of 37°C

may be used for the synthesis of specific proteins, including HSPs (Krebs and Feder, 1998). The influence of energy costs on heat-hardening effects emerged with the extension of recovery periods after initial heat stress because the levels of HSPs may have recovered to basal levels while the energy reserves could not. Therefore, although higher inducing temperatures can induce the response of HSPs, which may benefit the thermal tolerance of organisms to secondary thermal stress, it may also result in more energy being consumed during the response to stronger initial heat stress, which may affect the heat-hardening effects. This may explain the reason why a higher inducing temperature does not always lead to a more effective heat-hardening response.

To some extent, our study further explains why long-term acclimation/acclimatization matters for the rapid short-term heat-hardening response. Acclimation/acclimatization involves various physiological regulations, including basal levels or induction temperatures of stress proteins, and membrane lipid composition (Angilletta, 2009; Bowler, 2005). Acclimation/acclimatization to either warm or cold may therefore determine whether and how heat shock responses should be mounted in organisms encountering a specific thermal stress. Our results show that the mechanisms supporting the heat-hardening response overlap with those underpinning acclimation/acclimatization, suggesting that these two kinds of thermal plasticity are mechanistically linked. With the basal transcriptional levels of HSPs and regulation of membrane lipids, the heat-hardening response seems more effective in *S. constricta*; and when the inducing temperature rises further, the potential energy costs of a previous heat stress may restrict the heat-hardening effects even though HSPs were activated. The more effective inducing temperatures for the heat-hardening response should be related to the temperature range that the organisms were acclimated/acclimatized to. This suggests a mediating role of membrane lipid metabolism, HSPs and related energy costs in the interaction between long-term and short-term thermal plasticity. Acclimation/acclimatization can adjust the heat-hardening effects by affecting the magnitude and energy costs of the heat shock response to specific thermal stress. In turn, frequent heat hardening, especially that which is linked to upregulation of HSPs may have cumulative effects that can be regarded as acclimation/acclimatization (Phillips et al., 2016). This may also suggest that the reduced heat-hardening responses in organisms acclimating/acclimatizing to cold conditions were probably due to the extra energy costs in response to an initial heat stress (Sgrò et al., 2010; Zhang et al., 2021). Additionally, these findings also suggest that the heat-hardening response can benefit organisms with a greater long-term acclimation/acclimatization ability to survive and invade a wider temperature range.

In summary, the present study revealed the physiological mechanisms mediating the interaction of long-term acclimatization and short-term heat-hardening response in the intertidal bivalve *S. constricta*. Clams showed heat-hardening responses after acclimatization in summer and the inducing temperature closer to the maximum operative temperature the clams experienced resulted in a more effective heat-hardening effect (Zhang et al., 2021). After acclimatization in summer, heat-hardening responses of *S. constricta* may be more related to the regulation of membrane lipids. The content of phospholipids increased in *S. constricta* after the heat-hardening treatment at 33 and 37°C, and the duration of this effect matched the duration of heat-hardening responses. The initial heat stress at 33°C, which induced a more effective heat-hardening effect, did not increase expression of HSPs in *S. constricta*, suggesting that the heat-hardening response of *S. constricta* may not depend on extra induction but instead on basal levels of HSPs. The warmer inducing temperature, 37°C, led to an upregulation of HSPs in *S. constricta*, but may also result in more energy costs of the heat stress response, constraining heat-hardening effects. Our results contribute to our mechanistic understanding of evolutionary change and the thermal plasticity of organisms in the context of global climate change.

#### Acknowledgements
The authors thank Xiaoxu Li, Yongxu Sun, Yaqi Chen and Jieyu Yang of Xiamen University for assistance in the experiments.

#### Competing interests
The authors declare no competing or financial interests.

#### Author contributions
Conceptualization: Y.D.; Methodology: W.Z.; Investigation: W.Z.; Writing - original draft: W.Z.; Writing - review & editing: Y.D.; Supervision: Y.D.; Project administration: Y.D.; Funding acquisition: W.Z., Y.D.

#### Funding
The study was supported by the grants from Youth Science Foundation of the National Natural Science Foundation of China (no. 31901094).

