Intestinal metabolomics of juvenile lenok (*Brachymystax lenok*) in response to heat stress

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**Abstract** Changes in the metabolic profile within the intestine of lenok (*Brachymystax lenok*) when challenged to acute and lethal heat stress (HS) are studied using no-target HPLC–MS/MS metabonomic analysis. A total of 51 differentially expressed metabolites (VIP > 1, \( P < 0.05 \)) were identified in response to HS, and 34 occurred in the positive ion mode and 17 in negative ion mode, respectively. After heat stress, changes in metabolites related to glycolysis (i.e., alpha-D-glucose, stachyose, and L-lactate) were identified. The metabolites (acetyl carnitine, palmitoylcarnitine, carnitine, and erucic acid) related to fatty acid β-oxidation accumulated significantly, and many amino acids (L-tryptophan, D-proline, L-leucine, L-phenylalanine, L-aspartate, L-tyrosine, L-methionine, L-histidine, and L-glutamine) were significantly decreased in HS-treated lenok. The mitochondrial β-oxidation pathway might be inhibited, while severe heat stress might activate the anaerobic glycolysis and catabolism of amino acid for energy expenditure. Oxidative damage in HS-treated lenok was indicated by the decreased glycerophospholipid metabolites (i.e., glycerophosphocholine, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, and 1, 2-dioleoyl-sn-glycero-3-phosphatidylcholine) and the increased oxylipin production (12-HETE and 9R, 10S-EpOME). The minor oxidative pathways (omega-oxidation and peroxisomal beta-oxidation) were likely to be induced in HS-treated lenok.

**Keywords** Heat stress · Intestinal · Metabolomics

**Introduction**

Extreme and abrupt environmental changes are increasingly likely as a result of climate change. Not only have global temperature and precipitation patterns changed...
markedly in recent decades, but further change is predicted (Kibler et al. 2015; Yeo and Kim 2014). Increased climate variability and extreme high-temperature events at regional scales have impacted aquatic ecosystems, especially those of freshwater fish (Cqza et al. 2019; Newton et al. 2012; Clark et al. 2008; Susan et al. 2003). While fish can generally adapt to ranges of water temperature, acute and extreme fluctuations that exceed levels of tolerance will trigger series of stress-related responses, such as abnormal behavior, physiological dysfunction, biochemical reactions, and potentially death (Chen et al. 2021; Xia et al. 2017; Lu et al. 2016; Thorne et al. 2010).

The lenok (*Brachymystax lenok*) is a landlocked freshwater salmonid that has an extremely restricted distribution and small population size that occurs in upstream regions of cold rivers in East Asia (Liu et al. 2018). Although both artificial reproduction and breeding have been attempted for this species, natural lenok populations have decreased significantly with habitat degradation, and the species is now regarded to be endangered in Korea and China (Liu et al. 2018; Xu et al. 2014). Because lenok is very sensitive to fluctuation in water temperature, widespread declines in its populations may be a consequence of warming temperatures. Juvenile lenok function normally between 6 and 18 °C, but if climate events (e.g., high temperatures and droughts) in northern China become increasingly common and more extreme during summer, both cultured and wild fish will be affected (Liu et al. 2018; Mou et al. 2011). It is important to understand how lenok respond to heat stress (HS) and the effects of these temperature extremes on its survival, and therefore to maintain viable populations throughout its current distribution.

Systems biology approaches have been used to understand biological processes and metabolic changes in different tissues of salmonids following exposure to high temperature. Transcriptomics of the head kidney of rainbow trout *Oncorhynchus mykiss* in response to HS (between 18 and 24 °C, increased 1 °C per 24 h) revealed modulated pathways in the immune system, protein metabolism, and the spliceosome (Huang et al. 2018). Under acute HS (18–25 °C, increased by 2.5 °C h⁻¹), label-free quantification of protein expression in the rainbow trout liver changed in the estrogen signaling and platelet activation pathways, and complement and coagulation cascades (Kang et al. 2019). Regulation of DNA damage was reported in Chinook Salmon *Oncorhynchus tshawytscha* gill tissue after acute elevated temperature challenges (14–21 °C, increased by 4 °C h⁻¹) (Clark et al. 2008). It is apparent that HS induces tissue-specific responses in salmonids in these examples.

