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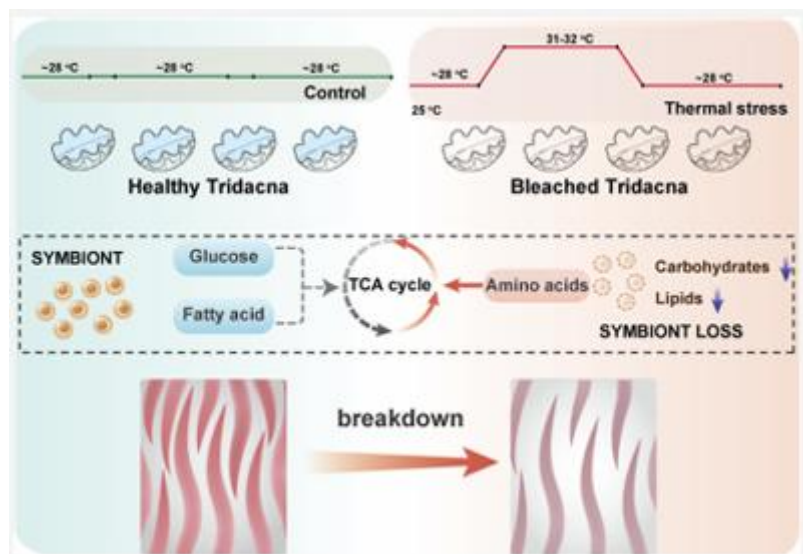
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Metabolic shifts and muscle remodeling as pro-survival and energy compensation strategies in photosymbiotic giant clams after bleaching

Fan Mao,# Shu Xiao,# Xin Dang,# Guoxin Cui, Juan Diego Gaitán-Espitia, Vengatesen Thiyagarajan, Jeremie Vidal-Dupiol, Wenjie Yi, Xiaoyang Jin, Ziniu Yu, and Yang Zhang*

ABSTRACT:

Tropical photosymbiotic giant clams are increasingly threatened by climate change, leading to widespread bleaching. Decline in density of symbionts caused mortality events in symbiotic organisms; however, giant clams appear to exhibit prior survival capacity against these detrimental effects. It remains unclear whether giant clams can mitigate the adverse impacts of bleaching. Herein, we found that bleaching events after chronic heat stress induce remarkable changes and remodeling in symbiotic tissue of the giant clam *Tridacna crocea*. The density and structure of unique muscle fibers with high collagen content, observed in siphonal mantle of these animals, were negatively altered after heat stress. These changes were associated with a metabolic shift from carbohydrates and fatty acids to amino acids as the breakdown of collagen-rich muscle fibers can partially compensate for energy loss during bleaching. Such a shift was proposed to be regulated by AMPK activated protein kinase (AMPK) signaling and FoxO-atrogin pathways. Overall, our study highlights a pro-survival mechanism in giant clams through plastic regulation, which likely contributes to their relatively high environmental resilience during bleaching.



KEYWORDS:

tropical photosymbiotic giant clams, climate change, bleaching, symbiotic tissue (siphonal mantle), pro-survival mechanism, collagen-rich muscle fibers

■ INTRODUCTION

Giant clams (subfamily Tridacninae) are conspicuous tropical bivalves distributed across the Indo-Pacific.^{1,2} Their unique symbiotic relationship with *Symbiodinium zooxanthellae* contributes to their ecological success in nutrient-limited tropical waters.¹ Meanwhile, giant clams play an essential role in ecosystem and marine biodiversity of coral reefs, including shaping the physical structure of the reefs, serving as fish nurseries,^{3,4} representing a significant biomass as predators, scavengers, and even epibionts.⁵⁻⁸ Despite the valuable services and functions provided by these animals to coral reefs and traditional fishery communities in this region,^{8,9} their populations have been drastically impacted by overexploitation and the increasing threat of global change stressors (e.g., ocean warming).¹⁰ Analogous to corals, giant clams are exceedingly susceptible to multiple climate change factors, including global warming, ocean acidification, and reduced availability of carbonate ions.¹¹⁻¹⁵ As a result, host giant clams could deteriorate to bleaching symptoms under extreme environmental stress, leading to population decline or mass mortality.

The functioning, survival, and growth of these animals rely heavily on the stability of their symbiotic relationships with photosynthetic dinoflagellates (family Symbiodiniaceae).¹⁸ This close partnership is not merely a simple coexistence between species but rather a complex network of energy and nutrient supply. As photosynthetic organisms in an extracellular symbiotic relationship, juvenile giant clams that have not yet established symbiosis initially filter *Symbiodinium* from seawater through their gills. These symbionts then migrate to the digestive gland before ultimately colonizing the mantle tissue, where they settle and conduct photosynthesis,¹⁹ releasing organic compounds (e.g., glucose) that are then absorbed by the host and converted into the energy needed for growth and survival.^{20,21} Through this process, giant clams can obtain the vast majority (more than 50%) of their carbon and nutrient requirements, with a minor contribution (8 to 14%) from their filter-feeding activity.²² Similar to other autotrophic marine animals (e.g., corals), giant clams can also support their nutritional requirements by “farming” and digesting their associated dinoflagellates.²³⁻²⁷ All of these diverse nutritional strategies contribute to the ecological success of these animals in tropical regions.²⁸ However, with the increasing pressure of global climate change, giant clams are experiencing warmer oceans and more frequent, intense, long-lasting heatwaves.^{3,10} The negative effects of these threats^{3,29,30} impact the fine-tuned nutritional interactions of giant clams with their photosynthetic dinoflagellates, inducing bleaching and massive die-off. It remains unclear how the mechanisms regulate the nutritional state, the physiological responses, and resilience of giant clams during thermal-induced bleaching events. Such information is critically important for conserving and restoring giant clam communities under global climate change.

High temperatures, beyond thermal tolerance limits, can induce multiple physiological impairments to giant clams, negatively affecting their respiration, biomineralization, symbiont density, and reproduction.^{31,32} These outcomes arise from excessive oxidative stress in giant clams, which leads to the inhibition of their antioxidant capacity, the activation of apoptotic pathways, and consequently, the disruption of their host-associated symbionts.^{31,33,34} Such negative effects can also be reflected in the alterations of fatty acid profiles and the nutritional conditions of the giant clams, aspects with profound implications for their physiological resilience and long-term acclimatization to elevated temperatures.^{35,36} Interestingly, it has been documented that the degradation of symbionts colonizing giant clams can deliver an additional energy source to the hosts, aiding them to cope with heat stress.³⁵ However, this physiological and nutritional strategy is constrained by the level of heat stress as elevated temperatures significantly reduce the density of symbiotic algae within bleached giant clams, impacting the capacity for “farming” algae as a crucial energy source. Despite these efforts to understand the functional and nutritional effects of heat stress on giant clams, there are still major knowledge gaps regarding the regulation of energy sources used when giant clams are completely bleached. Notably, during this period, the anomalies in sea surface temperature (SST) do not cause

immediate mortality of giant clams,^{37–40} even giant clams face physiological challenges to sustain their functional operation due to the limited energy supply. It is known that heterotrophy provides very limited energy to giant clams;²² therefore, further insights into the unique energy compensation strategies for these giant clams to overcome this energy limitation period are essential to explain pathways for acclimatization (e.g., adaptive plasticity) and adaptation to warming oceans.