#### Data availability
Data are available from the Dryad Digital Repository (Zhang and Dong, 2021): dryad.kh1893265

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**Table S1.** Primers used in the real-time PCR.

| Gene | Primers |
|---|---|
| *HSP19.5* | F: GAGACCAAAGACGACCAGCA |
| | R: TTGTCTGGTTCCTTCTCGGC |
| *HSP23* | F: GTGAACTCGCGGTACACTTTACTT |
| | R: GTATTCGAAAAGAAATGCACAAGAT |
| *HSP60* | F: GGCACGAAAACCCCTAGTCA |
| | R: CCCCGATTCCACCAAAGTCA |
| *HSP70* | F: GCAAGGCAGACATTGACAGA |
| | R: GTTCCCGTCTAGCCACTTCA |
| *HSP90* | F: CATCCTCGTCTTCCTCCAAA |
| | R: CCCAAATTACCCTGCACATC |
| *End* | F: CCGGTGGCTAGATACATCCAAG |
| | R: ACGCTAACAATCTGCTGACTCA |
| *Treh* | F: TGATGGTCCCCTGCTTCATG |
| | R: AGTTGACCTTTTGTTGCTGGC |
| *β-actin* | F: CACTTCATGATGCTGTTGTATGTG |
| | R: GATTGTCAGAGACATCAAGGAGAAG |

**Table S2.** Results of GO biological process category enrichment analysis for up-regulated or down-regulated differentially expressed genes (DEGs) and KEGG enrichment for DEGs in *Sinonovacula constricta* after heat hardening treatments.

| **33ºC-3 h vs. Control** | | | | |
|---|---|---|---|---|
| **Biological process categories enriched with up-regulated genes** | | | | |
| **GO.ID** | **Term** | **Significant value** | **Expected value** | **KS value** |
| GO:0006468 | protein phosphorylation | 3 | 2.96 | 0.00515 |
| GO:0060255 | regulation of macromolecule metabolic process | 6 | 5.94 | 0.00922 |
| GO:0010468 | regulation of gene expression | 6 | 5.23 | 0.01067 |
| GO:0051171 | regulation of nitrogen compound metabolic process | 6 | 4.95 | 0.01477 |
| GO:0032774 | RNA biosynthetic process | 6 | 5.54 | 0.0148 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 6 | 4.93 | 0.01551 |
| GO:0006351 | transcription, DNA-templated | 6 | 5.49 | 0.01582 |
| GO:0006835 | dicarboxylic acid transport | 1 | 0.11 | 0.01781 |
| GO:0006643 | membrane lipid metabolic process | 1 | 0.12 | 0.02512 |
| GO:0046467 | membrane lipid biosynthetic process | 1 | 0.08 | 0.0258 |
| GO:0009100 | glycoprotein metabolic process | 2 | 0.64 | 0.02888 |
| GO:0070085 | glycosylation | 1 | 0.61 | 0.03287 |
| GO:0051252 | regulation of RNA metabolic process | 6 | 4.27 | 0.03765 |
| GO:2001141 | regulation of RNA biosynthetic process | 6 | 4.17 | 0.04318 |
| GO:0006355 | regulation of transcription, DNA-templated | 6 | 4.17 | 0.04318 |
| GO:0009101 | glycoprotein biosynthetic process | 1 | 0.59 | 0.04971 |
| **Biological process categories enriched with down-regulated genes** | | | | |
| **GO.ID** | **Term** | **Significant value** | **Expected value** | **KS value** |

| | | | | |
|---|---|---|---|---|
| GO:0018298 | protein-chromophore linkage | 1 | 0.02 | 0.000069 |
| GO:0006464 | cellular protein modification process | 1 | 0.72 | 0.00421 |
| GO:0009765 | photosynthesis, light harvesting | 1 | 0.01 | 0.00608 |
| GO:1901135 | carbohydrate derivative metabolic process | 1 | 0.26 | 0.04418 |
| **Pathways enriched with differentially expressed genes** | | | | |
| No pathway significantly with DEGs | | | | |
| **33$^{o}$C-24 h vs. Control** | | | | |
| **Biological process categories enriched with up-regulated genes** | | | | |
| **GO.ID** | **Term** | **Significant value** | **Expected value** | **KS value** |
| GO:0031323 | regulation of cellular metabolic process | 10 | 7.79 | 0.0016 |
| GO:0080090 | regulation of primary metabolic process | 10 | 7.89 | 0.0036 |
| GO:0006468 | protein phosphorylation | 4 | 3.73 | 0.00496 |
| GO:0010468 | regulation of gene expression | 10 | 6.58 | 0.00796 |
| GO:0032774 | RNA biosynthetic process | 10 | 6.96 | 0.01154 |
| GO:0006351 | transcription, DNA-templated | 10 | 6.9 | 0.01279 |
| GO:0051171 | regulation of nitrogen compound metabolic process | 10 | 6.22 | 0.01444 |
| GO:0007606 | sensory perception of chemical stimulus | 1 | 0.06 | 0.01502 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 10 | 6.2 | 0.01518 |
| GO:0045944 | positive regulation of transcription from RNA polymerase II promoter | 2 | 0.3 | 0.01861 |
| GO:0006835 | dicarboxylic acid transport | 1 | 0.13 | 0.01882 |
| GO:0006886 | intracellular protein transport | 3 | 2.43 | 0.01947 |
| GO:0010628 | positive regulation of gene expression | 2 | 0.4 | 0.02501 |

