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Changes in metabolic profiling of whiteleg shrimp (*Penaeus vannamei*) under hypoxic stress

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ABSTRACT

Hypoxia is a common concern in shrimp aquaculture, affecting growth and survival. Although recent studies have revealed important insights into hypoxia in shrimp and crustaceans, knowledge gaps remain regarding this stressor at the molecular level. In the present study, a gas chromatography–mass spectrometry (GC–MS)-based metabolomics approach was employed to characterize the metabolic signatures and pathways underlying responses of Pacific white shrimp (*Penaeus vannamei*) to hypoxia and to identify associated candidate biomarkers. We compared metabolite profiles of shrimp haemolymph before (0 h) and after exposure to hypoxia (1 & 2 h). Dissolved oxygen levels were maintained above 85 % saturation in the control and before hypoxia, and 15 % saturation in the hypoxic stress treatment. Results showed 44 metabolites in shrimp haemolymph that were significantly different between before and after hypoxia exposure. These metabolites were energy-related metabolites (e.g., intermediates of citric acid cycle, lactic acid, alanine), fatty acids and amino acids. Pathway analysis revealed 17 pathways that were significantly affected by hypoxia. The changes in metabolites and pathways indicate a shift from aerobic to anaerobic metabolism, disturbance in amino acid metabolism, osmoregulation, oxidative damage and Warburg effect-like response caused by hypoxic stress. Among the altered metabolites, lactic acid was most different between before and after hypoxia exposure and had the highest accurate value for biomarker identification. Future investigations may validate this molecule as a stress biomarker in aquaculture. This study contributes to a better understanding of hypoxia in shrimp and crustaceans at the metabolic level and provides a base for future metabolomics investigations on hypoxia.

1. Introduction

The level of dissolved oxygen (DO) in water plays a key role in aquaculture production, especially in intensive aquaculture systems with active stock such as shrimp. While the ideal DO level for most aquaculture species is 4 or 5 $mg{\cdot}L^{-1}$ or higher (Boyd, 2003; Robertson, 2006), these levels may be difficult to maintain. Thus, low levels of DO in water (hypoxia) are a threat to production since they may reduce growth rates and lead to high mortalities (Ferreira et al., 2011). To cope with these conditions, marine organisms are known to have adaptation strategies that allow them to mitigate hypoxic effects, including enhancing the efficiency of oxygen uptake (e.g. increasing respiration rate, number of red blood cells, or oxygen binding capacity of hemoglobin), saving energy (e.g. lowering metabolic rate, down-regulation of protein synthesis, altering the expression of genes and proteins of certain regulatory enzymes), and eventually utilizing anaerobic metabolism (Childress and Seibel, 1998; Wu, 2002).

For the Pacific white shrimp (*Penaeus vannamei*), which is the most important Penaeid species in aquaculture, hypoxia appears to be a big concern in cultivation settings. Under hypoxic conditions, *P. vannamei* shrimp have been reported to turn to anaerobic metabolism which results in rapid increases of lactic acid and glucose in the haemolymph

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(Racotta et al., 2002; Soñanez-Organis et al., 2009). In addition to accumulations of lactic acid, Mugnier et al. (2008) also reported decreases in total haemocyte counts (THC) in shrimp exposed to hypoxia and reduction of total protein concentrations if exposed to both ammonia and hypoxia simultaneously. Lactate dehydrogenase (LDH) is a key enzyme of the anaerobic metabolism in all living cells that catalyzes the synthesis of lactate and pyruvate in a reversible reaction, as it converts NAD^+ to NADH and back to NAD^+. The structure of the LDH gene (LvanLDH) and its expression has been characterized as a response to hypoxia in *P. vannamei* (Soñanez-Organis et al., 2012). The silencing of the transcriptional activator hypoxia inducible factor 1 (HIF-1) has been shown to block the increase of LDH mRNA and activity produced by hypoxia in gills, suggesting expression of LvanLDH during hypoxia via the HIF-1 pathway (Soñanez-Organis et al., 2012). Under anaerobic conditions, shrimp require more glucose uptake in the cells by GLUT proteins - glucose transporters (Martínez-Quintana et al., 2014; Martínez-Quintana et al., 2015). The genes encoding for these proteins and gene expression during hypoxia have been characterized for *P. vannamei* which provides a better understanding of facilitative glucose transporters and gene regulation under hypoxic conditions in crustaceans. Phosphofructokinase (PFK) and fructose 1,6-bisphosphatase (FBP) are key enzymes in glycolytic and gluconeogenic metabolism. The cDNA of FBP and expression of PFK and FBP under normoxia (normal levels of oxygen) and hypoxia have been investigated in *P. vannamei* (Cota-Ruiz et al., 2015). In that study, the authors observed the expression of PFK and FBP in hepatopancreas, but not in gill and muscle tissues, suggesting tissue-specific expression of these genes. Although such investigations on shrimp hypoxia have revealed important information regarding the molecular responses of shrimp to hypoxia, there are still many unanswered questions. To address such questions, new approaches, such as high throughput metabolomics (study of biochemical processes of small molecules or metabolites) may be best suited to provide comprehensive mechanistic information about the complex biochemical processes that underpin the animal's responses to these physiological stresses.

Metabolomics is one of the newest and fastest growing omics. Metabolomics aims to characterize the metabolites, which are the end products of metabolism. Hence, metabolomics can be used as a tool to reveal insights into the molecular mechanisms underlying the responses of aquatic organisms to external stresses, diseases and developmental processes. Metabolomics investigations for molluscan species began in the early 2000, and the approach has expanded in recent years as applications continue to widen in scope. These studies cover diverse topics, including diet and nutrient (Grandiosa et al., 2018; Grandiosa et al., 2020), immunology and disease (Nguyen et al., 2019; Nguyen, 2020; Nguyen and Alfaro, 2020), environmental stress (Huo et al., 2019; Li et al., 2019; Nguyen and Alfaro, 2020), eco-toxicology (Li et al., 2017; Nguyen et al., 2018a), and post-harvest handling (Alfaro et al., 2019; Nguyen et al., 2020). Several studies have also employed metabolomics techniques to explore hypoxic effects in molluscan species, such as sea cucumbers (Huo et al., 2019; Li et al., 2019), abalone (Lu et al., 2016; Venter et al., 2018), mussels (Tuffnail et al., 2009; Haider et al., 2020) and prawn (Sun et al., 2018). In general, these studies have reported changes in metabolites from different tissues of the host under hypoxic stress conditions compared to controls. Most of the altered metabolites have been found to be involved in energy and osmoregulation. These studies have led to significant progress in understanding molecular response mechanisms of animals to hypoxic stress. However, no metabolomics investigation has been reported for hypoxic effects on Penaeid shrimp, even though this is a major production bottleneck in shrimp pond production. Hence, this study was conducted to reveal insights into the molecular pathways underlying the responses of Penaeid shrimp to hypoxic stress. For this purpose, we exposed Pacific white shrimp (*P. vannamei*) to different oxygen levels (85 % and 15 % saturation) and analyzed the haemolymph metabolite profiles at 0 h, 1 h and 2 h after exposure using a GC–MS-based metabolomics approach. Changes in haemolymph metabolite profiles were also used to identify biomarkers associated with hypoxic stress as a tool for on-farm management.

2. Materials and methods

2.1. Experimental design

The hypoxic stress experiment was conducted at the National Center for Aquaculture and Marine Research (CENAIM) of ESPOL University (Valdivia, Ecuador). Pacific white shrimp (*P. vannamei*) (5.6 ± 0.7 g) were obtained from CENAIM's Experimental Station (Palmar, Santa Elena Province, Ecuador) and were acclimatized in the lab for a week. Prior to the start of the experiment, 120 shrimp were randomly transferred to 20 3-L glass flasks (6 shrimp per flask) containing filtered and UV treated seawater (34.2 ppt) (Fig. 1). The DO in all tanks was maintained at 85 % saturation (5.8–6.7 $mg{\cdot}L^{-1}$) by bubbling air into the flasks through an air stone. DO was measured by a handheld oxygen meter (YSI EcoSense DO200A, YSI Company, Yellow Springs, Ohio, USA). Among these flasks, 10 flasks were used as control while another ten flasks were used for the hypoxia treatment. At the beginning of the experiment (0 h), two shrimp from each tank were randomly sampled for metabolomics analysis. For that purpose, 200 μL haemolymph from each animal were withdrawn and placed into 2 mL cyro-vials and immediately quenched into liquid nitrogen. Then, the DO level in ten flasks from the hypoxia treatment was reduced to 15 % saturation (0.9–1.2 $mg{\cdot}L^{-1}$) and the DO level in the control flasks was maintained above 85 % saturation (5.8–6.7 $mg{\cdot}L^{-1}$). DO was reduced by adding a pre-determined volume of a 2,000 $mg{\cdot}L^{-1}$ sodium sulfite stock solution to all 3-L experimental flasks. Control flasks were aerated while treatment flasks were not. Then, 10 shrimp from each treatment (one shrimp per flask) were randomly sampled at 1 h and 2 h and snap-frozen in liquid nitrogen, as described above. All haemolymph samples were stored at –80 °C until drying in a SpeedVac Concentrator with a Refrigerated Vapor trap (SavantTM SC250EXP, Thermo Scientific) for 4 h (0 °C, vacuum ramp 3,42 torr/min). Dried samples were then sent to the Auckland University of Technology (New Zealand) for metabolomics analysis (Permit no: 2019073018 issued by Ministry of Primary Industries). The shrimp were not fed during the experiment.