To understand the responsive mechanisms underpinning thermal stress and nutritional adjustments in giant clams during bleaching events, we experimentally assessed physiological and molecular phenotypes in *Tridacna crocea*. *T. crocea*, commonly referred to as the “boring clam”, is the smallest species within its genus and is typically found in shallow reef areas, including the South China Sea.⁴¹ This giant clam is strongly affected by abnormally high temperatures such as those experienced during El Niño events, which trigger bleaching and high mortalities not only for the giant clams but also for the coral reefs they inhabit.⁴² Given that *T. crocea* serves as a reef-building organism in the South China Sea, knowledge on the heat and bleaching stress responses of this species would be informative in assessing their survival and in developing conservation strategies. We experimentally induced bleaching in *T. crocea* through chronic heat stress (based on both projected global warming and the maximum SST experienced at South China Sea coral reefs during 2010–2021⁴³). Herein, we focus on the physiological phenotypes and histological alterations of *Tridacna* hosts to characterize their responses to prolonged thermal stress, revealing significant changes at the tissue level in the siphonal mantle, which houses symbiotic dinoflagellates. To perform comprehensive analyses of phenotypic changes, we further conducted multiple integrated omics analyses to identify the primary molecular and cellular changes underlying the mechanism of response to bleaching events. Consequently, a key mechanism that aids in energy compensation for bleached giant clams has been proposed as being essential for the survival of this species under thermal stress. This is particularly concerning as it represents a crucial step in understanding how giant clams contribute to healthy coral reefs ecosystem.

Brief Materials and Methods.

Visibly healthy giant clams *Tridacna crocea* (an average wet weight of approximately 200 g, 8–10 cm in length) were collected from the Xisha Islands (also known as the Paracel Islands) in the South China Sea in September 2021 and then transported to the Tropical Marine Biological Research Station in Hainan, China for experimentation. The giant clams were maintained in outdoor seawater flow-through systems under specific conditions. A thermal stress experiment (Figure 1A) was conducted on 80 individuals started on October 14, 2021, split into control and experimental groups, and was structured to include a 7 day acclimatization period at ambient temperature, followed by a 4 day exposure to a temperature increment of 3 °C above ambient temperature to simulate the effects of global warming. The recovery phase was extended for another 4 days, during which the temperatures were gradually lowered, and the survival status of each clam was meticulously recorded on a daily basis. The control group was kept at 28 °C, while the experimental group was exposed to 31–32 °C, reflecting projected global warming increases (IPCC2021) and recorded South China Sea coral reef temperatures (2010–2021).⁴³ Based on the mantle color post temperature treatment, the bleaching status of *T. crocea* individual is categorized as (a) healthy, (b) moderately bleached when < 50% of the mantle appears white, and (c) severely bleached when > 50% is white (Figure 1B).

Another 80 giant clams specifically underwent a replicated experimental design as the first mesocosm experiment to obtain bleached and healthy individuals for physiological parameters measurement and multiomics analysis. Tissue samples were procured from both healthy and bleached individuals to conduct a battery of tests including detailed histological assessments using hematoxylin and eosin staining, ultrastructural examinations with transmission electron microscopy (TEM) to visualize cellular damage, and metabolomic profiling to quantify metabolic perturbations. The number of samples for different experiments can be seen in Figure 1E. RNA extraction from different tissues was

followed by transcriptome sequencing using the Illumina platform to capture the gene expression landscape.

The photosynthetic efficiency was evaluated using pulse amplitude-modulated (PAM) fluorometry, which provided real-time measurements of photosystem II activity, while algal symbiont density was determined through standardized epifluorescence microscopy counting techniques. The metabolic rate was assessed by measuring oxygen consumption and ammonia excretion rates, which were monitored using the Winkler method⁴⁴ method,⁴⁵ and the sodium hypobromite oxidation respectively. These physiological metrics offered a comprehensive view of the giant clams' responses to thermal stress.

For the advanced omics analyses, the transcriptomic data underwent differential gene expression analysis using tools like DESeq2,⁴⁶ and pathway enrichment was conducted through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to unravel the molecular pathways affected by thermal stress. Moreover, we performed single-nucleus RNA sequencing (snRNA-seq) on the siphonal mantles of two healthy and two bleached giant clams using 10× Genomics Chromium Single-cell Gene Expression V3 technology. Nucleus isolation, library preparation, and sequencing were conducted by Gene Denovo Biotechnology Co. Ltd. Data were preprocessed using Cell Ranger (v3.1.0) and aligned to the *T. crocea* reference genome (CRA016517). UMI count matrices were analyzed with Seurat v3.1.4, and clusters were visualized using *t*-SNE. Significantly upregulated genes (≥ 1.28 -fold change, expressed in $>25\%$ of cells in the target cluster, $p < 0.05$) were subjected to GO and pathway enrichment analyses. Cluster-specific marker genes were manually identified, and functional annotation of each cluster was performed based on enrichment results.

Raw data for transcriptome have been submitted to Genome Sequence Archive (<https://ngdc.cncb.ac.cn/gsa/>), accession no. CRA015434. Raw data for snRNA-seq has been submitted to Genome Sequence Archive (<https://ngdc.cncb.ac.cn/gsa/>), accession no. CRA015534. Raw data for *T. crocea* reference genome has been submitted to Genome Sequence Archive (<https://ngdc.cncb.ac.cn/gsa/>), accession no. CRA016517 (the article for this has not been published). Data presentation and statistical analysis were conducted using GraphPad Prism 8.0 software, with detailed experimental materials and methods provided in the Supporting Information.

■ RESULTS

Severity and Survival of Bleached Giant Clams Subjected to Thermal Stress.

To examine the bleaching and recovery rates of giant clams, we designed a chronic heat stress exposure and recovery experiment (Figure 1A). During the 30 day exposure period, bleaching severity (Figure 1B) was significantly different between the thermal stress group (31–32 °C) and the control group (28 °C). The onset of *T. crocea* bleaching was observed on the fifth day of the chronic heat exposure period. By the end of the exposure period, 65% (26 individuals) of the thermal stress group exhibited bleaching (chi-square test, $p < 0.0001$), with 32.5% showing moderate bleaching and another 32.5% showing severe bleaching (Figure 1C).

