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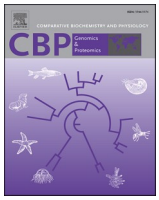

Research Article

Transcriptional and metabolomic investigation of the stress response in snow crab during simulated transport condition (*Chionoecetes opilio*)

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ABSTRACT

The molecular mechanisms underlying the stress response are poorly described in crustaceans. This includes the snow crab (*Chionoecetes opilio*), a commercially important stenotherm species distributed throughout the northern hemisphere. A better understanding of the stress response in *C. opilio* is desperately needed for commercial and conservation purposes. The purpose of this study was to investigate the transcriptional and metabolomic response of *C. opilio* exposed to stressors. Crabs were randomly assigned to 24 or 72 h treatment groups where they were exposed to conditions simulating live transport (handling and air exposure). A control group was kept in cold (2 °C) and well-oxygenated saltwater. The hepatopancreas of the crabs was sampled to perform RNA-sequencing and high-performance chemical isotope labeling metabolomics. Differential gene expression analyses showed that classic crustaceans' stress markers, such as crustacean hyperglycemic hormones and heat shock proteins, were overexpressed in response to stressors. Tyrosine decarboxylase was also upregulated in stressed crabs, suggesting an implication of the catecholamines tyramine and octopamine in the stress response. Deregulated metabolites revealed that low oxygen was an important trigger in the stress response as intermediate metabolites of the tricarboxylic acid cycle (TCA) accumulated. Lactate, which accumulated unevenly between crabs could potentially be used to predict mortality. This study provides new information on how stressors affect crustaceans and provides a basis for the development of stress markers in *C. opilio*.

1. Introduction

Metazoans have multiple strategies to defend their homeostasis when facing non-optimal biotic and abiotic conditions. In animals, the stress response is triggered by the rapid release of neuroendocrine factors such as catecholamines (e.g., epinephrine and norepinephrine). These cascades are best characterized in vertebrates, in which the hypothalamic-pituitary-adrenal (HPA) axis leads to the release of catecholamines and glucocorticoids (Sapolsky, 2002; Charmandari et al., 2005; Akin, 2011; Nelson and Kriegsfeld, 2017). These hormones, through physiological changes, signal adjustments to maintain homeostasis despite environmental disturbance. These changes generally include an increased oxygen uptake and transport (e.g., increased cardiac rate and output, increased hemoglobin affinity for oxygen), the mobilization of energy substrates, and energy reallocation to essential functions by inhibiting non-essential processes like growth, reproduction, and the immune system (Sapolsky, 2002; Nelson and Kriegsfeld, 2017). However, after prolonged or chronic exposure to a stressor, the stress response can become maladaptive (e.g., increased vulnerability to diseases) (Sapolsky, 2002; Charmandari et al., 2005).

Compared with vertebrates, invertebrates have different neuroendocrine organs but a similar stress response by releasing biogenic amines such as serotonin (5-hydroxytryptamine – 5-HT), dopamine and octopamine (Sneddon et al., 2000; Lorenzon et al., 2005; Adamo, 2008; Chen et al., 2008; Aparicio-Simón et al., 2010). Biogenic amines act as neurotransmitters and neuromodulators, both of which are released at the synapse, and sometimes as neurohormones by being secreted in the extracellular fluid (Fingerman et al., 1994). In crustaceans, two major neuroendocrine centers are conserved: the X-organ/sinus gland of the eyestalk (XO-SG) and the pericardial organ located along the pericardial chamber, the latter being the most studied (Christie, 2011; Tong et al., 2021). They release hormones (e.g., crustacean hyperglycemic hormones (CHH), molt-inhibiting hormones (MIH), and mandibular organ-inhibiting hormones (MOIH)), that regulate key physiological processes such as reproduction, molting, and stress and immune responses (Santos et al., 2001; Christie, 2011).

In aquatic animals, hyperglycemia is commonly used to provide energy to the organism in response to stressors (Durand et al., 2000;

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Lorenzon, 2005). In crustaceans, stress-induced hyperglycemia is controlled by pleiotropic CHH, mostly released by the XO-SG complex (Fanjul-Moles, 2006; Chung et al., 2010; Webster et al., 2012; Chen et al., 2020). CHH increases glycemia by stimulating glycogenolysis and inhibiting glycogenesis in tissues such as muscles and the hepatopancreas (Fanjul-Moles, 2006; Chen et al., 2020). Initially reported as a diabetogenic factor (Abramowitz et al., 1944; Kegel et al., 1989) the CHH is now described as a superfamily composed of several homologous genes modulating a variety of processes such as molting, reproduction, osmotic regulation, energy metabolism, and the stress response (Chung and Webster, 2005; Nakatsuji et al., 2009; Webster et al., 2012; Chen et al., 2020). The family can be divided into type 1 (CHH) and type 2 (MOIH/MIH/GIH), where type 1 has a precursor-related peptide (CPRP) that needs to be cleaved to produce a functional hormone, a peptide not found in type 2 (Lacombe et al., 1999; Chan et al., 2003; Chen et al., 2020, 2005). With its hyperglycemic effect, type 1 CHH is usually associated with the stress response and has been reported to increases in response to multiple stressors such as emersion, salinity changes, high temperature, and heavy metal exposure (Lorenzon et al., 2004; Chang, 2005; Chung and Zmora, 2008; Shinji et al., 2012).