| | | | | |
|---|---|---|---|---|
| GO:0050789 | regulation of biological process | 19 | 18.99 | 0.02742 |
| GO:0051252 | regulation of RNA metabolic process | 10 | 5.36 | 0.02889 |
| GO:0009100 | glycoprotein metabolic process | 3 | 0.8 | 0.02908 |
| GO:0034613 | cellular protein localization | 3 | 2.81 | 0.03253 |
| GO:0070085 | glycosylation | 3 | 0.76 | 0.03401 |
| GO:0044707 | single-multicellular organism process | 6 | 2.43 | 0.03445 |
| GO:0016050 | vesicle organization | 1 | 0.55 | 0.03543 |
| GO:0007275 | multicellular organism development | 5 | 2.17 | 0.04019 |
| GO:2001141 | regulation of RNA biosynthetic process | 10 | 5.25 | 0.04053 |
| GO:0006355 | regulation of transcription, DNA-templated | 10 | 5.25 | 0.04053 |
| GO:0032501 | multicellular organismal process | 6 | 2.62 | 0.04258 |
| GO:0070727 | cellular macromolecule localization | 3 | 2.85 | 0.04381 |
| GO:0044767 | single-organism developmental process | 5 | 2.78 | 0.0446 |
| GO:0009889 | regulation of biosynthetic process | 10 | 6.16 | 0.04914 |

**Biological process categories enriched with down-regulated genes**

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0018298 | protein-chromophore linkage | 1 | 0.04 | 0.000074 |
| GO:0044260 | cellular macromolecule metabolic process | 6 | 5.8 | 0.00085 |
| GO:0031323 | regulation of cellular metabolic process | 3 | 1.26 | 0.00171 |
| GO:0034645 | cellular macromolecule biosynthetic process | 4 | 2.46 | 0.00345 |
| GO:0080090 | regulation of primary metabolic process | 3 | 1.27 | 0.00382 |
| GO:0006464 | cellular protein modification process | 3 | 1.79 | 0.00421 |

| | | | | |
|---|---|---|---|---|
| GO:0046907 | intracellular transport | 2 | 0.68 | 0.00465 |
| GO:0006468 | protein phosphorylation | 1 | 0.6 | 0.00515 |
| GO:0015031 | protein transport | 1 | 0.49 | 0.00563 |
| GO:0009765 | photosynthesis, light harvesting | 1 | 0.02 | 0.00624 |
| GO:0010468 | regulation of gene expression | 2 | 1.06 | 0.00742 |
| GO:0032774 | RNA biosynthetic process | 2 | 1.12 | 0.01031 |
| GO:0016192 | vesicle-mediated transport | 1 | 0.48 | 0.0115 |
| GO:0010467 | gene expression | 3 | 2.81 | 0.01394 |
| GO:0016070 | RNA metabolic process | 3 | 2.12 | 0.01416 |
| GO:0006351 | transcription, DNA-templated | 2 | 1.11 | 0.01444 |
| GO:0051171 | regulation of nitrogen compound metabolic process | 2 | 1 | 0.0151 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 2 | 1 | 0.01586 |
| GO:0035556 | intracellular signal transduction | 2 | 0.58 | 0.016 |
| GO:0019220 | regulation of phosphate metabolic process | 1 | 0.22 | 0.01745 |
| GO:0051174 | regulation of phosphorus metabolic process | 1 | 0.22 | 0.01745 |
| GO:0006139 | nucleobase-containing compound metabolic process | 4 | 3.58 | 0.01791 |
| GO:0006886 | intracellular protein transport | 1 | 0.39 | 0.01999 |
| GO:0050789 | regulation of biological process | 5 | 3.06 | 0.02011 |
| GO:0050790 | regulation of catalytic activity | 1 | 0.29 | 0.02105 |
| GO:0065009 | regulation of molecular function | 1 | 0.31 | 0.02442 |
| GO:0009100 | glycoprotein metabolic process | 1 | 0.13 | 0.02708 |
| GO:1901360 | organic cyclic compound metabolic process | 4 | 3.77 | 0.02835 |
| GO:0051252 | regulation of RNA metabolic process | 2 | 0.86 | 0.02854 |