Metabolomics is a basic discipline in systems biology, the same as genomics, transcriptomics, and proteomics. Metabolomics approaches present new methods to study small endogenous metabolites and reveal changes in metabolites and metabolic pathways in response to external stimuli or disturbance (Maha et al. 2019; Sun et al. 2018; Lardon et al. 2013). Non-targeted metabolomics has been applied in studies of metabolic changes in tissue samples of salmonids in response to HS challenges. Nuclear magnetic resonance (NMR)-based metabolomics was conducted on plasma of Atlantic salmon (*Oncorhynchus* spp.) to reveal reprograming of amino acids, and energy and lipid metabolism following long-term (3 months) experimentation at high (18 °C) temperature (Kullgren et al. 2013). Liu et al. (2018) identified thermal stress-activated glutamate metabolism in lenok liver tissue and plasma using an NMR-based metabonomic strategy, and suggested that glutamate might be a biomarker associated with moderate thermal stress (24 °C for 7 days) (Liu et al. 2018). To our knowledge, no study has investigated the effect of acute HS on the intestinal metabolome of lenok despite HS induces tissue-specific responses in salmonids. We do so and suggest ways to improve survival of this endangered species at extreme high temperature. Our research will provide both practical and theoretical values for maintain populations of lenok affected by climate change.

**Materials and methods**

**Experimental design and sampling**

Healthy lenok were obtained from the Yanqing hatchery, Beijing Academy of Agriculture and Forestry Sciences, Institute of Fisheries Research (Beijing, China). Fish were first acclimated for 1 week at a mean temperature of 14±0.5 °C, pH 7.73±0.03, and dissolved oxygen 7.58–8.55 mg L⁻¹, with a light/dark photoperiod of 12:12 h. Following acclimation, 180 juvenile lenok (23.5±2.64 g in body weight and 13.2±0.59 cm in body length) were randomly selected and divided into two treatments (control, TC and HS), each containing
90 fish (with no significant difference in body weight). There were 3 replicates containing 30 fish for each treatment. Fish were placed into rectangular tanks and further acclimated for 3 days before experiment. During acclimation, fish were fed a commercial feed of 2% of their body weight twice a day (8 am, 4 pm).

Our previous studies demonstrated that the semi-lethal high temperature of lenok at 48 h was 26.3 °C. Water temperature in the TC treatment was maintained at 14 °C. The HS treatment was gradually increased from 14 to 26 °C at 1 °C h⁻¹, and then maintained at 26 °C for 48 h. No food was provided to fish during heat stress treatment. Fish were considered dead if immobile and non-responsive when probed with a glass rod. Dead fish were recorded and removed immediately.

After HS experimentation, nine fish (three from each replicate tank) from each treatment were collected and euthanized with a solution containing ~250 mg L⁻¹ ethyl 3-aminobenzoate methane sulfonate (MS-222; TCI, Tokyo, Japan). Intestinal tissues (without feces) were immediately removed from each fish, and intestinal samples were stored separately in 1.5-mL centrifuge tubes at −80 °C until metabolomics analyses.

Intestinal metabolomics analysis

Intestinal samples were homogenized. Metabolite extraction was performed using methanol and acetonitrile (volume ratio 1:1), with 20 μL of each sample taken for quality control, and the rest for LC–MS detection. Analyses were performed using an UHPLC (1290 infinity LC, Agilent Technologies) system coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) system at the Shanghai Applied Protein Technology Co., Ltd. The HILIC separation was accomplished using an ACQUITY UPLC BEH (2.1×100 mm, 1.7 μm, water, Ireland) column. LC–MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific). Data-dependent acquisition MS/MS experiments were performed with HCD scans. Dynamic exclusion was implemented to remove some unnecessary information in the MS/MS spectra. Mass spectrometry was operated in both positive and negative ion modes.