Several studies have focused on crustacean stress response (Lorenzon et al., 2005; Fotedar and Evans, 2011; Stoner, 2012; James et al., 2019), but much remains to be known compared with vertebrates. For example, little is known about how biogenic amines released in the hemolymph as neurohormones mediate the stress response (Aparicio-Simón et al., 2010). Because they are more diverse than vertebrates, it could be more difficult to understand and generalize the stress response in invertebrates (Adamo, 2012). However, recent breakthroughs have been made by studying the crustacean immune response, which is tightly intermingled with the stress response (Tong et al., 2021, 2020). However, these studies focused on stressors found in an aquaculture environment (e.g., ammonia exposure and diseases), and little is known about the transcriptional response and metabolism of crustaceans challenged by harvesting and live transport (e.g., air exposure, temperature change and handling), especially among larger species such as the snow crab (*C. opilio*).

C. opilio is distributed throughout the northern hemisphere at depths ranging from 200 to 1400 m and at temperature ranging from −1.5 °C to 4 °C (Dawe and Colbourne, 2002). Harvested in the north of the Atlantic and Pacific oceans, the snow crab supports a billion-dollar industry in Canada, the United States, China, and Japan (Gardner, 2014; Government of Canada, 2022). The industry relies on live transport of crabs and better animal welfare is needed to optimize the efficiency of live transport. Although the life cycle of the snow crab is well characterized (Sainte-Marie, 1993; Sainte-Marie et al., 1995; Comeau et al., 1998), little is known about its physiology and only a few studies have focused on its stress response (James et al., 2019; Hall et al., 2021). Efficient stress markers are needed to better quantify the stress response in snow crabs. Here, we leverage transcriptomics and metabolomics to identify differently expressed genes and deregulated metabolites during transport-induced stress in the hepatopancreas of mature male *C. opilio*. We focus on the hepatopancreas because it is a multifunctional organ known to respond to environmental stress, such as an osmotic shock (Gao et al., 2012) or hypoxia (Jiang et al., 2009).

2. Materials and methods

2.1. Treatments and sampling

Snow crabs were harvested in May 2020 from the Gulf of St. Lawrence by local fishermen and transported on ice to the Université de Moncton (Moncton, NB, Canada). Only mature males were obtained because females are released for conservation purposes (Government of Canada, 2016). The crabs were acclimated in a recirculating system with well-oxygenated artificial seawater (32 ppt, Crystal Sea Marinemix, Marine Enterprises International, Baltimore, MD, USA) kept at 2 °C for 4 weeks. Crabs were not fed during acclimation nor during the treatment. The crabs were then randomly assigned to 24 or 72 h treatment (air exposure at 4 °C) in loosely covered containers to allow air circulation to simulate transport conditions. These treatment durations were chosen based on realistic transport duration in the industry while minimizing mortality during the treatment. The remaining crabs were kept in the tanks throughout the experiment and used as a control group. At the end of the trial, the hepatopancreas was sampled, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. No mortality was reported during the trials.

2.2. RNA extraction

Total hepatopancreatic RNA was extracted from 6 individuals per group using a commercial kit, following the manufacturer's instructions (Monarch total RNA miniprep, New England BioLabs) with one minor modification. During RNA extraction, we noticed that *C. opilio*, as many other arthropods, appears to have a 28S ribosome composed of two subunits linked by a hydrogen bond known as the 'hidden break' (Sun et al., 2012; McCarthy et al., 2015; Natsidis et al., 2019). This bond can be broken by the denaturation step, so we mechanically homogenized the tissues using a micropestle instead of proteinase K. To remove genomic DNA, we performed a DNase I treatment (included in the kit), according to the manufacturer's instructions. Before sequencing, RNA quality was assessed using an Experion automated electrophoresis system (Bio-Rad, Hercules, CA) and quantified with Qubit (ThermoFisher Scientific). The samples all had an RNA quality indicator (RIN) > 8.

2.3. Library preparation and sequencing

Library preparation and sequencing were performed by the Atlantic Cancer Research Institute (ACRI, Moncton, NB, Canada). A total of 18 libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, E7760L) following the manufacturer's protocol. Paired-end sequencing (100 bp) was performed on the NovaSeq 6000 instrument (Illumina).