| | | | | |
|---|---|---|---|---|
| GO:0070085 | glycosylation | 1 | 0.12 | 0.02906 |
| GO:0034641 | cellular nitrogen compound metabolic process | 4 | 3.8 | 0.03071 |
| GO:0044700 | single organism signaling | 2 | 1.46 | 0.03272 |
| GO:0034613 | cellular protein localization | 1 | 0.45 | 0.0334 |
| GO:0090304 | nucleic acid metabolic process | 3 | 2.92 | 0.03618 |
| GO:0023052 | signaling | 2 | 1.46 | 0.03963 |
| GO:0007165 | signal transduction | 2 | 1.39 | 0.04019 |
| GO:2001141 | regulation of RNA biosynthetic process | 2 | 0.85 | 0.04034 |
| GO:0006355 | regulation of transcription, DNA-templated | 2 | 0.85 | 0.04034 |
| GO:0046483 | heterocycle metabolic process | 4 | 3.73 | 0.04304 |
| GO:1901135 | carbohydrate derivative metabolic process | 2 | 0.65 | 0.04418 |
| GO:0070727 | cellular macromolecule localization | 1 | 0.46 | 0.04494 |
| GO:0009101 | glycoprotein biosynthetic process | 1 | 0.12 | 0.04696 |
| GO:0034654 | nucleobase-containing compound biosynthetic process | 2 | 1.49 | 0.04854 |
| GO:0006725 | cellular aromatic compound metabolic process | 4 | 3.72 | 0.04914 |
| GO:1902582 | single-organism intracellular transport | 1 | 0.45 | 0.04954 |
| GO:0045944 | positive regulation of transcription from RNA polymerase II promoter | 1 | 0.05 | 0.04957 |

**Pathways enriched with differentially expressed genes**

| KO.ID | Pathway | Number of DEGs | $P_{-adjusted}$ |
|---|---|---|---|
| ko00512 | Mucin type O-Glycan biosynthesis | 5 | 0.046760734 |

**37°C-3 h vs. Control**

**Biological process categories enriched with up-regulated genes**

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0006004 | fucose metabolic process | 3 | 0.15 | 0.00091 |
| GO:0031323 | regulation of cellular metabolic process | 17 | 12.4 | 0.00171 |
| GO:0006457 | protein folding | 19 | 3.03 | 0.00307 |
| GO:0080090 | regulation of primary metabolic process | 17 | 12.56 | 0.00382 |
| GO:0010468 | regulation of gene expression | 16 | 10.47 | 0.0099 |
| GO:0032774 | RNA biosynthetic process | 16 | 11.07 | 0.01399 |
| GO:0051171 | regulation of nitrogen compound metabolic process | 17 | 9.89 | 0.0151 |
| GO:0006351 | transcription, DNA-templated | 16 | 10.98 | 0.01565 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 17 | 9.86 | 0.01586 |
| GO:0006835 | dicarboxylic acid transport | 2 | 0.21 | 0.02018 |
| GO:0045944 | positive regulation of transcription from RNA polymerase II promoter | 2 | 0.48 | 0.02369 |
| GO:0006643 | membrane lipid metabolic process | 1 | 0.24 | 0.02793 |
| GO:0046467 | membrane lipid biosynthetic process | 1 | 0.15 | 0.02805 |
| GO:0051252 | regulation of RNA metabolic process | 16 | 8.53 | 0.03106 |
| GO:0009100 | glycoprotein metabolic process | 3 | 1.27 | 0.03142 |
| GO:0010628 | positive regulation of gene expression | 2 | 0.64 | 0.03261 |
| GO:0044767 | single-organism developmental process | 7 | 4.42 | 0.03455 |
| GO:0070085 | glycosylation | 3 | 1.21 | 0.03686 |
| GO:0032502 | developmental process | 7 | 4.48 | 0.04215 |
| GO:2001141 | regulation of RNA biosynthetic process | 16 | 8.35 | 0.04394 |