The 38 surviving individuals in each experimental group (control and thermal stress) were subsequently subjected to a 40 day recovery period. A total of 14 individuals died in the thermal stress group, including two nonbleached ones. Specifically, 53.85% (7/13) of severely and 38.46% (5/13) of moderately bleached giant clams died during the recovery period in this group. Thus, during the recovery period, the mortality rate was approximately one-third (14/38) in total, evidencing a significant effect of the heat stress on survival postexposure (chi-square test, $p < 0.0001$, Figure 1D). Notably, nearly half of the bleached giant clams (12/26) regained their color and symbionts by day 40 of the recovery period. In particular, nine of the bleached giant clams exhibited full recovery of their mantle color, while three giant clams showed partial recovery, indicating moderate to high resilience

of *T. crocea* to bleaching (Figure S1). Since the individuals from the first mesocosm experiment required observation during the recovery period and were unavailable for sampling, a parallel repeated thermal stress experiment was conducted concurrently with the first for physiological and omics analysis. Following the heat stress exposure period, the results included 15 severely bleached giant clams, 10 moderately bleached individuals, one mortality, and 14 clams that remained visually healthy. This experiment was designed to investigate the mechanisms underlying bleaching at the cellular, tissue, and organ levels (Figure 1E).

Physiological and Histological Changes in Bleached Giant Clams.

To assess the impact of bleaching on the physiological status of *T. crocea*, we evaluated their oxygen production and consumption in both bleached and healthy giant clams. The latter group showed a consistent increase in oxygen production (2.30 ± 0.94 to 4.77 ± 1.95 mg/g·h) with increasing time duration. In contrast, severely bleached giant clams exhibited a decrease in oxygen production (-0.82 ± -0.34 to -0.99 ± -0.41 mg/g·h when counting for oxygen consumption, Figure 2A), suggesting the decrease of oxygen production by the photosynthesis. Photosynthesis in bleached giant clams was significantly compromised, showing a reduction in the maximum photochemical efficiency (F_v/F_m , two-way ANOVA, $p = 0.004$, Figure 2B), although the direct light photosynthetic efficiency (F_v'/F_m') remained unchanged. Symbiodiniaceae density drastically decreased from 3216 ± 307 in healthy giant clams to 100 ± 42 cells/mm² in bleached ones (Figure 2C; t -test, $p < 0.0001$). The histological analysis showed a depth-related decline in the presence of symbiotic algae from epidermal layer to inner tissue (Figure S2). These results indicate that bleaching in giant clams leads to significant changes in oxygen production, photosynthesis, and symbiont density, suggesting associated physiological and structural changes.

Interestingly, we found that bleaching also modified the fiber structures of the siphonal mantle of the giant clams, which is the main tissue of these animals colonized by symbionts. Such alteration resulted in marked morphological changes (Figures 2D and S3), and the significant decline of the amount of collagen-fiber-encapsulated myofibers in the bleached giant clams (i.e., 52% to less than 20%) compared to control giant clams (Figure 2E; t -test, $p < 0.001$). TEM imaging revealed fractured fibers in the siphonal mantle of bleached giant clams (Figure 2D). Histological analysis of giant clams recovered from severe bleaching revealed partial reconstitution of the fiber architecture in the siphonal mantle (Figure 2F), suggesting a plastic response, including the recolonization of symbiotic dinoflagellates and siphonal mantle reconstitution.

Reduction of Specific Muscle Cells Revealed by snRNA-Seq.

Considering that the siphonal mantle is the primary tissue for dinoflagellate colonization and the organ most affected by bleaching, we examined alterations in the cellular architecture of the siphonal mantle in giant clams using single-nucleus RNA sequencing (snRNA-seq). This allowed us to further investigate the complex cellular and potential functional changes associated with the morphological changes in the mantle following bleaching. We generated a cell atlas comprising 17,758 cells of siphonal mantle from both the healthy and bleached groups. Symbiont reads were not analyzed because the number of symbiont cells obtained from bleached clams was extremely low, making it challenging to robustly assess their gene expression profiles. Due to the fact that current single-cell cell marker databases primarily target humans and mice, we manually defined functional and marker genes for giant clams. This was based on the differentially expressed gene markers of the cell clusters and a combination of literature searches, following previous studies.^{47,48} As consequence, 14 unique cell clusters and key cell types were identified (Figure S4), including muscle cells (mantle-specific muscle cells and smooth

muscle cells), iridocytes, fibroblasts, epidermal cells, endothelial cells, and nutrition exchange cells (Figure 3A and Table S1).

Muscle cells, characterized by the expression of specific marker genes (Ttn, Myo, DOCK, and Cd109), were predominant, representing 46.81% of the cells in healthy giant clams but only 4.85% in bleached giant clams (Figure 3B; two-way ANOVA, $p < 0.0001$). This cell type simultaneously expressed fibroblast marker genes (Cdh23 and PCDH11Y) as significantly upregulated genes, which are cell adhesion molecules to produce connective tissue substance. More importantly, Col15a1 was also defined as specific marker genes of muscle cells, further suggesting the unique characteristic of muscle cells and their potential facilitation of muscle fiber contraction and relaxation. Interestingly, the reduction of this cell type parallels the significant decrease in myo-collagen fibers observed histologically in the mantle of the bleached giant clams (Figure S5). Concurrently, the number of epithelial cells identified by the ammonia transporter genes Rh family B glycoprotein (*Rhbg-a* and *Rhbg-b*) increased significantly during bleaching (Figure 3B; two-way ANOVA, $p < 0.0001$). In line with the bidirectionality of these transporters,⁴⁹ the ammonia excretion rates decreased from negative values in healthy giant clams (-1.35 ± -0.55 to $-0.31 \pm -0.13 \mu\text{g/g}\cdot\text{h}$) to positive values in bleached giant clams (6.25 ± 2.55 to $6.71 \pm 2.74 \mu\text{g/g}\cdot\text{h}$, Figure 3E), indicating a metabolic shift and a reduction in nitrogen assimilation during the bleaching process. Furthermore, it is noteworthy that there was a significant increase in the recovery of iridophores and immune cells in bleached clams. However, histological observations could not confirm an absolute increase in the number of cell types. Therefore, the observed increase in their proportion at the single-cell level may be attributed to the substantial reduction in muscle cell types.

Metabolic Shifts Associated with Giant Clam Bleaching.