2.4. Transcriptomic analysis

Quality filtering and trimming were performed with Trimmomatic v0.38 (Bolger et al., 2014) using default parameters except for HEADCROP:12 due to remaining adapters following demultiplexing. FastQC reports (Brown, Pirrung, and McCue2017) were compiled with MultiQC v1.9 (Ewels et al., 2016). An individual (chop-15) showed signs of contamination based on the GC content distribution, so we removed it and performed all other analyses on the remaining 17 individuals (Supplementary Fig. S1). Reads were mapped onto the reference genome of *C. opilio* (NCBI bioproject PRJNA602365, assembly ASM1658430v1) with STAR v2.7.7 using default parameters (Honarbakhsh et al., 2019, see Table 1). A count matrix was generated using Subreads's featurecounts v2.0.1 (Liao et al., 2014) and differential gene expression analysis was performed using DESeq2 v1.30.1 with default parameters (Love et al., 2014) in R (version 4.0.4, R Core Team, 2017).

2.5. Metabolomic analysis

Samples of hepatopancreas (~1 g) from 24 crabs (8 each group) were sent to the Metabolomics Innovation Centre, Edmonton, Alberta, Canada for a 2-channel analysis of high-performance chemical isotope labeling (LC-MS). Tissue samples were disrupted in 4:1, methanol: water (v:v), by vigorously shaking for 15 s in a bead beater, using ceramic beads. Following a 10-min centrifugation at 12,000 $\times g$ and 4 °C, the supernatant was transferred into a clean tube and dried down under a stream of nitrogen. The dried extracts were stored at −80 °C. The dried extracts were dissolved in 200 μL of distilled water and their total concentration determined using the Sample Normalization kit (NovaMT). Samples

Table 1
Summary of the sequencing data and mapping results.

Sample	Treatment	Raw reads	Trimmed reads	% GC	Reads mapped
chop-17	Ctl	40,470,244	38,341,396	50	32,895,081 (69.14 %)
chop-18	Ctl	54,558,564	52,561,898	50	47,213,601 (66.86 %)
chop-19	Ctl	61,863,800	58,938,032	49	51,044,148 (79.53 %)
chop-20	Ctl	57,469,874	55,139,852	50	48,268,577 (80.40 %)
chop-21	Ctl	60,319,212	57,850,928	48	49,470,753 (78.64 %)
chop-22	Ctl	68,021,650	65,507,910	49	57,837,871 (80.63 %)
chop-01	24 h	55,495,560	52,407,726	48	45,297,939 (77.99 %)
chop-02	24 h	60,489,914	58,265,940	51	53,243,937 (61.24 %)
chop-03	24 h	67,195,040	64,260,424	50	56,468,908 (63.23 %)
chop-04	24 h	55,938,268	53,726,144	49	47,083,691 (75.53 %)
chop-05	24 h	59,612,316	56,525,632	48	49,180,868 (75.88 %)
chop-06	24 h	60,930,380	58,701,266	49	51,380,408 (79.58 %)
chop-09	72 h	66,087,576	62,775,278	49	53,768,797 (77.68 %)
chop-10	72 h	62,350,012	59,596,904	49	52,546,810 (77.38 %)
chop-11	72 h	51,974,230	49,423,420	48	43,631,511 (76.16 %)
chop-12	72 h	57,920,086	55,649,910	51	50,001,742 (60.90 %)
chop-14	72 h	67,399,986	61,778,756	49	54,829,475 (81.80 %)
chop-15	72 h	623,58,966	55,014,440	48	46,951,579 (79,55 %)

with a total concentration higher than 8 mM were diluted to 8 mM using distilled water. The extracted metabolites were then labeled using the Dansyl Labeling Kit or the DmPA Labeling Kit (NovaMT Inc.). A pool of all samples was made to be used as a reference and quality control. Chemical isotope labeling was performed strictly following the standard operating procedure (SOP). Briefly, the metabolites for individual samples were labeled by adding $^{12}C_2$-dansyl chloride or $^{12}C_2$-dimethylaminophenacyl bromide ($^{12}C_2$-labeling) and the pooled samples by adding $^{13}C_2$ – dansyl chloride or $^{13}C_2$-dimethylaminophenacyl bromide ($^{13}C_2$-labeling), after which the pooled sample is mixed with the individual sample in a 1:1 mol ratio. The metabolites are then detected in a peak pair form (individual sample/reference sample). Based on peak pairs, metabolites were identified by a three-tier approach (Zhao et al., 2019), using IsoMS Pro 1.2.15 (NovaMT Inc.) and the NovaMT Metabolite Database v2.0. Results were analyzed using MetaboAnalyst (www.metaboanalyst.ca) to detect differences in the metabolite profile of the experimental groups (e.g., partial least square-discriminant analysis) based on the relative level of the metabolites.

3. Results

To investigate the transcriptional response to transport-induced stressors in *C. opilio*, we generated RNA sequencing data from the hepatopancreas in control individuals and crabs submitted to transport simulation for 24 h or 72 h. We obtained a total of 1,070,455,678 reads (535,227,839 pairs) among which 1,016,465,856 (94.96 %) remained after quality filtering and trimming (Table 1). We mapped the trimmed reads to the reference genome (ASM1658430v1) and obtained an average of 71.26 % uniquely mapped reads (total mapped, 60.90–81.80 % per library).