| | | | | |
|---|---|---|---|---|
| GO:0006355 | regulation of transcription, DNA-templated | 16 | 8.35 | 0.04394 |
| GO:0009889 | regulation of biosynthetic process | 16 | 9.8 | 0.04814 |
| GO:0006493 | protein O-linked glycosylation | 1 | 0.18 | 0.04952 |
| GO:0019933 | cAMP-mediated signaling | 1 | 0.12 | 0.04984 |

**Biological process categories enriched with down-regulated genes**

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0018298 | protein-chromophore linkage | 1 | 0.06 | 0.000076 |
| GO:0031323 | regulation of cellular metabolic process | 2 | 1.84 | 0.00171 |
| GO:0080090 | regulation of primary metabolic process | 2 | 1.87 | 0.00382 |
| GO:0006464 | cellular protein modification process | 3 | 2.63 | 0.00421 |
| GO:0046907 | intracellular transport | 2 | 1 | 0.00474 |
| GO:0008654 | phospholipid biosynthetic process | 1 | 0.08 | 0.00486 |
| GO:0006468 | protein phosphorylation | 1 | 0.88 | 0.00525 |
| GO:0015031 | protein transport | 1 | 0.72 | 0.00573 |
| GO:0009765 | photosynthesis, light harvesting | 1 | 0.02 | 0.00635 |
| GO:0016192 | vesicle-mediated transport | 2 | 0.71 | 0.01168 |
| GO:0006952 | defense response | 2 | 0.08 | 0.01623 |
| GO:0035556 | intracellular signal transduction | 3 | 0.85 | 0.01626 |
| GO:0019220 | regulation of phosphate metabolic process | 1 | 0.32 | 0.01762 |
| GO:0051174 | regulation of phosphorus metabolic process | 1 | 0.32 | 0.01762 |
| GO:0006886 | intracellular protein transport | 1 | 0.58 | 0.02025 |
| GO:0050789 | regulation of biological process | 5 | 4.49 | 0.02075 |
| GO:0050790 | regulation of catalytic activity | 1 | 0.43 | 0.02129 |
| GO:0065009 | regulation of molecular function | 1 | 0.45 | 0.0247 |

| | | | | |
|---|---|---|---|---|
| GO:0002376 | immune system process | 1 | 0.12 | 0.02653 |
| GO:0009100 | glycoprotein metabolic process | 1 | 0.19 | 0.02728 |
| GO:0070085 | glycosylation | 1 | 0.18 | 0.02947 |
| GO:0006021 | inositol biosynthetic process | 1 | 0.01 | 0.03166 |
| GO:0046173 | polyol biosynthetic process | 1 | 0.01 | 0.03166 |
| GO:0051607 | defense response to virus | 1 | 0.01 | 0.03181 |
| GO:0002252 | immune effector process | 1 | 0.01 | 0.03181 |
| GO:0009615 | response to virus | 1 | 0.01 | 0.03181 |
| GO:0044700 | single organism signaling | 3 | 2.14 | 0.03353 |
| GO:0034613 | cellular protein localization | 1 | 0.67 | 0.03384 |
| GO:0023052 | signaling | 3 | 2.14 | 0.04059 |
| GO:0007165 | signal transduction | 3 | 2.04 | 0.04113 |
| GO:1901135 | carbohydrate derivative metabolic process | 2 | 0.96 | 0.04485 |
| GO:0070727 | cellular macromolecule localization | 1 | 0.67 | 0.04551 |
| GO:0009101 | glycoprotein biosynthetic process | 1 | 0.18 | 0.04726 |

**Pathways enriched with differentially expressed genes**

| KO.ID | Pathway | Number of DEGs | $P_{\text{-adjusted}}$ |
|---|---|---|---|
| ko00512 | Mucin type O-Glycan biosynthesis | 8 | 0.000760336 |