To explore the organismal metabolic states associated with tissues and cellular alterations, we identified 1733 metabolites -comprising 1450 lipids, 65 amino acids and peptides, and 25 carbohydrates- from the siphonal mantle of healthy and bleached giant clams using targeted metabolomics (Figures 4A and S6). These metabolites represent the holobiont, encompassing contributions from both the host and its symbionts. Among the identified lipids, fatty acids, which serve as the main energy storage, were highly abundant and exhibited dramatic reductions following bleaching (Figure 4B). Moreover, the (O)PLS-DA model pinpointed 575 metabolites exhibiting significant difference ($\text{VIP} \geq 1$; Figures 4C and S7). The abundance of phosphatidylcholine (PC), a subclass of phospholipids essential for cell membrane structure, particularly the 22:6- or 20:4-containing PC, saturated PC, and lysophosphatidylcholines (LPCs), changed significantly following bleaching (Figure 4D). However, the overall abundance of PC remained relatively stable during the bleaching process (Figure 4B). Given the substantial metabolic overlap between host- and symbiont-derived compounds, the distinct phospholipid remodeling patterns were tightly coupled with heat stress/bleaching progression. These identified PCs and LPCs have the potential to act as biomarkers for bleaching-induced modifications in membrane lipid profiles.⁵⁰

Additionally, major energy sources, such as carbohydrates and amino acids, significantly decreased in the bleached giant clams, with carbohydrate levels decreasing by 80% (from $267.67 \pm 14.59 \mu\text{mol/g}$ in healthy giant clams to $54.62 \pm 15.64 \mu\text{mol/g}$ in bleached giant clams; Figure 4B). Interestingly, among 105 upregulated metabolites identified (fold change > 2 , p value < 0.05), only 20 were low-molecular weight metabolites. Half of these molecules (10/20) were amino acids and their metabolic intermediates and derivatives (Figure 4C, red), indicating that the bleaching-induced metabolic shift was predominantly driven by amino acids. Furthermore, four well-known metabolites (AMP, myo inositol, piperine, and folate) involved in regulating metabolic pathways were significantly upregulated after bleaching, responding to the metabolic stress state induced by the loss of symbionts. On the other hand, 247 metabolites exhibited a significant reduction (fold change < 0.5 , p value < 0.05). In addition to the three primary energy substances provided by the symbionts, there were also other 63

metabolites that showed a significant decrease following the bleaching of the giant clam. Considering that the metabolites are derived from the holobiont, those significantly reduced in the bleached giant clam may be attributed to substances originating from the symbionts.

Transcriptome Dynamics Explain the Metabolic Shift during Bleaching.

To understand metabolic dynamics during bleaching, we generated gene expression profiles of five different tissues (i.e., siphonal mantle, inner mantle, hemocytes, ctenidium, and digestive gland) from bleached and healthy giant clams, focusing on signaling and metabolic pathway alterations (Figures S8–S10). Strikingly, a set of genes involved in glucose and fatty acid metabolism (β oxidation) were identified across various tissues in the bleached giant clams (Figure 5). The expression changes of these genes vary across different tissues; however, overall, these genes are predominantly downregulated. Specifically, the expression levels of 6-phosphofructokinase (PFKM) and carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzymes in glycolysis, and fatty acid β -oxidation, respectively, were markedly reduced, highlighting the depletion of lipids and carbohydrates as primary energy sources in bleached giant clams.

We also observed significant activation of genes involved in amino acid mobilization. Isovalerylcarnitine, an intermediate of leucine deamination, exhibited a remarkable 9.47-fold increase in bleached giant clams (Figure 4C), which could be attributed to the upregulation of deaminase enzymes (BCAT and BCKAD) across various tissues. This activation pattern extended to glutamate, valine, glycine, and cysteine metabolism (Figure 5). In line with the upregulation of ammonia excretion (Figure 3D,E), these results further suggest an overall metabolic shift from carbohydrate and lipid metabolism to amino acid metabolism in bleached giant clams.

AMPK Governs Metabolic Shift and Muscle “Atrophy” during Bleaching.

In bleached giant clams, activation of the adenosine 5'-monophosphate triggered the protein kinase (AMPK) signaling pathway, with the AMP level increasing over 6-fold (Figures 6A and S10). This indicates that the AMPK pathway plays a key role in maintaining the energy balance. Based on regulatory network analysis, we detected potential interactions among the AMPK, the FoxO, and the PI3K-Akt pathways (Figure S11), both of which are crucial for metabolic regulation. Specifically, the FoxO pathway regulates the expression of atrogin, a muscle-specific F-box protein whose expression increased 9.03-fold in bleached giant clams, and thus might involve in myofiber size regulation. This highlights the central role of the AMPK pathway in metabolic control in the bleached giant clams.

■ DISCUSSION

Global climate change, particularly global warming, threatens the symbiotic relationship between giant clams and their symbionts. Although giant clams and corals belong to distinct evolutionary lineages and have diversified over more than 500 million years,^{51,52} both have evolved photosymbiosis through^{13,15,28,53} convergent evolution and hence suffer similar heat-induced bleaching events. Herein, we demonstrate that thermal stress leads to a sharp decrease in the symbiont algal density with cascade effects on the nutritional dynamics of the hosts. Furthermore, the three primary metabolic substrates (i.e., carbohydrates, lipids, and proteins) in the giant clams exhibited significant reductions, thereby inducing a state of host starvation. This starvation activates the conserved AMP AMPK energy regulation pathway, inhibiting the synthesis of muscle proteins. Moreover, elevated AMP levels activate AMPK, which in turn suppresses mTOR activity, thereby reducing energy consumption and promoting energy production. This is also a core network of cellular metabolic regulation in cnidarian-dinoflagellate symbiosis.⁵⁵ Under such conditions, the FoxO-atrogin pathway may facilitate muscle protein degradation, disrupting the balance between protein synthesis and degradation and leading

to a marked reduction in mantle muscle cell numbers. The resulting amino acids generate ketone bodies through deamination, potentially compensating for the TCA cycle. This process, in turn, can produce endogenous toxic NH_4^+ but may be excreted by Rhbg a and Rhbg-b in epithelial cells (Figure 6B). These cellular and molecular changes are thus proposed to be considerable adaptive modifications to cope with adverse environmental conditions, gradually alleviating the energy limitations caused by thermal stress and bleaching.

While this stress response parallels those observed in most symbiotic cnidarians,⁵⁶ our data indicate that certain mechanisms underlying the giant clams' response to bleaching stress reflect the unique characteristics of this bivalve group. Notably, the "atrophy" of the musculature in the symbiont colonized siphonal mantle suggests that the storage of structural proteins may function as an energy buffer pool, thereby counteracting bleaching-induced starvation and enhancing stress resilience. Taken together, these findings suggest a potential pro-survival mechanism involving muscle fiber degradation and shifts in amino acid metabolism in bleached giant clams.

Unique Myofibril Structure Serves as an Energy Buffer Pool.