To obtain a global picture of the experiment and determine whether individuals from each of the experimental groups clustered together, we performed a principal component analysis (PCA) on the normalized read counts obtained from DESeq2 (Fig. 1). Although the first principal component (PC1, 19.55 % of the variance) clearly separates the 72 h treatment group, the 24 h treatment group is not easily distinguishable from the control group. PC2 and PC3 also did not distinguish between the 24 h and control groups, suggesting a limited transcriptional response over a short transport time.

To identify differentially expressed genes (DEGs) among groups, we performed a pairwise differential gene expression analysis using DESeq2 (no fold-change filter, FDR < 0.1, adjusted p-value < 0.05). As expected, based on the PCA, there were fewer DEGs between the 24 h and control groups (N = 19) compared to between the 72 h and control group (N = 550) (Fig. 2, Table 2). We also found 416 DEGs between the 24 h and 72 h groups, 299 of which (71.8 %) were also differentially expressed between the control and 72 h groups. Only four genes were differentially expressed in all pairwise comparisons.

Because the most differentially expressed genes (that is, the most "responsive" genes) should be the best stress indicators, we selected DEGs with a $\log_2FC \geq 4$. With such a strict threshold, there were no DEGs between the 24 h and control groups (Fig. 2A). However, there were 21 DEGs remaining between the 24 h and 72 h, and 34 DEGs between the control and 72 h groups. Furthermore, most of these genes are up-regulated, except two genes that were down-regulated between the 72 h and control groups. Given the small number of DEGs between the 24 h and the control groups, we then focused on the comparison between the 72 h and control groups. We identified 34 DEGs (FDR < 0.1, adjusted p-value <0.05, $\log_2FC \geq 4$), of which 16 were annotated while the others are listed as "hypothetical proteins" (Table 3). To visualize the expression levels of these genes and thus identify potential stress markers capable of quantifying the stress response over time, we plotted the pseudocounts (log-transformed) for each of these 16 annotated DEGs (Fig. 3, pseudocounts = normalized read counts + 1).

The targeted metabolomics approach successfully detected 1791 metabolites in total of which 253 were deregulated by the treatment and identified with a high level of confidence (tier 1 and 2). We performed a partial least squares-discriminant analysis (PLS-DA) to investigate the metabolite profile of the experimental groups and target the metabolites that influence the model the most. PLS-DA clearly separated the three experimental groups (Fig. 4B). The individuals in the 72 h group seemed more dispersed among themselves, compared with the 24 h groups which were more consistent. Fig. 4 (A, C) showed the most influential metabolites in the model (i.e., those with a variable importance in projection (VIP) >1). Many of these metabolites were amino acids or related metabolites (Fig. 4A,C, Supplementary Table S5) (e.g., valine, glutamine, alanine, histidine, leucine, and isoleucine). However, it is worth noting that the derivatization techniques used selectively reacted with amines, phenols, hydroxyls, and organic acids, it is therefore not surprising that many amino acids were overrepresented.

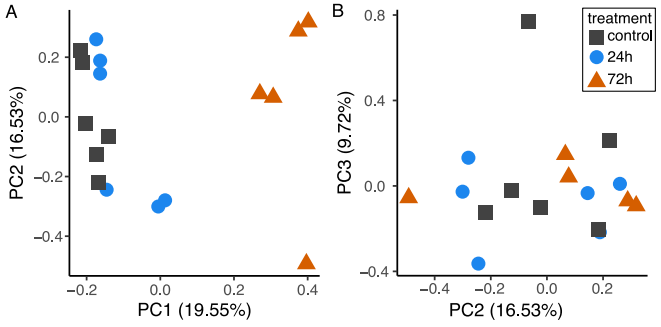

Fig. 1. PCA based on the RNA-seq read counts of *C. opilio* exposed to transport stress. The *prcomp* function was used to perform PCA in R. (A) PC1and PC2. (B) PC2 and PC3.