**37ºC-24 h vs. Control**

**Biological process categories enriched with up-regulated genes**

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0031323 | regulation of cellular metabolic process | 15 | 14.92 | 0.00171 |
| GO:0006004 | fucose metabolic process | 2 | 0.18 | 0.00488 |
| GO:0051234 | establishment of localization | 62 | 34.49 | 0.01107 |
| GO:0010468 | regulation of gene expression | 13 | 12.59 | 0.01284 |
| GO:0006810 | transport | 61 | 34.35 | 0.0133 |

| | | | | |
|---|---|---|---|---|
| GO:0051171 | regulation of nitrogen compound metabolic process | 13 | 11.9 | 0.0151 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 13 | 11.86 | 0.01586 |
| GO:0016050 | vesicle organization | 2 | 1.06 | 0.01664 |
| GO:0051179 | localization | 62 | 35.62 | 0.01744 |
| GO:0007268 | chemical synaptic transmission | 4 | 0.62 | 0.01751 |
| GO:0007606 | sensory perception of chemical stimulus | 1 | 0.11 | 0.0176 |
| GO:0044706 | multi-multicellular organism process | 1 | 0.11 | 0.0176 |
| GO:0023056 | positive regulation of signaling | 1 | 0.44 | 0.01884 |
| GO:0009967 | positive regulation of signal transduction | 1 | 0.44 | 0.01884 |
| GO:0010675 | regulation of cellular carbohydrate metabolic process | 1 | 0.11 | 0.02168 |
| GO:0006109 | regulation of carbohydrate metabolic process | 1 | 0.11 | 0.02168 |
| GO:0010906 | regulation of glucose metabolic process | 1 | 0.11 | 0.02168 |
| GO:0048511 | rhythmic process | 1 | 0.15 | 0.02218 |
| GO:0007623 | circadian rhythm | 1 | 0.15 | 0.02218 |
| GO:0006835 | dicarboxylic acid transport | 2 | 0.25 | 0.02248 |
| GO:0017156 | calcium ion regulated exocytosis | 2 | 0.07 | 0.02358 |
| GO:0017158 | regulation of calcium ion-dependent exocytosis | 2 | 0.07 | 0.02358 |
| GO:0006836 | neurotransmitter transport | 4 | 0.73 | 0.0253 |
| GO:0045944 | positive regulation of transcription from RNA polymerase II promoter | 2 | 0.58 | 0.02731 |
| GO:0032501 | multicellular organismal process | 10 | 5.02 | 0.02788 |

| | | | | |
|---|---|---|---|---|
| GO:0097305 | response to alcohol | 1 | 0.07 | 0.0281 |
| GO:0045475 | locomotor rhythm | 1 | 0.07 | 0.0292 |
| GO:0044707 | single-multicellular organism process | 9 | 4.66 | 0.03223 |
| GO:0009100 | glycoprotein metabolic process | 6 | 1.53 | 0.03299 |
| GO:0007275 | multicellular organism development | 8 | 4.15 | 0.03436 |
| GO:0010628 | positive regulation of gene expression | 2 | 0.76 | 0.03809 |
| GO:0051252 | regulation of RNA metabolic process | 13 | 10.26 | 0.04012 |
| GO:0070085 | glycosylation | 6 | 1.46 | 0.04017 |
| GO:0007346 | regulation of mitotic cell cycle | 1 | 0.47 | 0.04351 |
| GO:2001141 | regulation of RNA biosynthetic process | 13 | 10.04 | 0.04394 |
| GO:0006355 | regulation of transcription, DNA-templated | 13 | 10.04 | 0.04394 |
| GO:0048584 | positive regulation of response to stimulus | 1 | 0.47 | 0.04722 |
| GO:0010647 | positive regulation of cell communication | 1 | 0.47 | 0.04722 |
| GO:0044767 | single-organism developmental process | 8 | 5.31 | 0.04925 |