2.2. Metabolomics analysis and quality control

Metabolites from haemolymph (200 μL) were extracted with cold (−20 °C) methanol-water and derivatized with methylchloroformate (MCF), as described by Smart et al. (2010) with minor modifications (Nguyen, 2020) (Protocols are in the supplementary file). D_4-alanine (20 μL of 10 mM) was added to each sample before extraction as an internal standard. Blank samples containing only 20 μL of 10 mM d_4 alanine were extracted together with samples for quality control purposes. Another type of QC sample was amino acid mixtures (20 μL, 20 mM) that were derivatized as the protocol for samples. After derivatization, 10 μL from each sample were pooled to make pooled QC samples. The derivatized samples of shrimp haemolymph were analysed in a gas chromatograph – mass spectrometer (GC–MS) system. Details of the instruments, setting parameters and quality control are described in the supplementary file).

2.3. Spectra processing, data analysis and pathway analysis

Raw spectral data were processed using multiple software, including ChemStation (Agilent Technologies, Inc., US), Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (https://www.amdis.net/) and in-house MassOmics package (the University of Auckland) as previously described (Nguyen and Alfaro, 2019).

Data analyses including statistical analysis, pathway analysis and biomarker analysis were performed using the integrated web-based

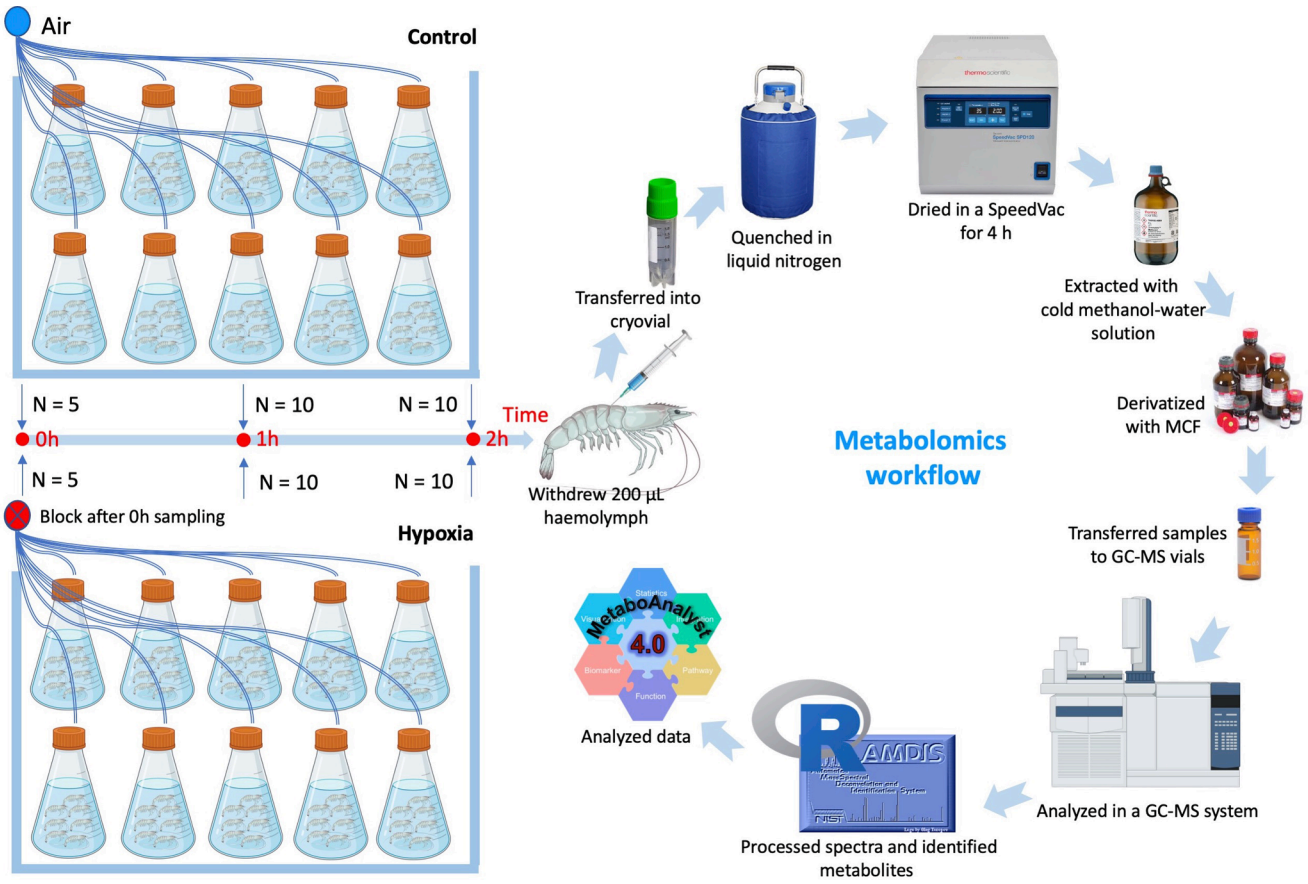


Fig. 1. Experimental design and metabolomics workflow.

platform MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) (Pang et al., 2021) as previously described by Alfaro et al. (2021). Data were normalized to the internal standard (d4 alanine) and then by auto scaling. Principal components analysis (PCA) and partial least squares - discriminant analysis (PLS-DA) were used to assess variability among samples and between sample groups as well as the accuracy of the classification and prediction model. Univariate analyses were performed using one-way analysis of variance (ANOVA) ($p < 0.05$ and post-hoc analysis: Fisher's LSD) to identify different metabolites in haemolymph samples among different treatments. A heatmap of altered metabolites identified via ANOVA was constructed to assess the abundance of these metabolites (hypoxia/control) via intuitive visualization.

Identification of pathways influenced by hypoxia was conducted via quantitative enrichment analysis (QEA) using global test algorithm (Xia and Wishart, 2010) and network topology analysis (NTA) using relative-betweeness centrality (Nikiforova and Willmitzer, 2007). The pathway library of *Drosophila melanogaster* (fruit fly) in the Kyoto encyclopedia of genes and genomes (KEGG) database (Kanehisa and Goto, 2000) was used as the reference pathway library. Pathways were considered as potential primary pathways of interest if they had at least two annotated metabolites matching with the KEGG database with simultaneous QEA p-values < 0.05 and with NTA pathway impact (PI) scores greater than 0.0.

Classical univariate receiver operating characteristic (ROC) analysis was performed to assess the specificity and sensitivity of metabolites for biomarker models based on the value of the area under the ROC curve (AUC).

3. Results

3.1. Metabolite profiles of shrimp haemolymph

Overall, we identified 81 metabolites including 75 annotated and 5 unknown metabolites from 480 components in the metabolite profiles of shrimp haemolymph. The majority of these metabolites were energy-related metabolites (e.g., intermediates of citric acid [TCA] cycle, lactic acid), amino acids, fatty acids and other organic acids. PCA score plots revealed clear separations between the hypoxia group and the control group (Fig. 2A). However, PCA score plots did not show differences between sampling times for any treatment or control. Similar patterns were observed in PLS-DA score plots with an exception in the control group where the 0 h sampling was clearly separated from the 1 h and 2 h sampling points (Fig. 2B).

In consistent with multivariate data analyses, univariate data analysis via one-way ANOVA revealed 39 metabolites that were significantly different among the control and treatment groups. These metabolites were visualized in a heatmap to reveal details of differences (Fig. 2C). Most of the differences were observed between the hypoxia group (1 and 2 h) and the control. Since there was no difference between different sampling times within each treatment, data from different sampling times were pooled together to achieve a bigger dataset to compare the effects of hypoxia on shrimp.