Data processing

Raw MS data (wiff.scan files) were converted to mzXML files using ProteoWizard msConvert and processed using XCMS for feature detection, retention time correction, and peak alignment. In extracted ion features, only variables with >50% nonzero measurement values in at least one group were kept. Compound identification of metabolites was performed by comparing the accuracy of m/z values (<25 ppm), retention time, molecular weight, secondary fragmentation spectrum, collision energy, and other information of the MS/MS spectra with the standard product (in-house database) built by Shanghai Applied Protein Technology. Results were checked and confirmed manually, to ensure that identification was at or better than structural level 2. All identified metabolites (combined positive and negative ion modes) were classified and counted according to their chemical classification information.

Data statistical analysis

The R package DEP 1.5.1 was used for statistical analyses of all metabolomics data (all metabolites, including unidentified ones, in both positive and negative ion modes). Data are expressed as log₂ (fold change) (log₂ FC) compared with control samples. Metabolites with FC > 1.5 or FC < 0.67 and P values less than 0.05 applied to Student’s t-test are graphed in volcano plots.

After normalizing to total peak intensity, processed data were analyzed using R. Multivariable data analyses (Pareto-scaled principal component analysis, PCA and orthogonal partial least-squares discriminant analysis, OPLS-DA) were performed. PCA was performed to show the distribution of origin data. OPLS-DA was applied to obtain a high level of group separation, and an understanding of variables responsible for a classification. A sevenfold cross-validation and response permutation test were conducted to estimate model robustness. Variable importance in projection (VIP) values for each variable in the OPLS-DA model were calculated to indicate their contribution to the classification. Metabolites with VIP values > 1 and P values < 0.05 that were applied to Student’s t-test at univariate level between the treatments were considered statistically significant.

Bioinformatic analysis of differentially expressed metabolites

To more comprehensively and intuitively display the metabolite expression patterns, data for the relative expression of metabolites were used to perform hierarchical clustering analysis using Cluster3.0 (http://
bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). A heat map is presented as a visual aid to visualize the differential metabolites of lenok in response to heat stress.

Metabolites were blasted against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/) to retrieve COs, and were subsequently mapped to pathways in KEGG. Corresponding KEGG pathways were extracted. The metabolic network of metabolites that differed significantly between CT and HS treatments was profiled based on KEGG annotation information and biological function.

Results

No death occurred in control group, and the morality of lenok was 57 ± 15% in HS group after 48 h heat stress.

Metabolite identification

The 396 metabolites included 333 identified in positive ion mode and 106 in negative ion mode. All metabolites were classified and counted according to their Chemical Taxonomy (Fig. 1). The number of metabolites in the following super classes was organic acids and derivatives (59), lipids and lipid-like molecules (29), nucleosides, nucleotides and analogs (25), organoheterocyclic compounds (25), organic oxygen compounds (24), organic nitrogen compounds (11), benzenoids (9), phenylpropanoids and polyketides (7), nucleosides, nucleotides, and analogs/organoheterocyclic compounds (1), organosulfur compounds (1), and undefined (205).

Differentially expressed metabolites (including unidentified ones) detected in both positive and negative ion modes were analyzed based on univariate analysis. Metabolites with FC > 1.5 or FC < 0.67 and P < 0.05 were visualized in volcano plots (Fig. 2).

PCA and OPLS-DA analysis

PCA was performed to identify intrinsic pattern within the data set. PCA score plots are shown in Fig. 3. Model evaluation parameters obtained after sevenfold cross-validation were $R^2_X = 0.571$ in the positive ion mode (Fig. 3A) model, and $R^2_X = 0.592$ in the negative ion mode (Fig. 3B) model. The closer the $R^2_X$ is to 1, the more reliable a model.