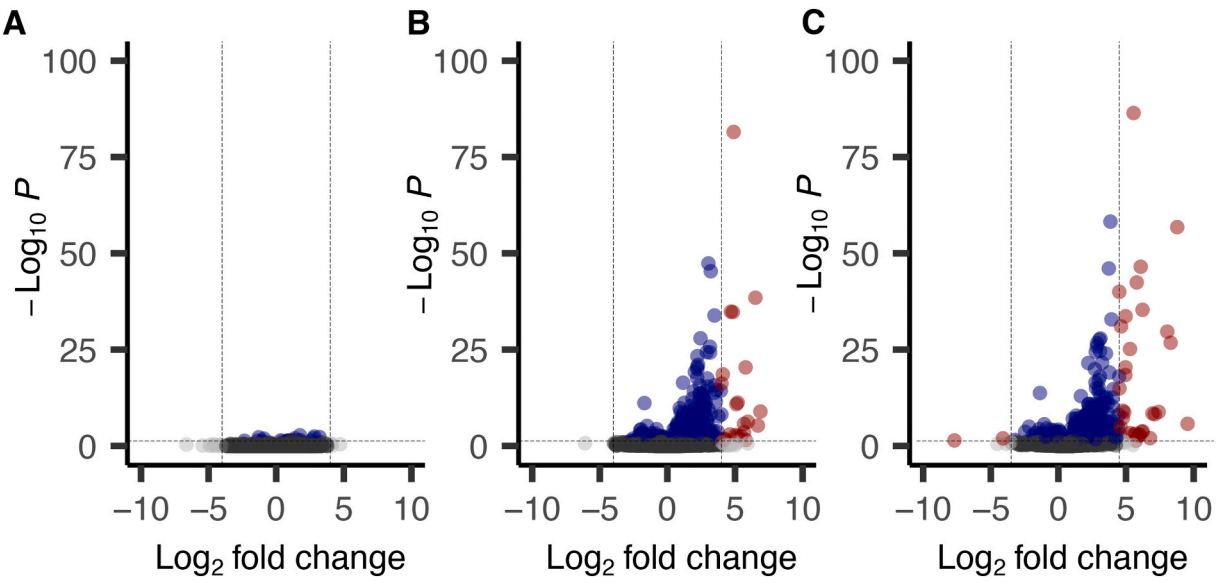

Fig. 2. Volcano plots of differentially expressed genes (DEGs) of *C. opilio* exposed to transport stress for all pairwise comparisons. Blue dots indicate significantly DEGs (FDR < 0.1, adjusted p-value <0.05) whereas red dots indicate significantly DEGs with a $Log_2FC \geq 4$. (A) 24 h versus control groups. (B) 24 h versus 72 h groups. (C) 72 h versus control groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of differential expression (FDR < 0.1, adjusted p-value <0.05, Supplementary Tables S1–S3).

Comparison	#DEGs up	#DEGs down	#DEGs total
24 h vs Ctl	14	5	19
24 h vs 72 h	338	78	416
72 h vs Ctl	422	128	550

Table 3
List of annotated DEGs between the 72 h and control groups (FDR < 0.1, adjusted p-value <0.05, $Log_2FC \geq 4$). Hypothetical proteins were removed (see Supplementary Table S3 for a complete list).

Locus tag	Log_2FC	Adjusted p-value	Protein name
GWK47_043225	9.05	1.79E-06	Transmembrane protein 205
GWK47_032395	7.80	1.65E-27	Heat shock protein cognate 4
GWK47_035755	5.72	4.65E-36	Regulator of G-protein signaling 2
GWK47_022009	5.59	3.08E-47	Leukocyte receptor cluster member 9
GWK47_044774	5.22	4.47E-02	Mandibular organ-inhibiting hormone
GWK47_002223	5.06	3.76E-87	Hsp70-binding protein 1
GWK47_009484	4.79	6.90E-26	Serpin B8
GWK47_041179	4.47	2.24E-34	CCAAT/enhancer-binding protein beta
GWK47_015185	4.46	3.96E-21	Sterile alpha and TIR motif-containing protein 1
GWK47_009678	4.45	3.97E-19	DNA-binding protein D-ETS-6
GWK47_002381	4.37	1.83E-09	Tyrosine decarboxylase
GWK47_009172	4.27	7.30E-10	Transcription factor E3
GWK47_033734	4.00	9.91E-41	Heat shock protein 1
GWK47_033791	4.00	9.43E-32	DnaJ subfamily B member 4
GWK47_054367	−8.19	3.76E-02	Acidic amino acid decarboxylase GADL1
GWK47_027099	−4.60	1.06E-02	Sodium-coupled monocarboxylate transporter 2

4. Discussion

The main goal of this study was to better understand the transcriptional and metabolic stress response of *C. opilio* exposed to transport stress. We performed RNA-sequencing on stressed and control individuals to identify DEGs among the three groups. We also performed a metabolomics analysis to identify differentially abundant metabolites. We specifically targeted the hepatopancreas, a multifunctional organ that plays key roles in the energy metabolism and is known to respond to environmental stress. Herein, we discuss the strongest candidate stress markers for future investigations.

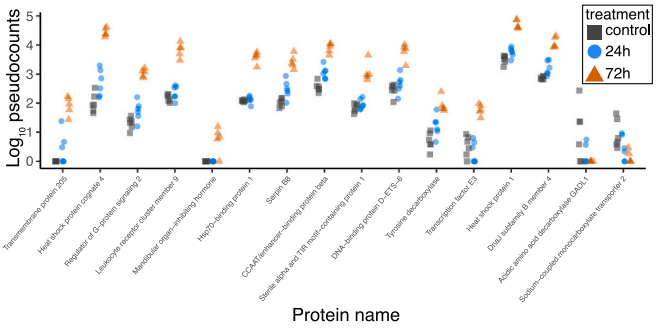

Fig. 3. Log_{10} pseudocounts (normalized read counts + 1) of the differentially expressed genes (DEGs) of *C. opilio* exposed to transport stress for 72 h and control groups (FDR < 0.1, adjusted p-value <0.05, $Log_2FC \geq 4$). Counts are shown for all three treatment groups (control, 24 h, 72 h).