3.2. Effects of hypoxia on shrimp haemolymph

A t-test analysis of haemolymph metabolite profiles before hypoxia (0 h) and after hypoxia exposure (1 & 2 h) revealed 44 metabolites that differed between these two groups (Table 1). Among these altered metabolites, there were 17 increased metabolites and 27 decreased

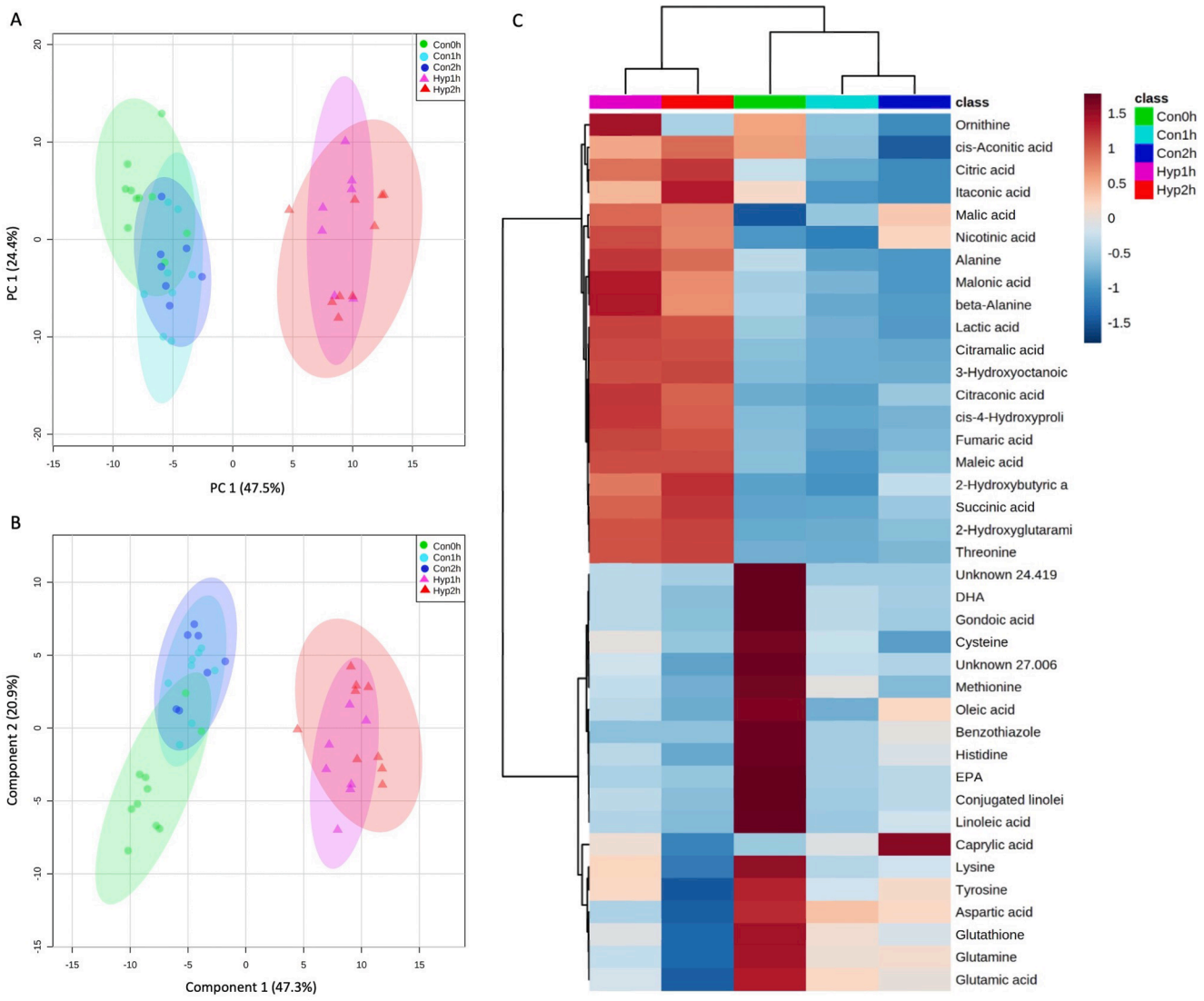


Fig. 2. Chemometrics and cluster analysis of metabolite profiles of shrimp haemolymph under the hypoxic stress and the control. A) PCA score plots. B) PLS-DA score plots. C) A heatmap of 39 metabolites identified as significantly different among the groups via one-way ANOVA. Abbreviations: con, control shrimp (no hypoxia exposure); hyp, hypoxia-exposed shrimp.

metabolites. Most of the increased metabolites were energy related metabolites (e.g., TCA cycle intermediates, lactic acids) some amino acids (e.g., alanine, threonine) and a few fatty acids (e.g., 2-hydroxyglutaramic acid, palmitic acid) while the decreased metabolites included mostly amino acids (e.g., asparagine, lysine, histidine) and fatty acids (palmitelaidic acid, conjugated linoleic acid, DHA).

Pathway analysis indicated 37 involved pathways. After filtering ($p < 0.05$ and IP > 0.01), 17 pathways were identified as significantly affected by the low oxygen stress (Fig. 3). Although glycolysis/gluconeogenesis had IP greater than 0.001, its *p*-value was great than 0.05. Hence, we included this pathway as a slightly affected pathway of interest.

3.3. Biomarker analysis

Classical univariate ROC curve analyses revealed that AUC of lactic acid in haemolymph was equal to 1 (Fig. 4). The very high value of AUC suggests that lactic acid could be an important and accurate biomarker for classification and prediction models. In addition to lactic acid, there were 8 metabolites with AUC equal to 1 and 13 metabolites with AUC greater than 0.9 (Fig. 4).

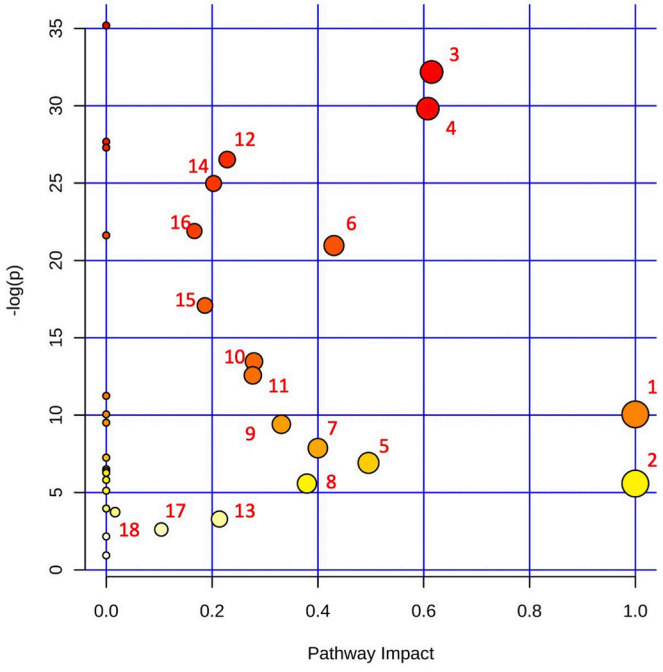
4. Discussion

In the present study, we applied a GC–MS-based metabolomics approach to reveal insights into the metabolic responses of *P. vannamei* to acute hypoxia. The metabolic change was observed in both control and hypoxia conditions. Under the control condition, there were significant decreases in some fatty acids and amino acids at 1 and 2 h post sampling compared to the 0 h sampling time (Fig. 2). The alteration of fatty acids and amino acids in this case may indicate the use of these metabolite classes for energy demands to maintain the normal physiological functions and basic metabolic activities of shrimp. The hypoxia exposure led to changes in many metabolites in the haemolymph of shrimp compared to the control (0 h). These metabolites are involved in many pathways associated with the response of shrimp to hypoxic stress, including a shift in energetic metabolism from aerobic to anaerobic, osmoregulation, oxidative damage, disturbance of amino acid metabolism and Warburg effect-like response.

Under the hypoxic condition, shrimp metabolite profile showed increased levels of many TCA cycle intermediates (citric acid, fumaric acid, succinic acid, *cis*-aconitic acid, itaconic acid). The TCA cycle is a major energy-producing catabolic pathway in all aerobic organisms, and

Table 1
List of altered metabolites due to the effects of hypoxia on shrimp haemolymph metabolome. Arrows (↑ and ↓) respectively indicate the increase and decrease of a metabolite in hypoxia-exposed shrimp compared to that of control shrimp.