The OPLS-DA model was prepared to obtain clear separation between the TC and HS treatments. Model evaluation parameters obtained after sevenfold cross-validation were $R^2_X = 0.362$, $R^2_Y = 0.938$, and $Q^2_Y = 0.598$ for the positive ion mode (Fig. 4A), and were $R^2_X = 0.452$, $R^2_Y = 0.934$, and $Q^2_Y = 0.706$ for the negative ion mode (Fig. 4B). All parameters were stable and effective for fitness and prediction. A permutation test was used to verify the model to avoid overfitting of supervised models to ensure
their effectiveness. $R^2$ and $Q^2$ intercept values determined after permutations were 0.804 and −0.336 in positive ion mode (Fig. 4C) and 0.644 and −0.449 in negative ion mode (Fig. 4D), respectively. Low $Q^2$ intercept values indicated that the robustness of the models presented low overfitting and reliability risks. All samples in score plots were within a 95% confidence ellipse prepared using Hotelling’s $T$-squared with clear separation and discrimination between pairwise groups. The OPLS-DA model identified differences between treatments and in subsequent analyses.

**Differentially expressed metabolites**

Based on the OPLS-DA and Student’s $t$-test analyses, significantly different metabolites occurred in TC and HS treatments. Of 51 differentially expressed metabolites, 34 were identified in the positive ion mode and 17 in the negative ion mode (Table S1). Hierarchical clustering

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**Fig. 2** Volcano plots of metabolites in lenok after heat stress with FC $>1.5$ or FC $<0.67$, and $P<0.05$ in positive (A) and negative (B) ion mode. Color denotes the abundance of metabolites from the highest (red) to the lowest (blue).

**Fig. 3** PCA score plots in positive (A) and negative (B) ion mode, showing separation between the control (blue) and heat stress (green) treatment. Each dot represents one intestinal sample from each treatment.
analysis was performed to visualize differences in the metabolome of these two treatments. In the HS treatment, 8 metabolites were upregulated (red) and 26 metabolites were downregulated (blue) in the positive ion mode (Fig. 5A), and 6 metabolites were upregulated and 11 metabolites were downregulated in the negative ion mode, respectively compared with the control (Fig. 5B).

To visualize the potential metabolic response of lenok to HS, the metabolic network of differentially expressed metabolites was built according to KEGG annotation information (Fig. 6). The metabolites are colored according to the type of change after the heat stress. The upregulated metabolites including N-acetyl-l-alanine, 4-oxoretinol, 12-HETE, L-palmitoylcarnitine, 9R, 10S-EpOME, acetyl carnitine, erucic acid, glutathione disulfide, creatine, lactate, and L-carnitine were colored into red. The downregulated metabolites including cytosine, D-proline, 1-stearoyl-2-oleoyl-sn-glycerol 3-phosphocholine (SOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, L-tryptophan, taurocholate, L-aspartate, 1-palmitoyl-sn-glycero-3-phosphocholine, L-leucine, uracil, cytidine, L-pyroglutamic acid, L-tyrosine, trans-cinnamate, alpha-D-glucose, dopamine, adenosine 3′-monophosphate,
**Fig. 5** Hierarchical clustering analysis of the significantly different metabolites in response to heat stress of positive (A) and negative (B) ion mode. Color denotes the abundance of metabolites, from the highest (red) to the lowest (blue).

**Fig. 6** The hypothetical framework based on the changed metabolites of heat-treated lenok compared with control group. The metabolites are colored according to the type of change after the heat stress (black, no change; red, upregulation; green, downregulation).
L-phenylalanine, N-acetylmannosamine, L-histidine, L-glutamine, L-methionine, stachyose, glycerophosphocholine, 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphethanolamine, 2-hydroxyadenine, taurochenodeoxycholate, and adenosine were colored into green.

**Discussion**

As poikilotherms, the effects of temperature on fish can be profound since their body temperature changes with ambient water (Mueller et al. 2015; Machado et al. 2014; Windisch et al. 2014; Scott and Johnston 2012). Predictions have been made about the increased frequency and severity of climate events in the future, and for them to occur for longer (Ikeda et al. 2012). The optimal temperature for lenok growth is between 14 and 18 °C (Liu et al. 2011). However, they may experience higher temperatures during seasonal changes and heat currents in more southern habitats. Metabolic regulation is an important strategy by which fish response to environmental stress (Melvin et al. 2018; Martyniuk and Simmons 2016). Metabolomic analysis provides an integrated description of HS-induced metabolic changes in the intestine of lenok and enables identification of differentially expressed metabolites resulting from acute HS.

