



Survival and Metabolic Modulation of Swimming Crab *Portunus trituberculatus* During Live Transport

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Demand from consumers for small quantities of live swimming crab *Portunus trituberculatus* is rising with the development of e-commerce. However, it is challenging to keep *P. trituberculatus* alive post-capture. In this study, a transport bag containing oxygen and seawater (24 ppt) was used to investigate the survival and metabolic changes of *P. trituberculatus* during transport. The results showed that more than 80% *P. trituberculatus* could survive at least 24 h in the transport bag. The ability of the crabs to survive may be attributed to adaptive metabolism, as suggested by the switch from an aerobic to an anaerobic pathway for energy generation and a decline in amino acid metabolism, nucleic acid metabolism, and osmoregulation. Our findings suggest that the transport bag could effectively extend the post-capture survival time of *P. trituberculatus*. Metabolic adaptation – especially energy homeostasis – is crucial for crab survival during transport. Our study provides a promising method for the transport of live *P. trituberculatus*.

Keywords: *Portunus trituberculatus*, transport, energy metabolism, metabolomics, survival

HIGHLIGHTS

- A transport bag was designed for *P. trituberculatus*.
- Changes in energy metabolism, amino acid metabolism, nucleic acid metabolism, and osmoregulation occurred during crab transport.
- Metabolic adaptation is crucial for crab survival during transport.

INTRODUCTION

Seafood is perishable, and its postmortem freshness reduces quickly (Grassi et al., 2019). Live crabs are appreciated in China because of their taste (Lu et al., 2015). The swimming crab *P. trituberculatus* (Crustacea, Decapoda, and Brachyura) is a commercially important fishery species in China, with a yield of 113,810 tons in 2019 (China Fishery Statistical Yearbook, 2020). However, the post-capture survival of the swimming crab is poor because they are a water-breathing species and are prone to suffer from stress because of factors such as handling, cannibalism, overcrowding, temperature fluctuation, low humidity, and oxygen (Fotedar and Evans, 2011; Dong et al., 2019). Although various tanks, containers, and vehicles equipped with aeration facilities are available for holding *P. trituberculatus* during transport, the mortality remains high, mainly

because of unavoidable air exposure and temperature fluctuation (Lu et al., 2015, 2016; Dong et al., 2019). As a result, it is hard to transport live *P. trituberculatus* over long distances (Lu et al., 2015). Nevertheless, with the development of e-commerce, consumers demand comparatively small quantities of *P. trituberculatus*, which necessitates long-distance transport. Therefore, it is necessary to develop a suitable method for the transport of live *P. trituberculatus*.

Accumulating evidence shows that the post-capture survival rate of crabs depends on the species and holding conditions. For example, the green mud crab *Scylla paramamosain* can survive without water for 36 h at 25°C (Gu et al., 2017), while the southern king crab *Lithodes santolla* can tolerate up to 30 h at 12°C (Urbina et al., 2013). Chinese mitten crab *Eriocheir sinensis* and the stone king crab *Paralomis granulosa* could survive without water only for 16 and 18 h, respectively (Romero et al., 2011; Bao et al., 2019). In addition, adverse environmental conditions such as suboptimal temperature also can lead to physiological effects on crabs. For example, temperature fluctuation and air exposure lead to abrupt changes in levels of energy-related metabolites such as glucose, lactate, and adenine nucleotides in hemolymph and muscle of *P. trituberculatus* (Lu et al., 2015, 2016). Because air exposure reduces the moisture of gills and leads to the accumulation of fatal metabolites in the hemolymph of *P. trituberculatus*, >90% of *P. trituberculatus* cannot survive for >24 h under air exposure even at the optimal temperature (Dong et al., 2019). Therefore, we made the following assumptions: (1) water is indispensable for long-distance transport of *P. trituberculatus*, and (2) metabolic modulation is vital for the survival of *P. trituberculatus* during live transport.

In this context, we designed a transport bag containing oxygen and seawater to investigate whether the survival of *P. trituberculatus* during transport could be improved. Furthermore, the metabolic changes over transport time in each of four tissues were investigated to obtain further insight into the survival mechanisms of *P. trituberculatus*. The present study aimed to provide an alternative method for small-scale, long-distance transport of live *P. trituberculatus*.

MATERIALS AND METHODS

Experimental Animal, Rearing, and Sampling

One hundred adult male *P. trituberculatus* weighing 187.5 ± 37.5 g (mean \pm SD) were purchased from a local crab farm in Ningbo, China, in November 2018. Only male crabs were used in the present study to avoid physiological differences caused by gender. After the crabs were transferred to the laboratory, they were kept in an aerated recycling seawater system (salinity 24 ppt) for 1 week. Crabs were fed with fresh manila clam *Ruditapes philippinarum* once daily at 18:00. Residual food and feces were removed, and approximately 20% seawater was exchanged each morning.

To investigate the survival time, crabs were randomly distributed into four groups (25 crabs per group). The crabs

were starved for 24 h before the experiment. A plastic box (17.5 cm \times 13.5 cm \times 7.5 cm) with holes on the bottom and top and a plastic bag (30 cm \times 17 cm) equipped with an air-tight type air tap were used to hold one live crab (Figure 1). First, a crab was placed into the plastic box to prevent the plastic bag from being punctured by the crab's hard shell. Next, the plastic box containing the crab was placed into the plastic bag containing 0.7 L seawater (salinity 24 ppt). Then, the air was extracted from the bag, and the bag was sealed with a heat sealer. Finally, 4.8 L of oxygen was added into the bag through the air tap, and the tap was closed to prevent gas leakage. After packaging, all crabs were transferred to 12°C with one replicate group per incubator. The temperature was set based on the results of Dong et al. (2019). The crabs were starved during the experiment. The number of dead crabs was recorded every 12 h. Mortality was determined based on the somatic response, as Barrento et al. (2011) and Lu et al. (2016) described and discarded.