Metabolites	*t*-test statistic	*p*-value	Hypoxia effect	Metabolites	*t*-test statistic	p-value	Hypoxia effect
Lactic acid	−22.405	0.000	↑	Asparagine	2.712	0.012	↓
2-Hydroxyglutaramic acid	−17.051	0.000	↑	*cis*-Vaccenic acid	2.833	0.009	↓
Citramalic acid	−15.999	0.000	↑	Palmitelaidic acid	2.902	0.008	↓
3-Hydroxyoctanoic acid	−14.097	0.000	↑	2,6-Diaminopimelic acid	3.232	0.004	↓
Threonine	−13.137	0.000	↑	Oleic acid	3.295	0.003	↓
Succinic acid	−12.032	0.000	↑	Tyrosine	3.303	0.003	↓
Fumaric acid	−11.019	0.000	↑	Lysine	3.582	0.002	↓
Maleic acid	−9.910	0.000	↑	Cysteine	3.881	0.001	↓
cis-4-Hydroxyproline	−9.061	0.000	↑	Histidine	4.124	0.000	↓
2-Hydroxybutyric acid	−6.923	0.000	↑	Glutamic acid	4.444	0.000	↓
Citraconic acid	−6.411	0.000	↑	Methionine	4.596	0.000	↓
Citric acid	−5.912	0.000	↑	Linoleic acid	4.711	0.000	↓
Malic acid	−5.091	0.000	↑	Glutamine	4.772	0.000	↓
Alanine	−4.472	0.000	↑	Glutathione	4.784	0.000	↓
Nicotinic acid	−3.488	0.002	↑	Conjugated linoleic acid	4.850	0.000	↓
Malonic acid	−2.939	0.007	↑	Benzothiazole	5.434	0.000	↓
beta-Alanine	−2.521	0.019	↑	Aspartic acid	5.825	0.000	↓
Stearic acid	2.408	0.024	↓	Unknown 24.419	5.830	0.000	↓
Unknown 9.255	2.467	0.021	↓	EPA	6.310	0.000	↓
Palmitic acid	2.515	0.019	↓	Gondoic acid	6.333	0.000	↓
Unknown 29.597	2.667	0.013	↓	DHA	6.953	0.000	↓
9-Heptadecenoic acid	2.672	0.013	↓	Unknown 27.006	6.973	0.000	↓

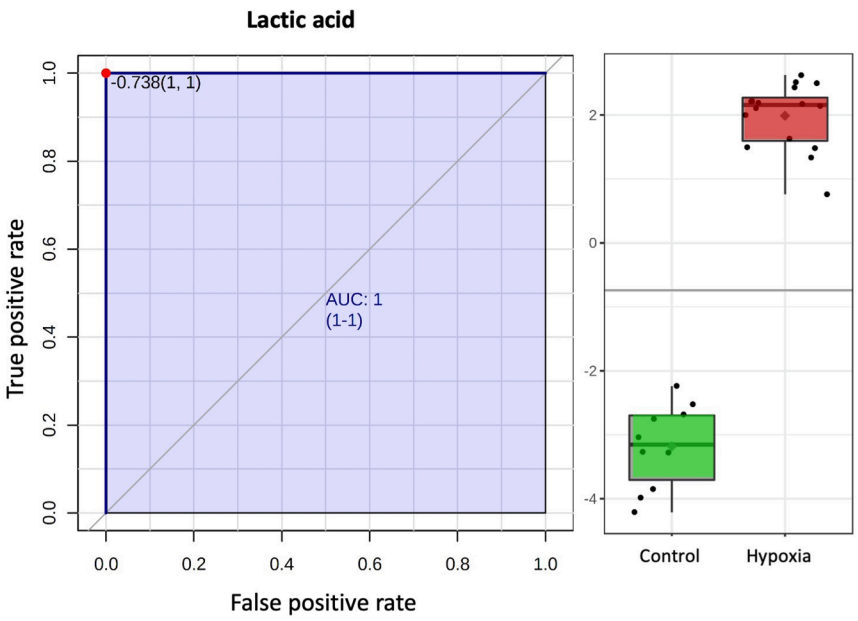


Order	Pathways	Hits/Total Compounds
1	D-Glutamine and D-glutamate metabolism	2/5
2	Phenylalanine, tyrosine and tryptophan biosynthesis	2/4
3	Alanine, aspartate and glutamate metabolism	7/23
4	Glycine, serine and threonine metabolism	4/30
5	Glutathione metabolism	6/26
6	Arginine and proline metabolism	5/31
7	Histidine metabolism	1/9
8	Phenylalanine metabolism	2/7
9	Cysteine and methionine metabolism	5/32
10	Glyoxylate and dicarboxylate metabolism	6/24
11	beta-Alanine metabolism	3/14
12	Arginine biosynthesis	5/12
13	Tryptophan metabolism	1/30
14	Citrate cycle (TCA cycle)	5/20
15	Tyrosine metabolism	2/33
16	Aminoacyl-tRNA biosynthesis	19/48
17	Glycolysis / Gluconeogenesis	1/26
18	Fatty acid biosynthesis	2/43

Fig. 3. List of pathways that were affected by hypoxia in shrimp haemolymph. The most impacted metabolic pathways are specified by the size and the colour of the spheres (yellow = least relevant; red = most relevant) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QTA) and Network Topology Analysis (NTA), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

does not function during hypoxia (Mimura and Furuya, 1995). A number of studies in both vertebrates and invertebrates have demonstrated that disturbances of the TCA cycle by stress conditions (e.g., pathogen infections, environmental stress) lead to accumulation of TCA cycle intermediates (Jha et al., 2015; Young et al., 2017; Alfaro et al., 2019; Nguyen, 2020). The accumulation of citric acid due to TCA cycle disturbance, in turn, leads to the generation of itaconic acid via the enzyme immune-responsive gene 1 (IRG1) (Michelucci et al., 2013). This has recently been observed in bivalves exposed to *Vibrio* sp. infections (Nguyen et al., 2018c; Nguyen and Alfaro, 2019), OsHV-1 virus (Young et al., 2017) and also in shrimp under immune stimulation with prebiotic and probiotics (Alfaro et al., 2022). Hence, the increases in many TCA cycle intermediates, itaconic acid and citraconic acid suggest the disturbance or inhibition of the aerobic TCA cycle pathway.

Along with the increase of metabolites involved in aerobic metabolism, there were accumulations of anaerobic end-products, including lactic acid, succinic acid, alanine and 2-hydroxyglutaramic acid (volatile fatty acid). Lactic acid is formed under insufficient oxygen conditions (anaerobiosis, onset of anaerobic metabolism) via LDH that covert pyruvic acid to lactic acid in a reversible reaction to generate ATP (Fig. 5). Hence, lactic acid is considered to be a final product of anaerobic metabolism and it has been used as a stress index (Plowman and Smith,



Compounds	AUC	P value
Lactic acid	1.000	< 0.001
Fumaric acid	1.000	< 0.001
Succinic acid	1.000	< 0.001
Maleic acid	1.000	< 0.001
Citramalic acid	1.000	< 0.001
3-Hydroxyoctanoic acid	1.000	< 0.001
Threonine	1.000	< 0.001
2-Hydroxyglutaramic acid	1.000	< 0.001
cis-4-Hydroxyproline	1.000	< 0.001
2-Hydroxybutyric acid	0.969	< 0.001
Unknown 27.006	0.963	< 0.001
Citraconic acid	0.956	< 0.001
Citric acid	0.944	< 0.001
Aspartic acid	0.938	< 0.001
Glutamine	0.938	< 0.001
Glutamic acid	0.931	< 0.001
Glutathione	0.925	< 0.001
EPA	0.925	< 0.001
Linoleic acid	0.919	< 0.001
Malic acid	0.913	< 0.001
Conjugated linoleic acid	0.906	< 0.001
DHA	0.906	< 0.001

Fig. 4. Biomarker analysis with univariate ROC curve of lactic acid and other metabolites with AUC greater than 0.9 in shrimp haemolymph.