4.1. Limited transcriptional response over short-term stress

Exposing *C. opilio* to air for 24 h led to a limited transcriptional response. This is supported by the lack of independent clustering of the 24 h and control groups in the PCA and the small number of DEGs (N = 19) identified. However, 24 h transport in the fishing industry is probably significantly harsher with rough handling, loud noise, and tons of crushed ice. Of note, there appears to be significant variation among individuals (PC2 axis, Fig. 1), which suggests that some crabs may be substantially more tolerant to air exposure than others.

4.2. Heat shock proteins are upregulated in stressed C. opilio

Several well conserved Heat Shock Proteins (HSP) such as HSP cognate 4, HSP70-binding protein 1, DnaJ subfamily B member 4 and

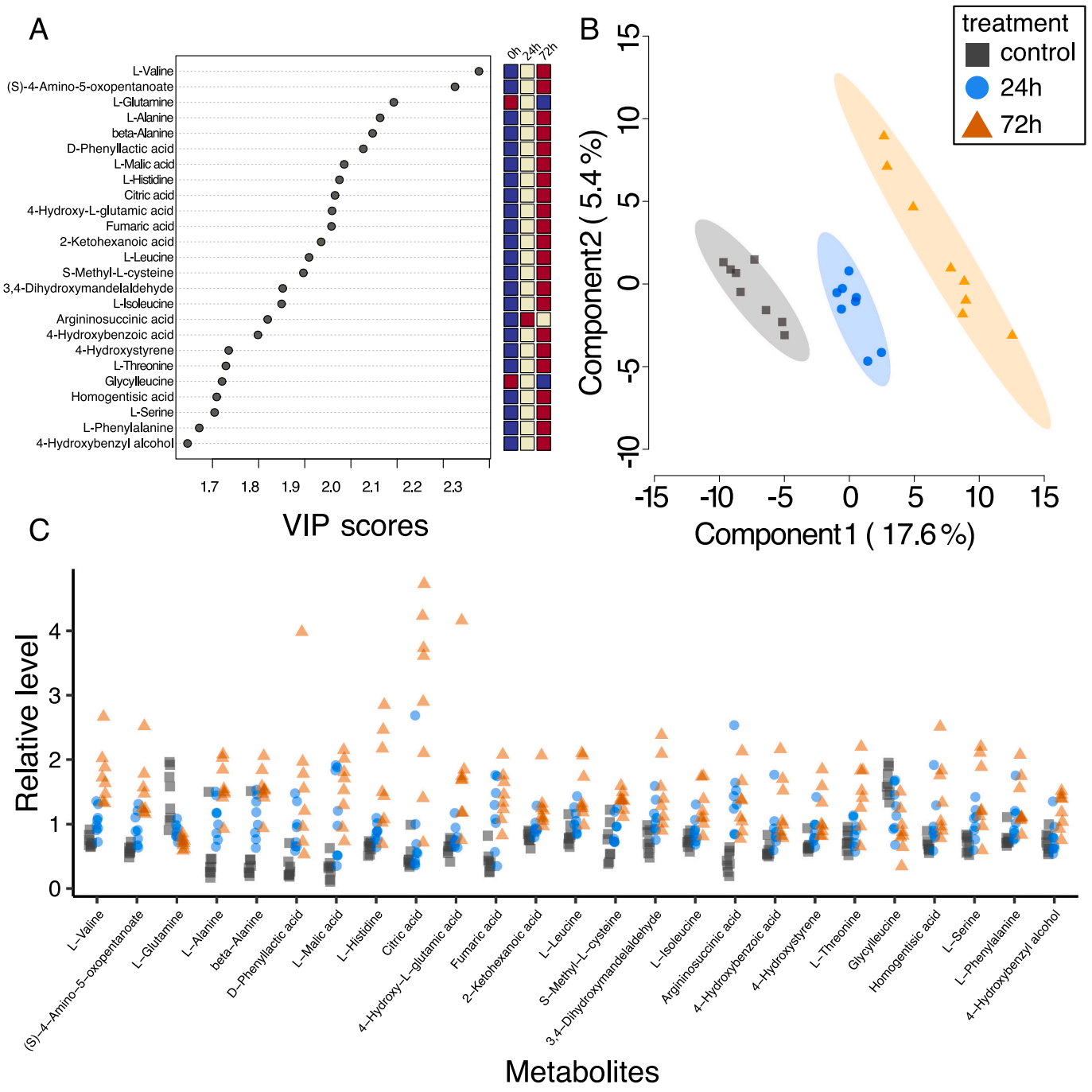

Fig. 4. Changes in the metabolite profile of *C. opilio* exposed to transport stress. We analyzed 253 metabolites (Supplementary Table S4) that were identified by liquid chromatography mass spectrometry (LC-MS) from hepatopancreas. (A) Top 25 metabolites with the highest VIP score (See Supplementary Tables S5–S6 for a complete list and cross validation data). (B) PLS-DA score plot. (C) Relative level of the 25 metabolites for each sample.