Histological analysis of molluscan mantle revealed that muscle fiber here is morphologically recognized as smooth muscle organized in a parallel and compact arrangement.⁵⁷⁻⁵⁹ In contrast, the muscle fiber in the siphonal mantle of giant clams exhibits distinct characteristics with a looser and more scattered arrangement, particularly at the mantle edge where a high density of symbiotic dinoflagellates is present. Our further investigation revealed that the spaces between the muscle fibers are filled with densely packed, regularly arranged collagen structures characterized as collagen-wrapped muscle fibers. While collagen is typically found in the intramuscular connective tissue of animals, helping to maintain structural integrity,⁶⁰ the content of this protein was remarkably elevated in the *Tridacna* mantle. This phenomenon is unusual and is more commonly associated with pathological conditions like smooth muscle tumors or cardiomyocytes in rheumatoid arthritis models.^{61,62} Given that the collagen fibers within the inner muscle membrane are arranged in a wave-like pattern, allowing for flexibility under tension,^{63,64} we propose that the abundant collagen fibers in the siphonal mantle plays a crucial role in enhancing its extensibility. This allows for an increased surface area for light reception, enabling the tissue to plastically adjust and maintain efficient photosynthesis within the symbiotic relationship. Correspondingly, the extensibility of the mantle in bleached giant clams may be diminished, potentially establishing a negative feedback loop that exacerbates the photosynthetic impairment. However, this hypothesis necessitates further empirical validation.

Collagen, besides providing structural elasticity, is some times regarded as a vital amino acid source under nutritional limitation, increasing fumarate levels, which may enter the tricarboxylic acid cycle to supply nutrients for cancer cell proliferation.⁶⁵ Despite the physiological differences between cancer and the mantle of *Tridacna*, both feature a rich collagen fiber composition, prompting us to investigate additional functions of the collagen network in *Tridacna*'s mantle. Furthermore, glycoproteins and collagen matrices metabolism in an autotrophic marine animals (e.g., the photosynthetic symbiotic jellyfish *Cassiopea*) supply energy to the host during thermal stress-induced carbon starvation, further leading to host shrinkage.⁶⁶ The extensive mesoglea of *Cassiopea* endows it with the capacity to delay starvation, thereby conferring a greater tolerance to starvation compared to other symbiotic cnidarians. Given *Tridacna*'s unique myofibrillar architecture and significant phenotypic changes after bleaching, we hypothesize that its collagen matrix may also serve as a crucial energy reservoir. Although the depletion of this structural protein in the mantle may compromise its extensibility and negatively affect the symbiotic relationship with dinoflagellates and overall photosynthetic efficiency, our result indicates that the myofibrillar structure in bleached giant clams is resilient if they regain their symbionts and health. Therefore, the underlying mechanisms involved in this recovery process warrant thorough investigation in future research.

Bleached *Tridacna* Is Trapped in a State of Starvation and Metabolic Shift.

In contrast to the control condition (i.e., 28 °C), heat-stress-induced bleaching resulted in energy limitation and starvation of the hosts with the drastic decrease of three primary fuel substances. Herein, we must consider that the diminished substances may be ascribed to the symbionts as their loss during the bleaching process leads to a reduced detection within the holobiont. However, the current data cannot elucidate the relationship between bleaching and metabolites other than the three primary energy substances. However, our metabolomics and transcriptomic findings revealed active physiological adjustments evidenced by metabolic shifts within the giant clam holobiont during bleaching. Specifically, multiple catabolic degradation pathways for various amino acids were activated, while the mobilization of glucose and fatty acids was downregulated. Generally, amino acids are utilized for fuel through deamination, yielding a carbon skeleton (usually α -keto acid) that enters the citric acid cycle.^{67,68} Consistently, we observed the accumulation of diverse intermediate metabolites in bleached giant clams, such as picolinic acid (a product of tryptophan catabolism) and isovalerylcarnitine (a secondary metabolite of leucine), with corresponding upregulation of the enzymes tryptophan 2,3 dioxygenase, BCKAD, and BCAT,⁶⁹⁻⁷¹ supporting the hypothesis of the metabolic shift from glucose and fatty acids to amino acids.

Recent studies suggest that disruptions in the nutritional cycle significantly lead to symbiont loss under thermal stress.^{56, 66} In other autotrophic marine animals (e.g., corals), increased energy demands during thermal stress trigger amino acid metabolism, releasing ammonia (NH_4^+) and promoting the rapid growth of symbiotic algae. This reduces the availability of carbon for the host and creates a positive feedback loop that accelerates bleaching.⁵⁶ While we observed activation of amino acid metabolic pathways in giant clams under nutrient limitation, our experiments did not confirm whether a similar disruption occurs during thermal stress in these animals. However, we noted an increased level of ammonia release linked to amino acid metabolism activation. Amino acid metabolism can generate significant ammonia (NH_4^+) through deamination, which is more soluble in lipids and can harm aquatic organisms, particularly bivalves, increasing their vulnerability to ammonia toxicity.⁷²⁻⁷⁴ It has been reported that the ability of giant clams to assimilate ammonium is diminished in bleached individuals,³⁸ a net increase in the release of NH_4^+ indicating during bleaching events. This is further supported by the significant upregulation of ammonia transporters *Rhbg-a* and *Rhbg-b* in the epidermal cells of the bleached giant clam mantle, suggesting that they may need to enhance NH_4^+ release to mitigate the toxicity associated with this energy compensation strategy.

Starvation Tolerance Might Determine the Environmental Resilience of Giant Clams.

While some coral reef symbiotic organisms such as sponges and cnidarians are highly sensitive to prolonged thermal stress and the associated bleaching and starvation effects,⁷⁵ bivalves tend to exhibit greater tolerance to such physiological challenges. For example, without energy replenishment, *Cassiopea* jellyfish experience increased mortality within short periods (e.g., 7 days) of thermal stress exposure.⁶⁶ Contrasting, we found that giant clams had low-to-moderate mortality after chronic (i.e., 30 days) exposure of thermal stress. Moreover, bleached animals evidenced low mortality (one-third), even without feeding during the recovery period. Higher starvation-tolerance in bivalves is supported by studies documenting their capacity to survive for more than 120 days without food supply.⁷⁶ It has been proposed that this capacity of marine bivalves to cope with long-term food restrictions relies on the regulation of various physiological processes, including the use of body components for energy.⁷⁷⁻⁷⁹ Given the limited heterotrophic capacity of bleached giant clams, activating amino acid metabolism through the consumption of tissue muscle and collagen fibers is crucial for their starvation and stress tolerance, enabling them to enhance their resilience and survival in warming oceans. In contrast to typical heterotrophic bivalves, *tridacna* -capable of both autotrophic and heterotrophic

feeding- exhibit a higher proportion of carbon deposition for tissue growth,⁸⁰ potentially enhancing their energy storage and stress resilience.³⁶

Apart from their tolerance to fuel restrictions, tridacna also exhibit strong bleaching recovery capabilities. Research has reported that different tridacna species can recover from bleaching after several months. *Tridacna gigas*, for example, can even regain the color of its mantle after being bleached for eight months.³⁷⁻³⁹ Our experiments also indicate that *T. crocea* can recover (partially to complete) from bleaching after heat stress exposure (42 day recovery period), similar to other giant clam species (e.g., *Tridacna maxima* after a 24 day recovery period⁴⁰). These data suggest that when temperatures return to normal, these animals can rebuild their dinoflagellate populations to restore mantle color and photosynthesis.⁸¹ Although bleached giant clams appear completely white, our analyses reveal that their mantle tissues still contain small amounts of dinoflagellates, which may serve as a seed for recovery. Additionally, the well-recovered fibrous structures, which are evidenced to correlate with energy expenditure, further attest to their resilience. Hence, Tridacna exhibit heightened resistance and recovery from bleaching events, indicative of robust environmental resilience.