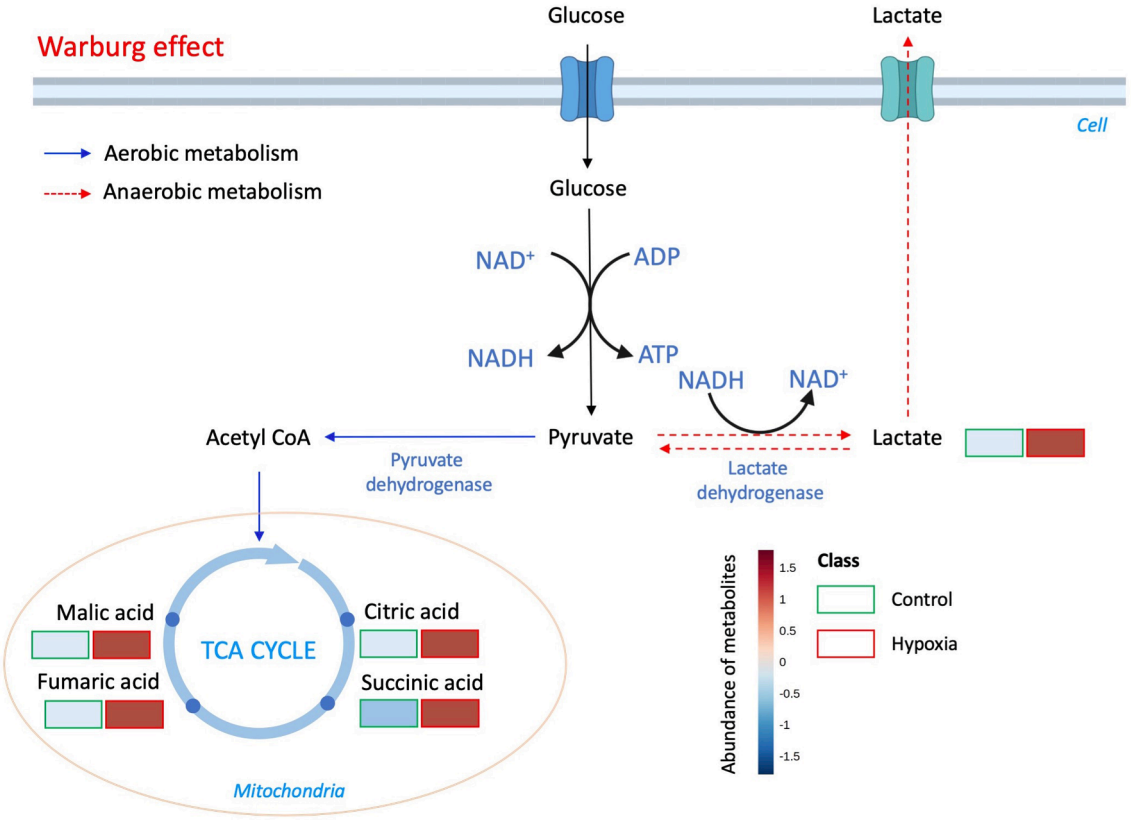


Fig. 5. Anaerobic glycolysis and Warburg effect-like response of shrimp under the hypoxia stress. The hypoxia exposure led to the significant increases of lactic acid, citric acid, succinic acid, fumaric acid and malic acid in shrimp haemolymph, which may indicate the presence of a Warburg effect-like response.

2007). Indeed, an increase of lactic acid has often been observed in bivalves under different stress conditions (Enomoto et al., 2000; Strahl et al., 2011; Giacomin et al., 2014; Alfaro et al., 2019; Nguyen and Alfaro, 2019). In shrimp, accumulation of lactic acid has been recorded during out of water storage stress (Paterson, 1993) and elevated temperatures (Sánchez et al., 2001). Interestingly, increased levels of lactic acid as a consequence of hypoxic stress have been recently reported in *M. nipponense* (Sun et al., 2018) and *P. vannamei* (Racotta et al., 2002; Soñanez-Organis et al., 2009; Soñanez-Organis et al., 2010; Aparicio-Simón et al., 2018). In addition to lactic acid, other metabolites, such as succinic acid, alanine and volatile fatty acids are also anaerobic end-products in invertebrates (de Zwaan and Wijsman, 1976b; Zurburg

and Ebberink, 1980; Ellington, 1983). Increased levels of these metabolites in hypoxic shrimp are probably initial end products of anaerobic metabolism. Together, our findings suggest that hypoxia inhibited aerobic energy metabolism and resulted in a shift from aerobic to anaerobic metabolism in shrimp. Among these metabolites, lactic acid was the most altered metabolite after the hypoxia exposure (Table 1) and its AUC value was very high (=1). This suggests that lactic acid could be a very accurate biomarker for hypoxia and stress responses in shrimp.

Interestingly, the shift in energetic metabolism from aerobic to anaerobic may indicate the presence of a Warburg effect-like response. The Warburg effect is an abnormal metabolic shift from the oxidative phosphorylation to the rapid aerobic glycolysis by upregulation of several major glycolytic enzymes to facilitate the production of more energy and building blocks (Schröder, 2020). The Warburg effect was first found in cancer cells (Warburg, 1956), but has also been described in vertebrate cells infected by viruses (Zwerschke et al., 1999; Munger et al., 2006; Munger et al., 2008; Delgado et al., 2010; Diamond et al., 2010). In addition, Warburg effect-like responses have been reported in invertebrate species, such as shrimp (*P. vannamei*) infected with white spot syndrome virus (WSSV) (Chen et al., 2011; Su et al., 2014), prawn (*Macrobrachium nipponense*) exposed to hypoxia (Sun et al., 2018), oysters (*Crassostrea gigas*) infected with Oyster Herpesvirus type 1 (OsHV1) (Young et al., 2017) and mussels (*P. canaliculus*) challenged with *Vibrio* sp. (Nguyen et al., 2018c).

Decreases of amino acids and fatty acids in hypoxia-exposed shrimp may be involved in energy metabolism. Fatty acids are components of cell membranes and have diverse roles in all cells, including source of metabolic energy, signalling molecules, precursors for the synthesis of eicosanoids (Yaqoob, 2003). Hence, the reduction of fatty acids has been commonly observed in marine bivalves as a consequence of high energy demands in responses to external stresses, such as semi-anhydrous living-preservation (Chen et al., 2015), aerial and heat exposure stress (Alfaro et al., 2019) and pathogen infections (Nguyen et al., 2018b; Nguyen and Alfaro, 2019). Such reduction may be due to the depression in the glycogen pathway and activation of gluconeogenesis that generates glucose from non-carbohydrate carbon substrates. In this pathway, fatty acids can be oxidized to yield propionyl CoA, which enters into gluconeogenesis through Acetyl- CoA (de Zwaan and Wijsman, 1976a). Sun et al. (2018) also observed the decrease of linoleic acid in hypoxic prawns compared to control animals, suggesting the use of fatty acid metabolism to supply energy. Hence, the decrease of many fatty acids in the shrimp haemolymph indicates the use of fatty acids as an energy source and the elevated gluconeogenesis under the hypoxic condition.

Similar to fatty acids, amino acids are also a source of energy and their decreases in shrimp haemolymph may indicated degradation of proteins to provide energy for shrimp under hypoxic conditions. Indeed, pathway analysis revealed 13 amino acid pathways that were significantly affected by the hypoxia. This suggests the disturbance of amino acid metabolisms by the hypoxia. In agreement with this study, Sun et al. (2018) observed decreases of some amino acids (valine, leucine, isoleucine, lysine, glutamate and methionine) in prawn under hypoxia and reoxygenation conditions. In sea cucumbers under the combination of heat and hypoxia, most metabolites involved in amino acid metabolism were significantly decreased compared to the controls (Huo et al., 2019). Furthermore, decreases of amino acids to supply energy have been reported in mussels (*P. canaliculus*) exposed to pathogenic *Vibrio* sp. (Nguyen et al., 2018b; Nguyen and Alfaro, 2019). Among the decreased amino acids, glutamine is an important α-amino acid that serves as an energy substrate, and a building block of glucose, peptides and protein (Souba, 1991). Under hypoxic conditions, cells use glutamine as an alternative fuel to generate citrate and support proliferation (Sun and Denko, 2014). Aspartic acid is a constituent of most proteins, and also play an important role in the metabolism of nitrogen and neurotransmission (Johnson, 2017). A study by Graham and Ellington (1985) who investigated the metabolism of aspartate by *in vitro* preparations of the ventricle of the whelk (*Busycon contrarium*) under anoxic conditions found that there was a substantial conversion of aspartate to succinate with no detectable enrichment of other metabolites. Tyrosine, also known as 4-hydroxyphenylalanine, is an aromatic amino acid that is used for protein synthesis. Tyrosine is also an antioxidant that can scavenge free radicals and contribute to reducing oxidative damage caused by hypoxic stress (Cui et al., 2017). Lysine is an essential amino acid for protein synthesis and a precursor for carnitine, a substance that transports fatty acids to the mitochondria to be oxidised for the release of energy (Vaz and Wanders, 2002; Flanagan et al., 2010). Hence, the common observation in reduction of these amino acids in marine molluscs exposed to external stressors (Lu et al., 2017; Nguyen et al., 2018b; Sun et al., 2018; Huo et al., 2019; Li et al., 2019) as well as in shrimp under the hypoxia in this study may suggest the key role of these amino acids in energy metabolism and other unknown functions which require future investigations.

On the other hand, there were accumulations of some fatty acids (2-hydroxyglutaramic acid, 3-hydroxyoctanoic acid, 9-heptadecenoic acid, stearic acid and palmitic acid) and amino acids (alanine, beta-alanine, *cis*-4-hydroxyproline and threonine) in hypoxic shrimp. It has been shown that short-term exposure to hypoxia can have a negative effect on shrimp osmoregulation (Charmantier and Soyez, 1994; Mugnier and Soyez, 2005). The lipid in the gill tissues of marine molluscs is known to have an important role in osmoregulation and ion exchange (Palacios et al., 2004; Chen et al., 2014; Ray, 2018). Hence, the increases in fatty acids observed in shrimp under the hypoxic condition in the present study may suggest the involvement of these compounds in osmoregulation or ion exchange. Aquatic invertebrates are able to use high concentrations of amino acids to regulate the intracellular osmolarity with that of their surrounding environment (Viant et al., 2003; Fasulo et al., 2012; Cappello et al., 2013). Thus, the significant increase of many amino acids in this study may also be involved in osmoregulation of the host. In addition, alanine and proline are among the most abundant osmolytes in molluscs and crustaceans (Carr et al., 1996). The accumulation of alanine, beta-alanine and *cis*-4-hydroxyproline (a proline derivative) may also be indicative of osmoregulation in shrimp exposed to the hypoxia.