**Biological process categories enriched with down-regulated genes**

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0018298 | protein-chromophore linkage | 1 | 0.09 | 0.000083 |
| GO:0031323 | regulation of cellular metabolic process | 4 | 2.85 | 0.00171 |
| GO:0034645 | cellular macromolecule biosynthetic process | 6 | 5.58 | 0.00381 |
| GO:0080090 | regulation of primary metabolic process | 4 | 2.88 | 0.00382 |
| GO:0006464 | cellular protein modification process | 5 | 4.07 | 0.00421 |

| | | | | |
|---|---|---|---|---|
| GO:0046907 | intracellular transport | 2 | 1.55 | 0.00474 |
| GO:0006221 | pyrimidine nucleotide biosynthetic process | 1 | 0.1 | 0.00582 |
| GO:0009765 | photosynthesis, light harvesting | 1 | 0.03 | 0.00658 |
| GO:0010468 | regulation of gene expression | 3 | 2.4 | 0.00839 |
| GO:0035556 | intracellular signal transduction | 2 | 1.32 | 0.01653 |
| GO:0006952 | defense response | 2 | 0.12 | 0.01695 |
| GO:0050789 | regulation of biological process | 7 | 6.94 | 0.01713 |
| GO:0009894 | regulation of catabolic process | 1 | 0.38 | 0.01716 |
| GO:0019220 | regulation of phosphate metabolic process | 1 | 0.5 | 0.01762 |
| GO:0051174 | regulation of phosphorus metabolic process | 1 | 0.5 | 0.01762 |
| GO:0006886 | intracellular protein transport | 1 | 0.89 | 0.02025 |
| GO:0050790 | regulation of catalytic activity | 1 | 0.66 | 0.02129 |
| GO:0031329 | regulation of cellular catabolic process | 1 | 0.36 | 0.02335 |
| GO:0065009 | regulation of molecular function | 1 | 0.7 | 0.0247 |
| GO:0002376 | immune system process | 2 | 0.18 | 0.02713 |
| GO:0009100 | glycoprotein metabolic process | 1 | 0.29 | 0.02767 |
| GO:0070085 | glycosylation | 1 | 0.28 | 0.03008 |
| GO:0051252 | regulation of RNA metabolic process | 2 | 1.96 | 0.03134 |
| GO:0016485 | protein processing | 1 | 0.24 | 0.03209 |
| GO:0051607 | defense response to virus | 1 | 0.01 | 0.03239 |
| GO:0002252 | immune effector process | 1 | 0.01 | 0.03239 |
| GO:0009615 | response to virus | 1 | 0.01 | 0.03239 |
| GO:0044248 | cellular catabolic process | 4 | 2.02 | 0.03739 |
| GO:0009263 | deoxyribonucleotide biosynthetic process | 1 | 0.05 | 0.04176 |

| | | | | |
|---|---|---|---|---|
| GO:0009129 | pyrimidine nucleoside monophosphate metabolic process | 1 | 0.03 | 0.04391 |
| GO:0009130 | pyrimidine nucleoside monophosphate biosynthetic process | 1 | 0.03 | 0.04391 |
| GO:2001141 | regulation of RNA biosynthetic process | 2 | 1.92 | 0.04394 |
| GO:0006355 | regulation of transcription, DNA-templated | 2 | 1.92 | 0.04394 |
| GO:1901361 | organic cyclic compound catabolic process | 1 | 0.66 | 0.04515 |
| GO:1901135 | carbohydrate derivative metabolic process | 3 | 1.48 | 0.04554 |
| GO:0009101 | glycoprotein biosynthetic process | 1 | 0.27 | 0.04786 |
| GO:0006278 | RNA-dependent DNA biosynthetic process | 1 | 0.04 | 0.04812 |

**Pathways enriched with differentially expressed genes**

| KO.ID | Pathway | Number of DEGs | $P_{-adjusted}$ |
|---|---|---|---|
| ko00512 | Mucin type O-Glycan biosynthesis | 11 | 0.00000467 |
| ko00520 | Amino sugar and nucleotide sugar metabolism | 11 | 0.010131728 |
| ko00480 | Glutathione metabolism | 10 | 0.019769695 |

**37ºC-3 h vs. 33ºC-3 h**

**Biological process categories enriched with up-regulated genes**

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0044260 | cellular macromolecule metabolic process | 12 | 7.35 | 0.00029 |
| GO:0031323 | regulation of cellular metabolic process | 2 | 1.59 | 0.0016 |