Currently, despite sustainable fisheries management measures to reduce overfishing and habitat degradation of coral reefs,^{82,83} changes in environmental conditions remain the primary threat to giant clam populations. In such scenarios, assessments of mortality and resilience under heat stress and bleaching can help us to understand which animals are better equipped to cope with these threats. While the host's metabolic shift and breakdown of collagen-rich muscle fibers can partially compensate for energy loss during bleaching in giant clams, not all individuals in our study showed strong resistance and recovery. Environmental resilience varies by species, and the survival rates observed here align with those of previous research. This variation within the species might stem from its capacity to adapt through metabolic changes and muscle remodeling or from genetic differences in coping with stress. It is important to note that the observed differences in thermal adaptability may also arise from variations in symbiont composition as studies show symbiotic communities are diverse, comprising multiple algal species that vary by giant clam species, size, and environmental conditions like seawater temperature.^{84,85} Notably, artificial breeding of giant clams has been successfully established in several countries, easing pressure on wild populations and providing a sustainable source. Building on this success, further research into genetic variations in stress response, coupled with the development of molecular breeding, could significantly aid in managing population decline. Our insights into the molecular basis of giant clam's bleaching response offer potential targets for future breeding programs. Therefore, additional studies are needed to bridge this gap and enhance the resilience of giant clam populations in their natural habitats.

ASSOCIATED CONTENT

Supporting Information The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.5c00474>.

Detailed experimental materials and methods, additional data, and visual representations to support the research (PDF)

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Figure 1. Schematic illustration of the thermal stress procedure for *T. crocea* and the different bleaching severities. (A) Experimental design and setup. (B) Different bleaching characteristics of giant clams: (a) healthy, (b) moderately bleached, and (c) severely bleached. (C) Bleaching progress in giant clams *T. crocea* during the exposure period. (D) Kaplan–Meier survival curves of the healthy group and thermal stress group. (E) Severely bleached giant clams from the thermal stress group and healthy giant clams from the control group in another repeated thermal stress exposure experiment were selected for further physiological and molecular analyses.

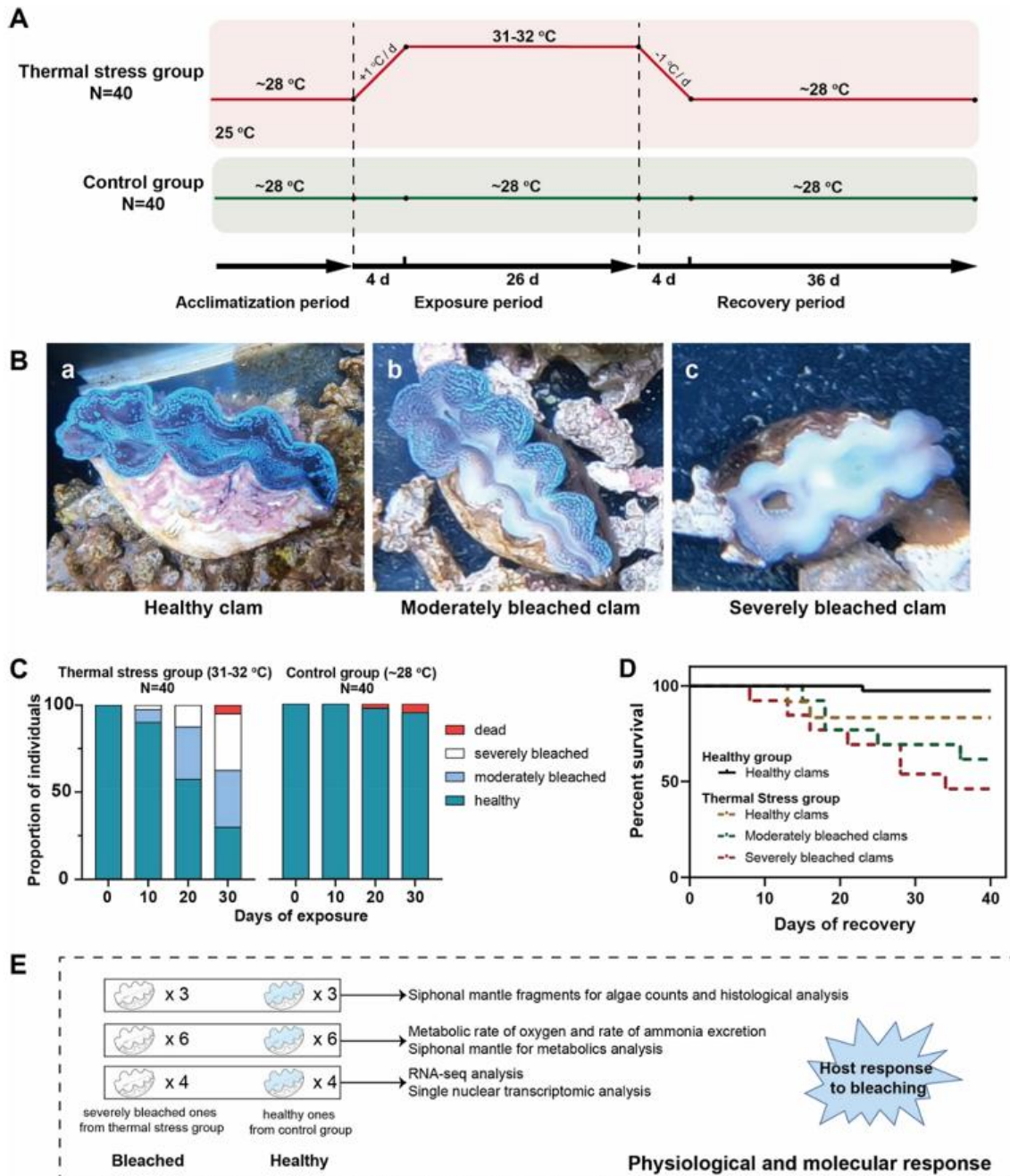


Figure 2. Photosynthetic capacity of *T. crocea* and its histological features. (A) Metabolic rate of oxygen of healthy and bleached *T. crocea* (per dry weight of biomass) at different durations. The data are presented as the mean \pm SEM, and statistical analysis was performed using two-way ANOVA. (B) Maximum quantum yield of chlorophyll, represented by Fv/Fm; photochemical efficiency of chlorophyll in the light, represented by Fv'/Fm' (right panel). Chl, chlorophyll; Fv/Fm, Chl fluorescence ratios in the dark-adapted state; Fv'/Fm', Chl fluorescence ratios in the light adapted state. The data are presented as min to max, and statistical analysis was performed using two-way ANOVA. (C) Density of zooxanthellae in giant clams was calculated by using equally sized fragments of siphonal mantle tissue. The data are presented as min to max, and statistical analysis was performed using the t-test. (D) Masson-stained histological observation of the edge siphonal mantle of *T. crocea* by TEM imaging: (a–c) healthy group, (d–f) bleached group. EL, epidermal layer; Zx, zooxanthellae. (E) The fiber content obtained from the histological slices was quantified as the proportion of the total area (%). The data are presented as the mean \pm SD, and statistical analysis was performed using the t-test ($p < 0.001$). (F) Comparative histological analyses of the symbiotic mantle in healthy, bleached, and recovered giant clams. The upper panels show slices stained with HE (63 \times image), and the bottom panels show the same tissue slices stained with Masson (63 \times image).