We also observed a reduction of many metabolites in the glutathione pathway (cysteine, glutathione, glutamic acid and methionine) and an accumulation of 4-hydroxyproline in hypoxic shrimp which may be signatures of oxidative stress caused by hypoxia. Acute hypoxia was found to induce oxidative stress due to the excessive production of reactive oxygen species (ROS) in *P. vannamei* (Zenteno-Savín et al., 2006; Li et al., 2016). Oxidative damage due to the excessive ROS production is considered a major contributor to oxidative injury during hypoxia-reoxygenation stress (Dröse et al., 2016; Cadenas, 2018; Groehler et al., 2018). Glutathione is an antioxidant that effectively scavenges free radicals such as ROS and reactive nitrogen species (RNS) (e.g., hydroxyl radical, hydrogen peroxide, lipid peroxyl radical and superoxide anion) directly and indirectly through the glutathione cycle (Aquilano et al., 2014). The regulation of ROS by the glutathione pathway and the up-regulation of glutathione metabolism have been recently demonstrated in marine bivalves under stress conditions (Nguyen et al., 2018c). However, this process requires oxygen to convert glutathione in reduced form (GSH) into its oxidized form (GSSG). Under the hypoxic condition in this study, we observed decreases of many key metabolites in glutathione metabolism (cysteine, methionine, glutamic acid and glutathione) in hypoxia-exposed shrimp compared to the control shrimp, and glutathione metabolism itself was also identified as significantly affected by the hypoxic conditions. This suggests that hypoxic conditions depress the GSH pathway in ROS regulation. This in turn may lead to oxidative damage caused by the increase of ROS. Indeed, we observed the accumulation of 4-hydroxyproline which is known to be a major component of collagen, and marker for collagen degradation caused by oxidative stress (Monboisse and Borel, 1992; Fisher et al., 2009). In agreement, the accumulation of 4-hydroxyproline in gill tissues of mussels after long-term (6 days) hypoxia exposure has

been suggested to be an indicator of oxidative injury (Haider et al., 2020). Together, our results suggest oxidative damage caused by oxidative stress and the depression of the GSH pathway in ROS regulation in shrimp under hypoxic conditions.

In conclusion, this study provides the first metabolomics investigation into acute hypoxia effects on Penaeid shrimp. The study revealed metabolic pathways underlying the shift in energetic metabolism from aerobic to anaerobic in shrimp under the hypoxic conditions. In addition, we observed changes in metabolites and pathways associated with amino acid metabolism and fatty acids metabolism, osmoregulation, oxidative damage and Warburg effect-like response in hypoxia-exposed shrimp. The study also demonstrated the sensitivity and applicability of metabolomics approaches to investigate molecular mechanism of stress (e.g., hypoxia) responses in aquatic organisms and to develop biomarkers for health assessment in crustacean aquaculture. Future metabolomics studies may combine different analytical platforms to characterize more metabolites classes for a broader and more detailed analysis of host responses to hypoxia.

CRediT authorship contribution statement

Thao V. Nguyen: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Validation, Writing – original draft. **Andrea Alfaro:** Project administration, Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Investigation. **Jenny Rodríguez:** Conceptualization, Methodology, Writing – review & editing. **Bonny B. Arroyo:** Conceptualization, Methodology, Writing – review & editing. **Stanislaus Sonnenholzner:** Project administration, Conceptualization, Methodology, Resources, Funding acquisition, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2022.107798.

References

Alfaro, A., Nguyen, V.T., Mellow, D., 2019. A metabolomics approach to assess the effect of storage conditions on metabolic processes of New Zealand surf clam (*Crassula aequilatera*). Aquaculture 498, 315–321. https://doi.org/10.1016/j.aquaculture.2018.08.065.

Alfaro, A.C., Nguyen, T.V., Venter, L., Ericson, J.A., Sharma, S., et al., 2021. The effects of live transport on metabolism and stress responses of abalone (Haliotis iris). Metabolites 11, 748. https://doi.org/10.3390/metabo11110748.

Alfaro, A.C., Nguyen, T.V., Rodríguez, J.A., Bayot, B., Domínguez-Borbor, C., Sonnenholzner, S., Azizan, A., Venter, L., 2022. Evaluation of immune stimulatory products for whiteleg shrimp (Penaeus vannamei) by a metabolomics approach. Fish Shellfish Immunol. 120, 421–428. https://doi.org/10.1016/j.fsi.2021.12.007.

Aparicio-Simón, B., Piñón, M., Racotta, R., Racotta, I.S., 2018. Neuroendocrine and metabolic responses of Pacific whiteleg shrimp *Penaeus vannamei* exposed to hypoxia stress. Latin Am. J. Aquatic Res. 46, 364–376.

Aquilano, K., Baldelli, S., Ciriolo, M.R., 2014. Glutathione: new roles in redox signaling for an old antioxidant. Front. Pharmacol. 5 https://doi.org/10.3389/fphar.2014.00196.

Boyd, C.E., 2003. Guidelines for aquaculture effluent management at the farm-level. Aquaculture 226, 101–112.

Cadenas, S., 2018. ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection. Free Radical Biol. Med. 117, 76–89. https://doi.org/10.1016/j.freeradbiomed.2018.01.024.

Cappello, T., Mauceri, A., Corsaro, C., Maisano, M., Parrino, V., Paro, G.L., Messina, G., Fasulo, S., 2013. Impact of environmental pollution on caged mussels Mytilus galloprovincialis using NMR-based metabolomics. Mar. Pollut. Bull. 77, 132–139.

Carr, W.E., Netherton, I., JAMES C, Gleeson, R.A., Derby, C.D., 1996. Stimulants of feeding behavior in fish: analyses of tissues of diverse marine organisms. Biol. Bull., 190, 149-160.

Charmantier, G., Soyez, C., 1994. Effect of molt stage and hypoxia on osmoregulatory capacity in the peneid shrimp Penaeus vannamei. J. Exp. Mar. Biol. Ecol. 178, 233–246.

Chen, I.-T., Aoki, T., Huang, Y.-T., Hirono, I., Chen, T.-C., Huang, J.-Y., Chang, G.-D., Lo, C.-F., Wang, H.-C., 2011. White spot syndrome virus induces metabolic changes resembling the warburg effect in shrimp hemocytes in the early stage of infection. J. Virol. 85, 12919–12928.

Chen, K., Li, E., Gan, L., Wang, X., Xu, C., Lin, H., Qin, J.G., Chen, L., 2014. Growth and lipid metabolism of the pacific white shrimp *Litopenaeus vannamei* at different salinities. J. Shellfish Res. 33, 825–832.

Chen, S., Zhang, C., Xiong, Y., Tian, X., Liu, C., Jeevithan, E., Wu, W., 2015. A GC-MS-based metabolomics investigation on scallop (*Chlamys farreri*) during semi-anhydrous living-preservation. Innovative Food Sci. Emerg. Technol. 31, 185–195.

Childress, J.J., Seibel, B.A., 1998. Life at stable low oxygen levels: adaptations of animals to oceanic oxygen minimum layers. J. Exp. Biol. 201, 1223–1232.

Cota-Ruiz, K., Peregrino-Uriarte, A.B., Felix-Portillo, M., Martínez-Quintana, J.A., Yepiz-Plascencia, G., 2015. Expression of fructose 1,6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia. Mar. Environ. Res. 106, 1–9. https://doi.org/10.1016/j.marenvres.2015.02.003.

Cui, S., Wang, L., Qiu, J., Liu, Z., Geng, X., 2017. Comparative metabolomics analysis of *Callosobruchus chinensis* larvae under hypoxia, hypoxia/hypercapnia and normoxia. Pest Manag. Sci. 73, 1267–1276.

de Zwaan, A., Wijsman, T.C.M., 1976a. Anaerobic metabolism in bivalvia (Mollusca) Characteristics of anaerobic metabolism. Compar. Biochem. Physiol. Part B: Compar. Biochem. 54, 313–323. https://doi.org/10.1016/0305-0491(76)90247-9.

de Zwaan, A., Wijsman, T.C.M., 1976b. Anaerobic metabolism in bivalvia (Mollusca). Characteristics of anaerobic metabolism. Compar. Biochem. Physiol Part B: Compar. Biochem. 54, 313–323. https://doi.org/10.1016/0305-0491(76)90247-9.

Delgado, T., Carroll, P.A., Punjabi, A.S., Margineantu, D., Hockenbery, D.M., Lagunoff, M., 2010. Induction of the Warburg effect by Kaposi's sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. Proc. Natl. Acad. Sci. 107, 10696–10701.

Diamond, D.L., Syder, A.J., Jacobs, J.M., Sorensen, C.M., Walters, K.-A., Proll, S.C., McDermott, J.E., Gritsenko, M.A., Zhang, Q., Zhao, R., 2010. Temporal proteome and lipidome profiles reveal hepatitis C virus-associated reprogramming of hepatocellular metabolism and bioenergetics. PLoS Pathog. 6, e1000719.