Changes in metabolites (i.e., alpha-D-glucose, stachyose, and L-lactate), which were related to carbohydrate and glycolysis (Fig. 6), suggested that energy metabolism of lenok was strongly influenced by heat stress. Decreased glucose and stachyose stores in the intestine of HS-treated lenok may indicate an increased energy expenditure with increased temperature. Stimulation of glycogenosis to meet energy demands led to the decreases in glucose and carbohydrate levels in several fish species when exposed to extreme high temperatures (Forgati et al. 2017; Xia et al. 2017; Lu et al. 2016). Lactate, a major end-product of anaerobic metabolism (Lu et al. 2016), accumulated in the intestine of HS-treated lenok. Previous studies have been reported that heat stress induced significant reduction of oxygen concentration and PO2 in fish species, and the induced functional hypoxia promoted expression of hypoxia inducible factor 1 (HIF-1) to maintain oxygen homeostasis (Islam et al. 2020; Thomsen et al. 2017; Semenza 2012). In conjunction with the decreased glucose and carbohydrate levels, the accumulation of lactate may suggest that anaerobic glycolysis was activated in lenok to meet energy demands under acute heat stress.

Differentially expressed metabolites (acetyl carnitine, palmitoylcarnitine, carnitine, 12-HETE, 9R, 10S-EpOME, and erucic acid) appeared to be linked to fatty acid metabolism. In animals, the carnitine pool comprises L-carnitine and acylcarnitine ester, which played important roles in mitochondrial β-oxidation of long-chain fatty acids and ATP production (Wang et al. 2016; Ozorio et al. 2010). Major physiological functions of carnitine involve transferal of long-chain fatty acids by conjugation of acyl residues to the β-hydroxyl group on the carnitine molecule. The carnitine derivative of the long-chain fatty (usually palmitoylcarnitine), formed by carnitine palmitoyltransferase-I (CPT-I) in the mitochondrial outer membrane, enters the mitochondrial matrix in exchange for a free carnitine (Sabzi et al. 2017; Neto et al. 2012; Ozorio et al. 2010). As the most common carnitine ester, acetyl-L-carnitine transports acetyl groups to different regions. Accumulation of L-carnitine and the acylcarnitine ester might indicate the inhibition of β-oxidation, which led to the accumulation of the unsaturated fatty (erucic) acid (Vetter et al. 2020; Sharma and Black 2009). Unlike previous studies which demonstrated that metabolic rate, including fatty acid metabolism, was promoted to feed energy demand following the increasing environmental temperature, we suggested that the energy source from fatty acid β-oxidation might be inhibited in an acute and lethal heat stress condition (Chen et al. 2021; Hermann et al. 2019; Forgati et al. 2017; Thorne et al. 2010).