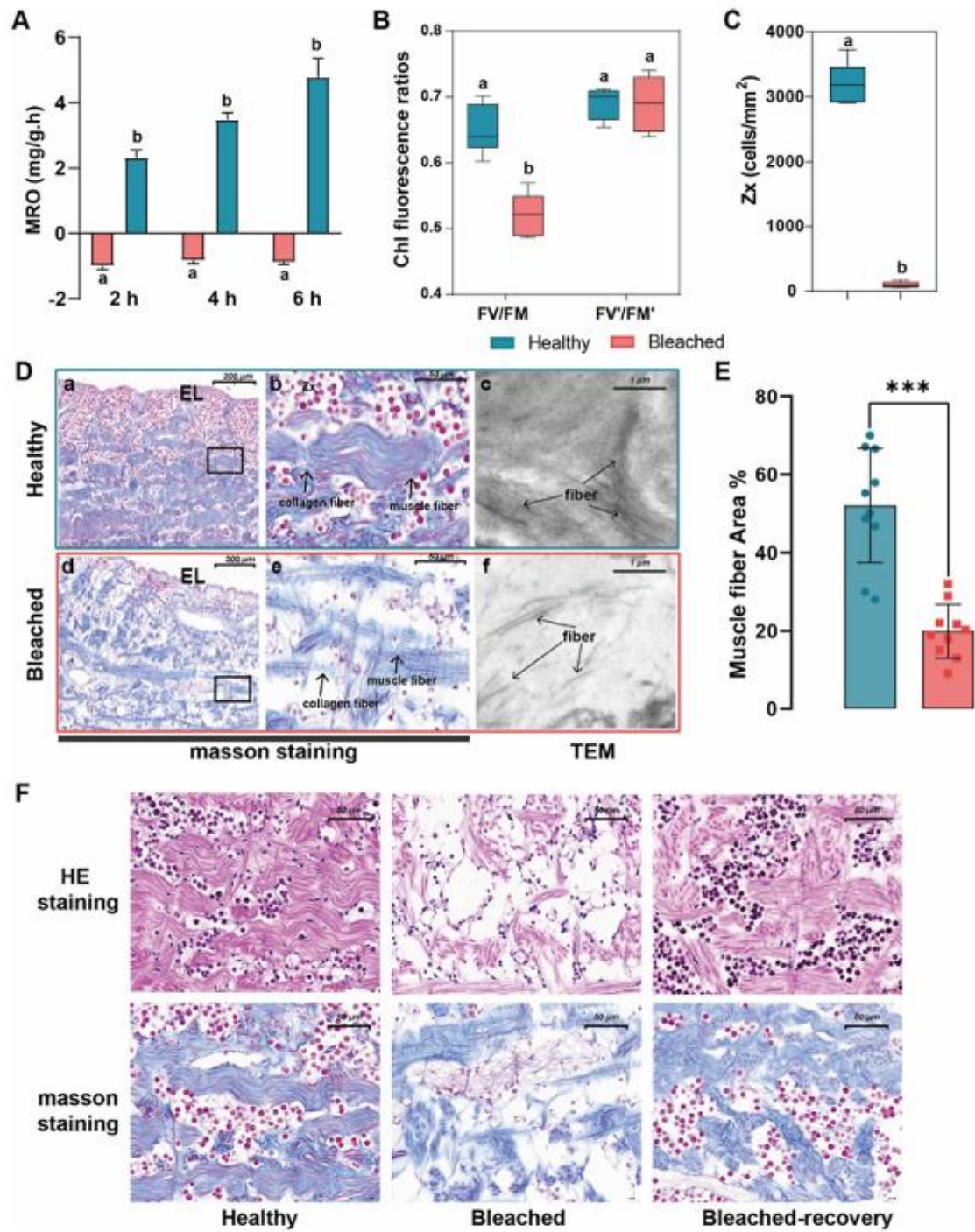


Figure 3. Single-nucleus transcriptome profiling contrasts healthy and bleached siphonal mantles. (A) t-SNE plots revealing distinct cell types; additional details are provided in Figure S4A. (B) Bubble charts highlighting marker genes across clusters, merging similar cell types (clusters 2 and 5, 11, and 12). The cell type proportions in the healthy and bleached groups are indicated in the upper histogram. (C) t-SNE-based unsupervised clustering differentiates single-cell transcriptomes in healthy and bleached states. (D) Expression of *Rhbg-a* and *Rhbg-b* in the tissue transcriptome and single-nucleus transcriptome, respectively, indicating that genes were significantly upregulated ($*p < 0.05$). (E) Ammonia metabolism rates in *T. crocea* were quantified (per dry weight of biomass). The data are presented as the mean \pm SEM, and statistical analysis was performed using two way ANOVA.