HSP22 were upregulated in stressed crabs. HSPs, also called molecular chaperones, are the guardians of cellular homeostasis (Welch, 1992; Hendrick and Hartl, 1993; Sanders, 1993). Divided into several families, they are involved in the folding and refolding of proteins, in the degradation of damaged proteins, and in the prevention of protein aggregation (Kampinga and Craig, 2010; Li, 2017; Junprung et al., 2021). Thus, they contribute to the protection of the cell against many stressors. HSP levels increase in crustaceans exposed to temperature stress, hypoxia, osmotic stress, pH, heavy metal exposure, pathogenic infection, and more (Li, 2017). Heat shock protein cognate 4 and Heat shock protein 1 are members of the HSP70 family. While HSP70 family members are ubiquitously expressed, their concentration has been shown to increase in response to several stressors in crustaceans. From cell growth to protein folding and translocation, the HSP70 family has evolved a diversity of functions (Kampinga and Craig, 2010; Li, 2017), and can be described as a hub in the chaperone network (Finka et al., 2015). Heat shock protein cognates are constitutively expressed, and the results suggest that HSP cognate 4, with a fold change of 7 (in the 72 h group), is also involved in the stress response of *C. opilio*.

The DnaJ subfamily B member 4 is part of the HSP40 family which stimulates HSP70 to hydrolyze ATP and adopt a close configuration to stabilize the binding to the peptide (Cyr et al., 1994; Kampinga and Craig, 2010). Once the protein substrate is properly folded, a nucleotide exchange factor (e.g., heat shock protein binding protein 1 (HSPBP1))

exchanges the ADP for ATP, which again modifies the configuration of HSP70 and releases the peptide. The results suggest that the complex formed by HSP70, DnaJ domain and HSPBP1 plays an important role in the refolding of proteins in snow crabs in response to stressors (Table 3, Fig. 3).

HSP22 is a small HSP which has been shown to be up-regulated in crustaceans exposed to environmental stressors (Shekhar et al., 2013; Kim et al., 2014; Shi et al., 2016). Small HSPs are regulated by post-transcriptional phosphorylation, and HSP22 is known to be phosphorylated during stress conditions (Bakthisaran et al., 2015). HSP22 appears to be involved in the early stress response of *C. opilio* (Supplementary Table S1).

4.3. The CHH family

So far, two CHH genes have been described in *C. opilio* (KAG0710697 and AHM93480.1) and the results indicated that CHH1 (GWK47_022272) was upregulated with a fold change of 3.8 while CHH2 was not expressed in the hepatopancreas. The mandibular organ-inhibiting hormone (MOIH), which is a member of the CHH superfamily, was also upregulated with a fold change of 5.22. However, after a multiple sequence alignment (Supplementary Fig. S2) of *C. opilio* CHH/MIH/MOIH with those of other crustaceans (*Cancer pagurus, Libinia emarginata, Homarus americanus and Carcinus maenas*), we suspect that the gene annotated as the mandibular organ-inhibiting hormone (MOIH) of *C. opilio* is a paralog of the CHH2. This is further supported by the observation that the snow crab family (Oregoniidae) does not possess MOIH (Webster et al., 2012). The gene annotated as MOIH (GWK47_044774) seems to be involved in the prolonged stress response (72 h) in *C. opilio* (Fig. 3, Supplementary Fig. S3).

4.4. Catecholamine synthesis is a strong stress indicator pathway candidate

The results show that tyrosine decarboxylase (TDC) (Table 3, Fig. 3) was upregulated which suggests an implication of the biogenic amine tyramine (TA) and octopamine (OA) in the transport-induced stress response of *C. opilio*, but further investigation is needed. Tyrosine is the metabolic precursor of the epinephrine and octopamine synthesis pathways. In the octopamine pathway, tyrosine decarboxylase (TDC) catalyzes the conversion of tyrosine into tyramine (TA), a precursor of octopamine (OA). In invertebrates, these catecholamines are involved in a variety of physiological processes such as behavior and metabolism. With similar effects to epinephrine and norepinephrine in vertebrates, TA and OA modulate the fight or flight response in insects (Verlinden et al., 2010; Roeder, 2020). In crustaceans, TA/OA appears to promote immunity by stimulating hemocyte proliferation and phenoloxidase activity (Kuo et al., 2019; Kuo and Cheng, 2021, 2018). Further investigation is needed to confirm that the biogenic amine TA/OA are involved in the stress response of snow crabs.