Dröse, S., Stepanova, A., Galkin, A., 2016. Ischemic A/D transition of mitochondrial complex I and its role in ROS generation. Biochim. Biophys. Acta (BBA) - Bioenergetics. 1857, 946–957. https://doi.org/10.1016/j.bbabio.2015.12.013.

Ellington, W.R., 1983. The recovery from anaerobic metabolism in invertebrates. J. Exp. Zool. 228, 431–444.

Enomoto, T., Nakao, C., Ohyama, H., 2000. Regulation of glycolysis during acclimation of scallops (*Patinopecten yessoensis* Jay) to anaerobiosis. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 127, 45–52. https://doi.org/10.1016/S0305-0491(00)00235-2.

Fasulo, S., Iacono, F., Cappello, T., Corsaro, C., Maisano, M., D'Agata, A., Giannetto, A., De Domenico, E., Parrino, V., Paro, G.L., 2012. Metabolomic investigation of *Mytilus galloprovincialis* (Lamarck 1819) caged in aquatic environments. Ecotoxicol. Environ. Saf. 84, 139–146.

Ferreira, N., Bonetti, C., Seiffert, W., 2011. Hydrological and water quality indices as management tools in marine shrimp culture. Aquaculture 318, 425–433.

Fisher, G.J., Quan, T., Purohit, T., Shao, Y., Cho, M.K., He, T., Varani, J., Kang, S., Voorhees, J.J., 2009. Collagen Fragmentation Promotes Oxidative Stress and Elevates Matrix Metalloproteinase-1 in Fibroblasts in Aged Human Skin. Am. J. Pathol. 174, 101–114. https://doi.org/10.2353/ajpath.2009.080599.

Flanagan, J.L., Simmons, P.A., Vehige, J., Willcox, M.D., Garrett, Q., 2010. Role of carnitine in disease. Nutrit. Metabol. 7, 1–14.

Giacomin, M., Jorge, M.B., Bianchini, A., 2014. Effects of copper exposure on the energy metabolism in juveniles of the marine clam *Mesodesma mactroides*. Aquat. Toxicol. 152, 30–37. https://doi.org/10.1016/j.aquatox.2014.03.025.

Graham, R.A., Ellington, W.R., 1985. Anaerobic aspartate metabolism and the formation of alanine in molluscan cardiac muscle: A 13C Nmr study. J. Exp. Zool. 236, 365–370.

Grandiosa, R., Mérien, F., Young, T., Van Nguyen, T., Gutierrez, N., Kitundu, E., Alfaro, A.C., 2018. Multi-strain probiotics enhance immune responsiveness and alters metabolic profiles in the New Zealand black-footed abalone (*Haliotis iris*). Fish Shellfish Immunol. 82, 330–338. https://doi.org/10.1016/j.fsi.2018.08.034.

Grandiosa, R., Young, T., Nguyen, V.T., Mérien, F., Alfaro, A.C., 2020. Immune response in probiotic-fed New Zealand black-footed abalone (*Haliotis iris*) under Vibrio