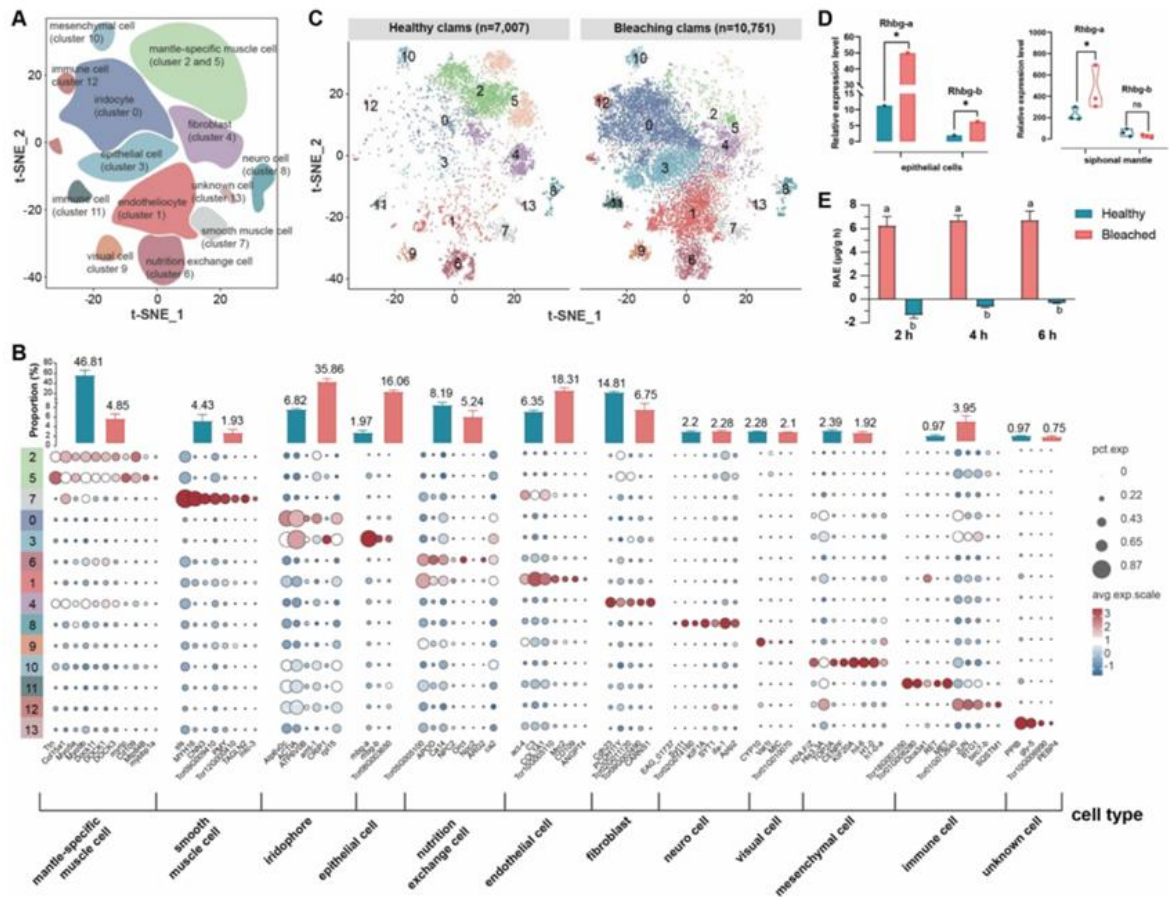


Figure 5. Metabolic shift in bleached giant clams. Metabolic map of carbohydrates, fatty acids, and amino acids with gene expression depicted as log₂ (fold change) values (bleached group vs control group). The blocks from left to right represent different tissues, listed in the following order: siphonal mantle, inner mantle, hemocytes, ctenidium, and digestive gland. Genes differentially expressed are marked with *. The metabolic map is based on KEGG pathways (<http://www.genome.jp/kegg/>). Genes (*italicized*) located between metabolites encode enzyme(s) for the corresponding reaction. The upregulated metabolites are marked in orange.

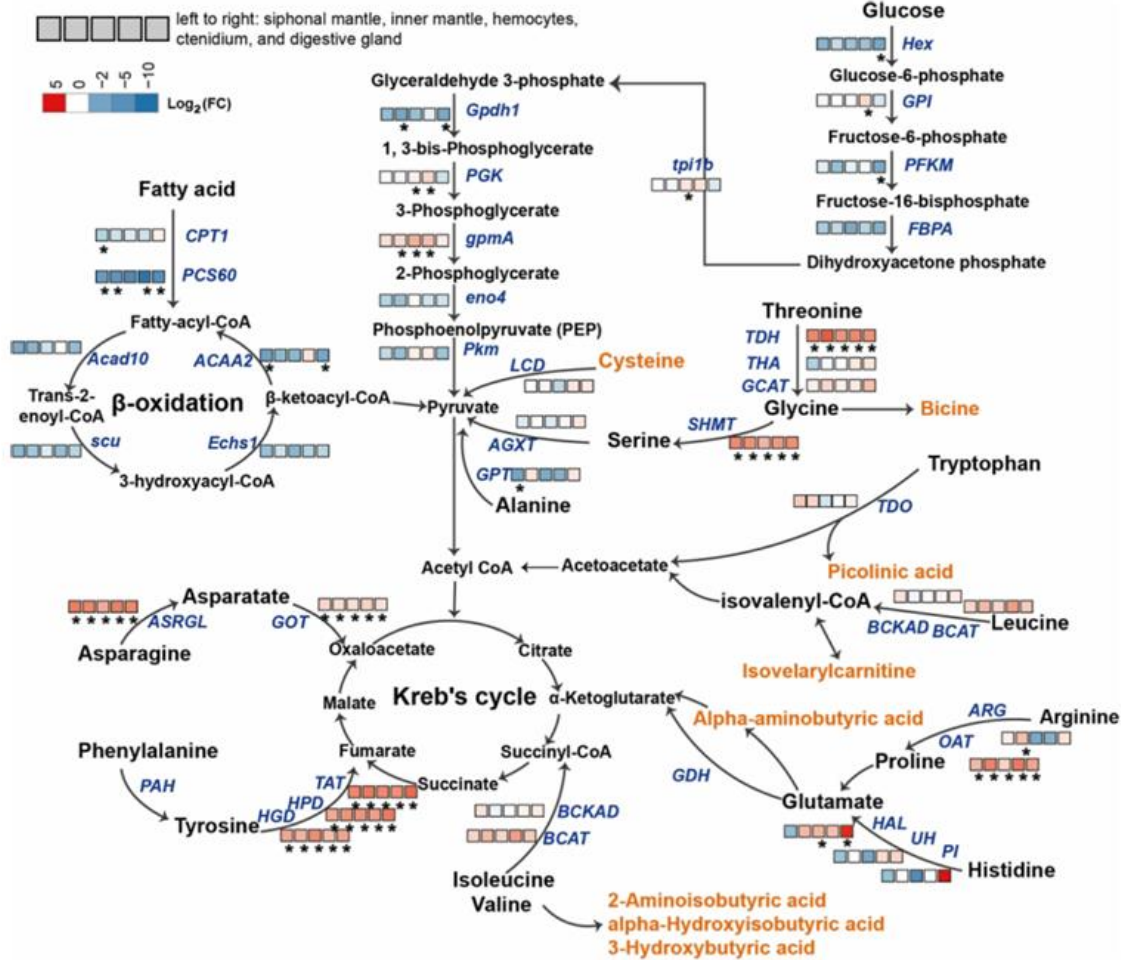


Figure 6. Metabolic shift and muscle fiber “atrophy” in bleached giant clams centered on the AMPK signaling pathway. (A) Gene regulatory network centered on AMPK underlying the molecular response in bleached giant clams. Gene expression levels are indicated in the blocks from left to right and represent different tissues, as shown in Figure 5. The histograms represent the levels of the compound AMP. (B) Model outlining muscle degradation and metabolic shifts in bleached giant clams. Glucose and fatty acids are typically utilized by algae symbionts for healthy giant clams.