4.5. Immune response

The important fold change of serpinB8 (Table 3, Fig. 3), which is thought to be an inhibitor of the immune response, suggests that transport stress response also modulates the immune response. Previous work has revealed that environmental factors affect the immune system of invertebrates (Chen and He, 2019). Invertebrates do not have an adaptive immune system as vertebrates do, and thus rely on their innate immune system to fight infections (Cerenius et al., 2010). In crustaceans, three types of hemocytes (hyaline, semi-granular, and granular) are involved in the immune defense (Söderhäll and Cerenius, 1992; Söderhäll, 2016). Differential expression of genes involved in immunity have been observed in response to ammonia (Lu et al., 2016), osmotic stress (Zhao et al., 2015), and hypoxia (Jiang et al., 2009; Li and Brouwer, 2013). In general, stress has a negative impact on the immune system (Tong et al., 2021). For instance, a decrease in hemocytes count and phenoloxidase activity may be observed in response to hypoxia (Tanner et al., 2006; Hu et al., 2009; Paschke et al., 2010). Some metabolites play a key role in both the immune response and stress response (Adamo, 2008). For example, hemocytes express an octopamine receptor which is known to stimulate immune functions (increase PO activity and hemocyte count) (Kuo and Cheng, 2018; Kuo et al., 2019). Therefore, we speculate that octopamine released in response to stress modulates the immune response. However, octopamine was not detected in the metabolomics study and would need to be quantified using a targeted approach to further support this hypothesis. In general, the stress response has both negative and positive effects on the immune system. We did not investigate the effect of stressors on the immune system, but some DEGs appear to be related to immunity. Serpins are a superfamily of proteins that are known to have inhibitory effects on the immune system of crustaceans (Shekhar et al., 2014). However, serpin members of clade B have been barely described in invertebrates. Although it is difficult to know the exact role of serpinB8, we speculate that it controls the immune response during the stress response, which could be harmful to the individual if left out of control. Other studies have also observed that serpin B was upregulated in response to salinity stressors (Shekhar et al., 2014) and air exposure (Bao et al., 2019).

4.6. Anaerobic metabolism in response to hypoxemia

Accumulation of intermediate metabolites of the TCA cycle such as citric acid, succinate, and fumarate in snow crabs (Fig. 4, Supplementary Table S4) is a consequence of limited oxygen (Nguyen et al., 2022). During emersion, crabs may become hypoxemic, as oxygen uptake is limited during air exposure. Under such stress, aerobic metabolism is directly affected, as oxygen is needed for oxidative phosphorylation. Anaerobic metabolism-related pathways (e.g., Warburg effect) as well as amino acid-related pathways were identified in an enrichment metabolic pathway analysis, another indication of hypoxic stress. With limited oxygen, organisms must rely on anaerobic metabolism to produce ATP. Surprisingly, lactate, the main end product of the anaerobic metabolism in crustaceans, was not higher compared with what is found in another crustacean (Mota et al., 2021; Nguyen et al., 2022). This is explained by the fact that lactate seems to accumulate in some individuals while it does not accumulate in others (Supplementary Fig. S4). Lactate is possibly a potential stress marker as James et al. (2019) found that snow crabs with low reflex action mortality predictor (RAMP) had a high lactate concentration, while the most responsive crabs had a lower lactate concentration after air exposure. Why some crabs have lower lactate concentration than others is unclear. Some crustaceans that adapt to hypoxia have been reported to turn lactate into glucose (Ulaje et al., 2019), but such a strategy has not yet been described in *C. opilio*. It is also possible that some crabs were better able to obtain oxygen in the simulated transport stress. In either case, crabs with lower lactate concentrations are probably the most resistant to air exposure, which could be interesting to investigate further.

4.7. Use of amino acids as an energy source

Several deregulated metabolites in stressed snow crabs were related to amino acid metabolism. Crustacean tissues usually have a higher concentration of free amino acids compared with vertebrate tissues (Li et al., 2021). Free amino acids (FAA) are absorbed by the hepatopancreas and then released in the hemolymph where it is distributed to peripheric tissues to be used as substrates for protein synthesis, for ATP production or as precursors of low molecular weight metabolites (Wu, 2020, 2013). With multiple purposes, amino acids are certainly involved in one way or another in the stress response. In this experiment, several amino acids concentrations (e.g., valine, glutamine, alanine, histidine, leucine, isoleucine, threonine.) responded to transport stress. Mota et al., (2021) have found similar results in king crab exposed to transport

stress. FAA are thought to be an important metabolic fuel for crustaceans, particularly in the hepatopancreas (Li et al., 2021). The branched-chain amino acid valine has the highest VIP score of the 253 metabolites analyzed. Tripp-Valdez et al. (2019) suggested that the accumulation of valine is an indication of altered protein turnover.

5. Conclusion

Overall, this study provides new insight into the transcriptional and metabolic stress responses of *C. opilio* exposed to emersion. Hypoxemia seem to be an important trigger of the stress response as metabolomic analyses reveal deregulation of free amino acid and fatty acid pathway. In general, this study provides potential targets for the future development of stress markers such as lactate accumulation and the tyramine/octopamine pathway.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2023.101079.

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.

Data availability

Raw data is available on NCBI SRA (PRJNA809949).

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