The expression of many amino acids (D-proline, and L-tryptophan, L-leucine, L-phenylalanine, L-aspartate, L-tyrosine, L-methionine, L-histidine and L-glutamine) decreased in HS-treated lenok. In fish, amino acids represented a major substratum for energy production (Li et al. 2020; Jia et al. 2017). With increasing energy loss in stress conditions, amino acids could function as an immediate source of fuel to produce energy to maintain pathway function (Lu et al. 2017; Li et al. 2009). Energy expenditure accounted for large-scale decreases in amino acids of fish in severe conditions, because these pools of oxidizable amino acids were used extensively in energy metabolism (Maha et al. 2019; Kullgren et al. 2013). Additionally, adenosine is a basic component of synthesis energy substances, such as adenosine triphosphate (ATP), coenzyme nicotinamide adenine dinucleotide
Oxidative stress is a common response associated with acute heat stress in fish because of the imbalance between production of reactive oxygen species (ROS) and the capacity of ROS scavenging (Chen et al. 2021; Xia et al. 2017; Qian and Xue 2016). Fish exposed to acute heat have increased the lipid and protein oxidation, DNA damage, and cellular injury (Banh et al. 2016; Logan and Somero 2011; Kaur et al. 2010). Oxidative damage in HS-treated lenok was indicated by decreased metabolites of glycerophospholipid (i.e., glycerophosphocholine, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) and increased oxylipin production (i.e., 12-HETE and 9R, 10S-EpOME) in the present study (Fig. 6). Glycerophospholipids are the main cell membrane lipids and play important roles in regulating membrane potential, curvature, ion transport, etc. (Zhang et al. 2020; Melvin et al. 2019; Shimura et al. 2016). Down-regulation of glycerophospholipids along with oxidative stress might indicate changes in the structure and function of cellular membranes in fish species (Melvin et al. 2019; van Meer et al. 2008). Hydroperoycicosatetraenoic acids (HETEs) and epoxyoctadecamonoenoic acids (EpOMEs) derived from arachidonic acid (AA) and linoleic acid (LA), respectively, were associated with oxidative stress and pathological conditions in organisms, including fish (Hildreth et al. 2020; Zarini et al. 2014). AA, found in a bound form (rather than free) as a cell membrane phospholipid, could be released by lipases like phospholipase A2 (PLA2) after the disruption of cell membrane. In the presence of a lipoxygenase (LOs)-mediated pathway, AA was oxidized into hydroperoycicosatetraenoic acid (HETE) (Zarini et al. 2014; Yang et al. 2006; Rocha et al. 2003). Leukotoxin (9R, 10S-EpOME), an epoxide derivative of linoleic acid, was synthesized by cytochrome P450 monooxygenases (Vatanparast et al. 2020). Increased 12-HETE and 9R, 10S-EpOME revealed the activation of arachidonic acid released from membrane phospholipids and oxidative stress in HS-treated fish, consistent with previous findings for fish under stress (Maha et al. 2019; Baldissera et al. 2018).

Severe heat stress triggered series dynamic metabolic changes including heat shock, immune response, repression of energy metabolism, catabolism of amino acids, and biosynthesis of glutamate and glutamine in tissues of salmonids according to the analysis of systems biology (Li et al. 2021; Liu et al. 2018). Our data provided a new insight of metabolism changes in intestine of lenok and suggested that oxidative stress increased formation of both eicosanoids and dicarboxylic acids, and overwhelmed the mitochondrial β-oxidation pathway. On the other hand, the minor oxidative pathways (omega-oxidation and peroxisomal beta-oxidation) were likely to be activated in HS-treated fish. Series of metabolic reactions might be induced in an attempt to reduce inflammatory tissue damage. Except for increased cellular glutathione (GSH) to scavenge ROS, L-carnitine and acylcarnitine ester played protective roles against oxidative stress in fish (Li et al. 2019; Wang et al. 2016; Guzman-Guillon et al. 2013). Accumulation of L-carnitine and acylcarnitine ester may act to inhibit aerobic oxidation of lipids to reduce indices of oxidative stress. As main components of phospholipids in cell membranes, decreases in glycerophospholipids could significantly affect the permeability and polarity of cell membranes—regarded to be a defense mechanism to prevent ROS from entering cells and reducing oxidative stress (Zhang et al. 2020; Melvin et al. 2019).

Author contribution Yan Chen was responsible for experimental design and manuscript writing. Yang Liu took part in sampling and funding acquisition. Yucen Bai supervised the research project and supervised the writing of the manuscript. Shaogang Xu was responsible for preliminary investigation and funding acquisition. Xiaofei Yang took part in experimental procedures. Bo Cheng was responsible for supervising the research project.

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Data availability The data used to support the findings of this study are available from the corresponding author upon request.
Declarations

Ethics approval  This study was approved by the Institutional Animal Care and Use Committee of Beijing Fisheries Research Institute, Beijing Academy of Agriculture and Forestry Sciences. All experimental procedures were in compliance with the guidelines of Yangqing site of the Beijing Academy of Agriculture and Forestry Sciences, Institute of Fisheries Research, Beijing, China.

Consent to participate  This paper does not contain any studies with human participants by any of the authors.

Consent for publication  Not applicable.

Conflict of interest  The authors declare no competing interests.

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