splendidus challenge. Fish Shellfish Immunol. 104, 633–639. https://doi.org/10.1016/j.fsi.2020.06.007.
Groehler, A., Kren, S., Li, Q., Robledo-Villafane, M., Schmidt, J., Garry, M., Tretyakova, N., 2018. Oxidative cross-linking of proteins to DNA following ischemia-reperfusion injury. Free Radical Biol. Med. 120, 89–101. https://doi.org/10.1016/j.freeradbiomed.2018.03.010.
Haider, F., Falfushynska, H.I., Timm, S., Sokolova, I.M., 2020. Effects of hypoxia and reoxygenation on intermediary metabolite homeostasis of marine bivalves Mytilus edulis and *Crassostrea gigas*. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 242, 110657. https://doi.org/10.1016/j.cbpa.2020.110657.
Huo, D., Sun, L., Zhang, L., Ru, X., Liu, S., Yang, H., 2019. Metabolome responses of the sea cucumber *Apostichopus japonicus* to multiple environmental stresses: Heat and hypoxia. Mar. Pollut. Bull. 138, 407–420. https://doi.org/10.1016/j.marpolbul.2018.11.063.
Jha, A.K., Huang, S.-C.-C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E., Chmielewski, K., Stewart, K.M., Ashall, J., Everts, B., 2015. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity 42, 419–430.
Johnson, E.C., 2017. Aspartic Acid, Reference Module in Biomedical Sciences. Elsevier.
Kanehisa, M., Goto, S., 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucl. Acids Res. 28, 27–30.
Li, L., Chen, M., Storey, K.B., 2019. Metabolic response of longitudinal muscles to acute hypoxia in sea cucumber *Apostichopus japonicus* (Selenka): A metabolome integrated analysis. Comp. Biochem. Physiol. D: Genomics Proteomics 29, 235–244. https://doi.org/10.1016/j.cbd.2018.12.007.
Li, T., Li, E., Suo, Y., Xu, Z., Jia, Y., Qin, J.G., Chen, L., Gu, Z., 2017. Energy metabolism and metabolomics response of Pacific white shrimp *Litopenaeus vannamei* to sulfide toxicity. Aquat. Toxicol. 183, 28–37. https://doi.org/10.1016/j.aquatox.2016.12.010.
Li, Y., Wei, L., Cao, J., Qiu, L., Jiang, X., Li, P., Song, Q., Zhou, H., Han, Q., Diao, X., 2016. Oxidative stress, DNA damage and antioxidant enzyme activities in the pacific white shrimp (*Litopenaeus vannamei*) when exposed to hypoxia and reoxygenation. Chemosphere 144, 234–240. https://doi.org/10.1016/j.chemosphere.2015.08.051.
Lu, J., Shi, Y., Cai, S., Feng, J., 2017. Metabolic responses of Haliotis diversicolor to Vibrio parahaemolyticus infection. Fish Shellfish Immunol. 60, 265–274.
Lu, J., Shi, Y., Wang, S., Chen, H., Cai, S., Feng, J., 2016. NMR-based metabolomic analysis of Haliotis diversicolor exposed to thermal and hypoxic stresses. Sci. Total Environ. 545, 280–288.
Martínez-Quintana, J.A., Kikuta, S., Felix-Portillo, M., Peregrino-Uriarte, A.B., Yepiz-Plascencia, G., 2015. A novel functional glucose transporter in the white shrimp *Litopenaeus vannamei*-LvGLUT2-is up-regulated during hypoxia in hepatopancreas. Mar. Environ. Res. 112, 61–67.
Martínez-Quintana, J.A., Peregrino-Uriarte, A.B., Gollas-Galván, T., Gómez-Jiménez, S., Yepiz-Plascencia, G., 2014. The glucose transporter 1-GLUT1-from the white shrimp *Litopenaeus vannamei* is up-regulated during hypoxia. Mol. Biol. Rep. 41, 7885–7898.
Michelucci, A., Cordes, T., Ghelfi, J., Pailot, A., Reiling, N., Goldmann, O., Binz, T., Wegner, A., Tallam, A., Rausell, A., 2013. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc. Natl. Acad. Sci. 110, 7820–7825.
Mimura, Y., Furuya, K., 1995. Mechanisms of adaptation to hypoxia in energy metabolism in rats. J. Am. Coll. Surg. 181, 437–443.
Monboisse, J.C., Borel, J.P., 1992. Oxidative damage to collagen. In: Emerit, I., Chance, B. (Eds.), Free Radicals and Aging. Birkhäuser Basel, Basel, pp. 323–327.
Mugnier, C., Soyez, C., 2005. Response of the blue shrimp *Litopenaeus stylirostris* to temperature decrease and hypoxia in relation to molt stage. Aquaculture 244, 315–322.
Mugnier, C., Zipper, E., Goarant, C., Lemonnier, H., 2008. Combined effect of exposure to ammonia and hypoxia on the blue shrimp *Litopenaeus stylirostris* survival and physiological response in relation to molt stage. Aquaculture 274, 398–407.
Munger, J., Bajad, S.U., Coller, H.A., Shenk, T., Rabinowitz, J.D., 2006. Dynamics of the cellular metabolome during human cytomegalovirus infection. PLoS Pathog. 2, e132.
Munger, J., Bennett, B.D., Parikh, A., Feng, X.-J., McArdle, J., Rabitz, H.A., Shenk, T., Rabinowitz, J.D., 2008. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. Nat. Biotechnol. 26, 1179–1186.
Nguyen, V.T., 2020. Metabolomics Applications in Immunological Studies of Marine Molluscs. Auckland University of Technology, PhD Thesis, Auckland, New Zealand, 329 pp. 10.13140/RG.2.2.10327.21929.
Nguyen, V.T., Alfaro, A., 2019. Targeted metabolomics to investigate antimicrobial activity of itaconic acid in marine molluscs. Metabolomics 15, 97. https://doi.org/10.1007/s11306-019-1556-8.
Nguyen, V.T., Alfaro, A., 2020. Applications of omics to investigate responses of bivalve haemocytes to pathogen infections and environmental stress. Aquaculture 518, 734488. https://doi.org/10.1016/j.aquaculture.2019.734488.
Nguyen, V.T., Alfaro, A., Merien, F., 2019. Omics approaches to investigate host-pathogen interactions in mass mortality outbreaks of Crassostrea gigas. Review in Aquaculture. 11, 1308-1324. https://www.doi.org/10.1111/raq.12294.
Nguyen, V.T., Alfaro, A., Merien, F., Lulijwa, R., Young, T., 2018a. Copper-induced immunomodulation in mussel (Perna canaliculus) haemocytes. Metallomics. 10, 965–978. https://doi.org/10.1039/c8mt00092a.
Nguyen, V.T., Alfaro, A., Young, T., Merien, F., 2018b. Tissue-specific immune responses to Vibrio sp. infection in mussels (*Perna canaliculus*): A metabolomics approach. Aquaculture 500, 118–125. https://doi.org/10.1016/j.aquaculture.2018.09.061.
Nguyen, V.T., Alfaro, A., Young, T., Ravi, S., Merien, F., 2018c. Metabolomics study of immune responses of New Zealand greenshell™ mussels (Perna canaliculus) infected with pathogenic Vibrio sp. Mar. Biotechnol. 20, 396–409. https://doi.org/10.1007/s10126-018-9804-x.
Nguyen, V.T., Ragg, N.L.C., Alfaro, A., Zamora, L.N., 2020. Physiological stress associated with mechanical harvesting and transport of cultured mussels (*Perna canaliculus*): A metabolomics approach. Aquaculture 529, 735657. https://doi.org/10.1016/j.aquaculture.2020.735657.
Nikiforova, V.J., Willmitzer, L., 2007. Network visualization and network analysis. in: Baginsky, S., Fernie, A.R. (Eds.), Plant Systems Biology. Birkhäuser Basel, Basel, pp. 245-275.
Palacios, E., Bonilla, A., Luna, D., Racotta, I.S., 2004. Survival, Na+/K+-ATPase and lipid responses to salinity challenge in fed and starved white pacific shrimp (*Litopenaeus vannamei*) postlarvae. Aquaculture 234, 497–511.
Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., et al., 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucl. Acids Res. 3, 4. https://doi.org/10.1093/nar/gkab382.
Paterson, B., 1993. The rise in inosine monophosphate and L-lactate concentrations in muscle of live penaeid prawns (*Penaeus japonicus*, *Penaeus monodon*) stressed by storage out of water. Compar. Biochem. Physiol. B. Compar. Biochem. 106, 395–400.
Plowman, S.A., Smith, D.L., 2007. Anaerobic Metabolism during Exercise. In: Donatelli, R. (Ed.), Sports-Specific Rehabilitation. Churchill Livingstone, Saint Louis, pp. 213-230.
Racotta, I.S., Palacios, E., Méndez, L., 2002. Metabolic responses to short and long-term exposure to hypoxia in white shrimp (*Penaeus vannamei*). Mar. Freshwater Behav. Physiol. 35, 269–275.
Ray, S., 2018. Biological Resources of Water. In: Ray, S. (Ed.). BoD – Books on Demand, pp. 340.
Robertson, C., 2006. Australian prawn farming manual: health management for profit. The State of Queensland, Department of Primary Industries and Fisheries.
Sánchez, A., Pascual, C., Sánchez, A., Vargas-Albores, F., Le Moullac, G., Rosas, C., 2001. Hemolymph metabolic variables and immune response in *Litopenaeus setiferus* adult males: the effect of acclimation. Aquaculture 198, 13–28. https://doi.org/10.1016/S0044-8486(00)00576-7.
Schröder, K., 2020. Chapter 27 - Redox signaling in cellular differentiation. In: Sies, H. (Ed.), Oxidative Stress. Academic Press, pp. 539-563.
Smart, K.F., Aggio, R.B., Van Houtte, J.R., Villas-Bôas, S.G., 2010. Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography–mass spectrometry. Nat. Protoc. 5, 1709.
Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., López-Zavala, A., Forman, H.J., Yepiz-Plascencia, G., 2009. Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue-specific expression under hypoxia. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 150, 395–405.
Soñanez-Organis, J.G., Racotta, I.S., Yepiz-Plascencia, G., 2010. Silencing of the hypoxia inducible factor 1–HIF-1-obliterates the effects of hypoxia on glucose and lactate concentrations in a tissue-specific manner in the shrimp *Litopenaeus vannamei*. J. Exp. Mar. Biol. Ecol. 393, 51–58.
Soñanez-Organis, J.G., Rodriguez-Armenta, M., Leal-Rubio, B., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Yepiz-Plascencia, G., 2012. Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp *Litopenaeus vannamei*. Biochimie 94, 1250–1260. https://doi.org/10.1016/j.biochi.2012.02.015.
Souba, W.W., 1991. Glutamine: A Key Substrate for the Splanchnic Bed. Annu. Rev. Nutr. 11, 285–308. https://doi.org/10.1146/annurev.nu.11.070191.001441.
Strahl, J., Dringen, R., Schmidt, M.M., Hardenberg, S., Abele, D., 2011. Metabolic and physiological responses in tissues of the long-lived bivalve *Arctica islandica* to oxygen deficiency. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 158, 513–519.
Su, M.-A., Huang, Y.-T., Chen, I.-T., Lee, D.-Y., Hsieh, Y.-C., Li, C.-Y., Ng, T.H., Liang, S.-Y., Lin, S.-Y., Huang, S.-W., 2014. An invertebrate Warburg effect: a shrimp virus achieves successful replication by altering the host metabolome via the PI3K-Akt-mTOR pathway. PLoS Pathog. 10, e1004196.
Sun, R.C., Denko, N.C., 2014. Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. Cell Metab. 19, 285–292.
Sun, S., Guo, Z., Fu, H., Ge, X., Zhu, J., Gu, Z., 2018. Based on the metabolomic approach the energy metabolism responses of oriental river prawn *Macrobrachium nipponense* hepatopancreas to acute hypoxia and reoxygenation. Front. Physiol. 9, 1–11. https://doi.org/10.3389/fphys.2018.00076.
Tuffnail, W., Mills, G.A., Cary, P., Greenwood, R., 2009. An environmental 1 H NMR metabolomic study of the exposure of the marine mussel *Mytilus edulis* to atrazine, lindane, hypoxia and starvation. Metabolomics 5, 33–43.
Vaz, F.M., Wanders, R.J., 2002. Carnitine biosynthesis in mammals. Biochem. J 361, 417–429.
Venter, L., Loots, D.T., Mienie, L.J., Jansen van Rensburg, P.J., Mason, S., Vosloo, A., Lindeque, J.Z., 2018. The cross-tissue metabolic response of abalone (*Haliotis midae*) to functional hypoxia. Biol. Open. 7, bio031070. https://doi.org/10.1242/bio.031070.
Viant, M.R., Rosenblum, E.S., Tjeerdema, R.S., 2003. NMR-based metabolomics: A powedul approach for characterizing the effects of environmental stressors on organism health. Environ. Sci. Technol. 37, 4982–4989.
Warburg, O., 1956. On the origin of cancer cells. Science 123, 309–314.
Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Mar. Pollut. Bull. 45, 35–45. https://doi.org/10.1016/S0025-326X(02)00061-9.
Xia, J., Wishart, D.S., 2010. MetPA: a web-based metabolomics tool for pathway analysis and visualization. Bioinformatics 26, 2342–2344.
Yaqoob, P., 2003. Fatty acids as gatekeepers of immune cell regulation. Trends Immunol. 24, 639–645. https://doi.org/10.1016/j.it.2003.10.002.
Young, T., Kesarcodi-Watson, A., Alfaro, A.C., Merien, F., Nguyen, T.V., Mae, H., Le, D. V., Villas-Bôas, S., 2017. Differential expression of novel metabolic and

immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. Dev. Comp. Immunol. 73, 229–245. https://doi.org/10.1016/j.dci.2017.03.025.
Zenteno-Savín, T., Saldierna, R., Ahuejote-Sandoval, M., 2006. Superoxide radical production in response to environmental hypoxia in cultured shrimp. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 142, 301–308. https://doi.org/10.1016/j.cbpc.2005.11.001.
Zurburg, W., Ebberink, R., 1980. Flexibility in anaerobic metabolism in Mytilus edulis L.I organ specific differences in ATP-generating systems. In: Gilles, R. (Ed.), Animals and Environmental Fitness: Physiological and Biochemical Aspects of Adaptation and Ecology. Elsevier, pp. 55-56.
Zwerschke, W., Mazurek, S., Massimi, P., Banks, L., Eigenbrodt, E., Jansen-Dürr, P., 1999. Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. Proc. Natl. Acad. Sci. 96, 1291–1296.