| | | | | |
|---|---|---|---|---|
| GO:0080090 | regulation of primary metabolic process | 2 | 1.61 | 0.0036 |
| GO:0010468 | regulation of gene expression | 2 | 1.34 | 0.00927 |
| GO:0032774 | RNA biosynthetic process | 2 | 1.42 | 0.01263 |
| GO:0016070 | RNA metabolic process | 3 | 2.68 | 0.0133 |
| GO:0010467 | gene expression | 4 | 3.56 | 0.01337 |
| GO:0051171 | regulation of nitrogen compound metabolic process | 2 | 1.27 | 0.01444 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 2 | 1.27 | 0.01518 |
| GO:0006351 | transcription, DNA-templated | 2 | 1.41 | 0.01759 |
| GO:0051252 | regulation of RNA metabolic process | 2 | 1.1 | 0.03588 |
| GO:0016072 | rRNA metabolic process | 1 | 0.28 | 0.03862 |
| GO:2001141 | regulation of RNA biosynthetic process | 2 | 1.07 | 0.04243 |
| GO:0006355 | regulation of transcription, DNA-templated | 2 | 1.07 | 0.04243 |
| GO:0034470 | ncRNA processing | 1 | 0.4 | 0.0486 |
| **Biological process categories enriched with down-regulated genes** | | | | |
| No biological process categories significantly with down-regulated DEGs | | | | |
| **Pathways enriched with differentially expressed genes** | | | | |
| **KO.ID** | **Pathway** | **Number of DEGs** | ***P*-*adjusted*** | |
| ko00500 | Starch and sucrose metabolism | 3 | 0.010740943 | |
| ko04141 | Protein processing in endoplasmic reticulum | 6 | 0.000999716 | |
| **37ºC-24 h vs. 37ºC-3 h** | | | | |
| **Biological process categories enriched with up-regulated genes** | | | | |
| No biological process categories significantly with up-regulated DEGs | | | | |
| **Biological process categories enriched with Down-regulated genes** | | | | |

| GO.ID | Term | Significant value | Expected value | KS value |
|---|---|---|---|---|
| GO:0044260 | cellular macromolecule metabolic process | 19 | 11.22 | 0.00011 |
| GO:0031323 | regulation of cellular metabolic process | 4 | 2.43 | 0.0016 |
| GO:0080090 | regulation of primary metabolic process | 4 | 2.46 | 0.0036 |
| GO:0010468 | regulation of gene expression | 4 | 2.05 | 0.00973 |
| GO:0032774 | RNA biosynthetic process | 3 | 2.17 | 0.01325 |
| GO:0010467 | gene expression | 6 | 5.44 | 0.01365 |
| GO:0051171 | regulation of nitrogen compound metabolic process | 3 | 1.94 | 0.01444 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | 3 | 1.93 | 0.01518 |
| GO:0009894 | regulation of catabolic process | 1 | 0.33 | 0.01672 |
| GO:0006351 | transcription, DNA-templated | 3 | 2.15 | 0.01842 |
| GO:0031329 | regulation of cellular catabolic process | 1 | 0.31 | 0.02279 |
| GO:0016485 | protein processing | 1 | 0.2 | 0.0311 |
| GO:0044248 | cellular catabolic process | 2 | 1.73 | 0.03537 |
| GO:0006457 | protein folding | 13 | 0.59 | 0.03613 |
| GO:0051252 | regulation of RNA metabolic process | 3 | 1.67 | 0.03721 |
| GO:0016072 | rRNA metabolic process | 1 | 0.42 | 0.04102 |
| GO:2001141 | regulation of RNA biosynthetic process | 3 | 1.64 | 0.04243 |
| GO:0006355 | regulation of transcription, DNA-templated | 3 | 1.64 | 0.04243 |

**Pathways enriched with differentially expressed genes**

| KO.ID | Pathway | Number of DEGs | $P_{\text{-adjusted}}$ |
|---|---|---|---|
| ko04010 | MAPK signaling pathway | 3 | 0.006702441 |

| | | | |
|---|---|---|---|
| ko04141 | Protein processing in endoplasmic reticulum | 9 | 3.4188E-05 |
| **37°C-24 h vs. 33°C-24 h and 33°C-24 h vs. 33°C-3 h** | | | |
| **Biological process categories enriched with up-regulated genes** | | | |
| No biological process categories significantly with up-regulated DEGs | | | |
| **Biological process categories enriched with down-regulated genes** | | | |
| No biological process categories significantly with down-regulated DEGs | | | |
| **Pathways enriched with differentially expressed genes** | | | |
| No pathway significantly with DEGs | | | |

**Table S3.** All differential metabolites in *Sinonovacula constricta* after heat hardening treatments.

Click here to download Table S3