To measure the metabolomics changes during transport, two live crabs per group were randomly collected at 0, 1, 2, and 3 days for nuclear magnetic resonance (NMR) analysis (eight crabs in total per time point). The crabs were removed from the bags, anesthetized with iced seawater, and sacrificed to collect tissues, including gills, hepatopancreas, muscle, and intestine. All the samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Nuclear Magnetic Resonance Measurements

Metabolite Extraction From Crab Tissues

Each tissue sample (approximately 100 mg) was treated twice with an ice-cold aqueous methanol extraction solution (2:1, v/v) using a TissueLyser (QIAGEN, Hilden, Germany) at 20 Hz for 90 s. The resultant supernatants were pooled and lyophilized. The lyophilized powder was reconstituted in 600 μL of phosphate buffer ($\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.1 M, pH 7.46) containing 50% deuterated water (D_2O) and 0.001% sodium 3-trimethylsilyl [2,2,3,3- $^2\text{D}_4$] propionate (TSP). Here, D_2O and TSP were, respectively, used as a field lock signal and an internal quantitative reference for NMR analysis. Following 10 min of centrifugation ($6037.2 \times g$, 4°C), 550 μL of supernatant from each sample were transferred into an NMR tube (5 mm).

Nuclear Magnetic Resonance Spectroscopy

All NMR spectra were acquired at 298 K on a Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Germany) equipped with an inverse cryogenic probe according to the procedure described in a previous study (Ye et al., 2016). In brief, one-dimensional ^1H NMR spectra were acquired using a standard noesygppr1d pulse sequence with recycle delay time of 2 s and mixing time of 100 ms. The 90° pulse length was set to approximately 10 μs , and 64 transients were collected into 32,768 data points for each ^1H NMR spectrum with a spectral width of 20 ppm. Several two-dimensional NMR spectra were recorded for the NMR signal assignment for gill, hepatopancreas, muscle, and intestine extracts, including ^1H - ^1H COSY, ^1H - ^1H TOCSY, ^1H - ^{13}C HSQC, and ^1H - ^{13}C HMBC.



FIGURE 1 | The transport bag for live *Portunus trituberculatus*.

Data Analyses

The survival rate was analyzed using a log-rank (Mantel–Cox) test with the Kaplan–Meier approach (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA, United States). The phase and baseline of all ^1H NMR spectra were corrected using TOPSPIN software (V2.0, Bruker Biospin) with chemical shifts referenced to the TSP peak as δ 0.00. The spectral region δ 0.8–9.2 without residual water and methanol signals was bucketed into bins with equal width of 0.004 ppm (2.4 Hz) for all tissue samples. Wet weight normalization was conducted for each bucketed region of gill, hepatopancreas, muscle, and intestine extracts.

Multivariate data analysis was conducted on the normalized NMR data using SIMCA-P⁺ (12.0, Umetrics, Umeå, Sweden) as previously described (Ye et al., 2014). In brief, principal component analysis (PCA) was performed to provide groupings, trends, and outliers of all tissue samples. Subsequently, orthogonal projection to latent structures with discriminant analysis (OPLS-DA) was conducted using unit-variance scaled NMR data. The quality of all OPLS-DA models was evaluated by sevenfold cross-validation with CV-ANOVA ($p < 0.05$) (Eriksson et al., 2008). After back-transformation of the NMR data, color-coded loading plots of OPLS-DA were generated to mine information on the metabolites that significantly contributed to intergroup differences using a MATLAB script (V7.1, The MathWorks Inc., Natick, MA, United States). Herein, the absolute value of correlation coefficient ($|r|$) > 0.666 for a metabolite was statistically significant based on Pearson correlation analysis. The changing rate of lactate in four tissues with transport time was calculated as $(C_i - C_0)/C_0$, where C_i represents the lactate concentration in the tissues at day 1, 2, and 3 and C_0 represents the lactate concentration in the tissues at day 0.

RESULTS

Survival Rate During Transport

To investigate the survival of *P. trituberculatus* in the transport bag, the survival rates at 12°C were recorded at different time points (Figure 2). More than 80% crabs could survive the first day, and then a sharp decline was observed on day 2, during which the survival rate dropped below 40% (Figure 2).

Metabolite Changes During Transport

Untargeted ^1H NMR spectra for low-molecular-weight metabolites in crab tissues were acquired to investigate the metabolic changes in gills, hepatopancreas, muscle, and intestine

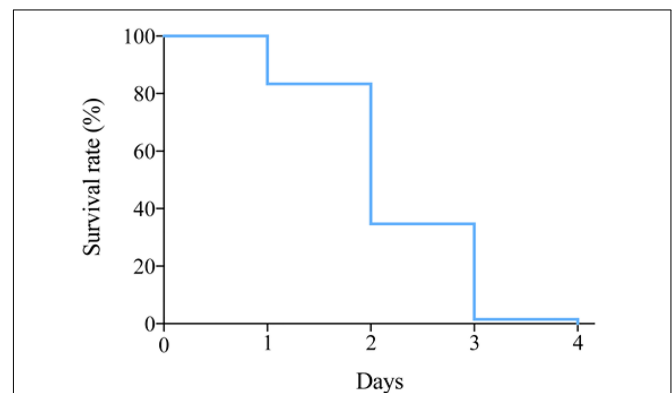
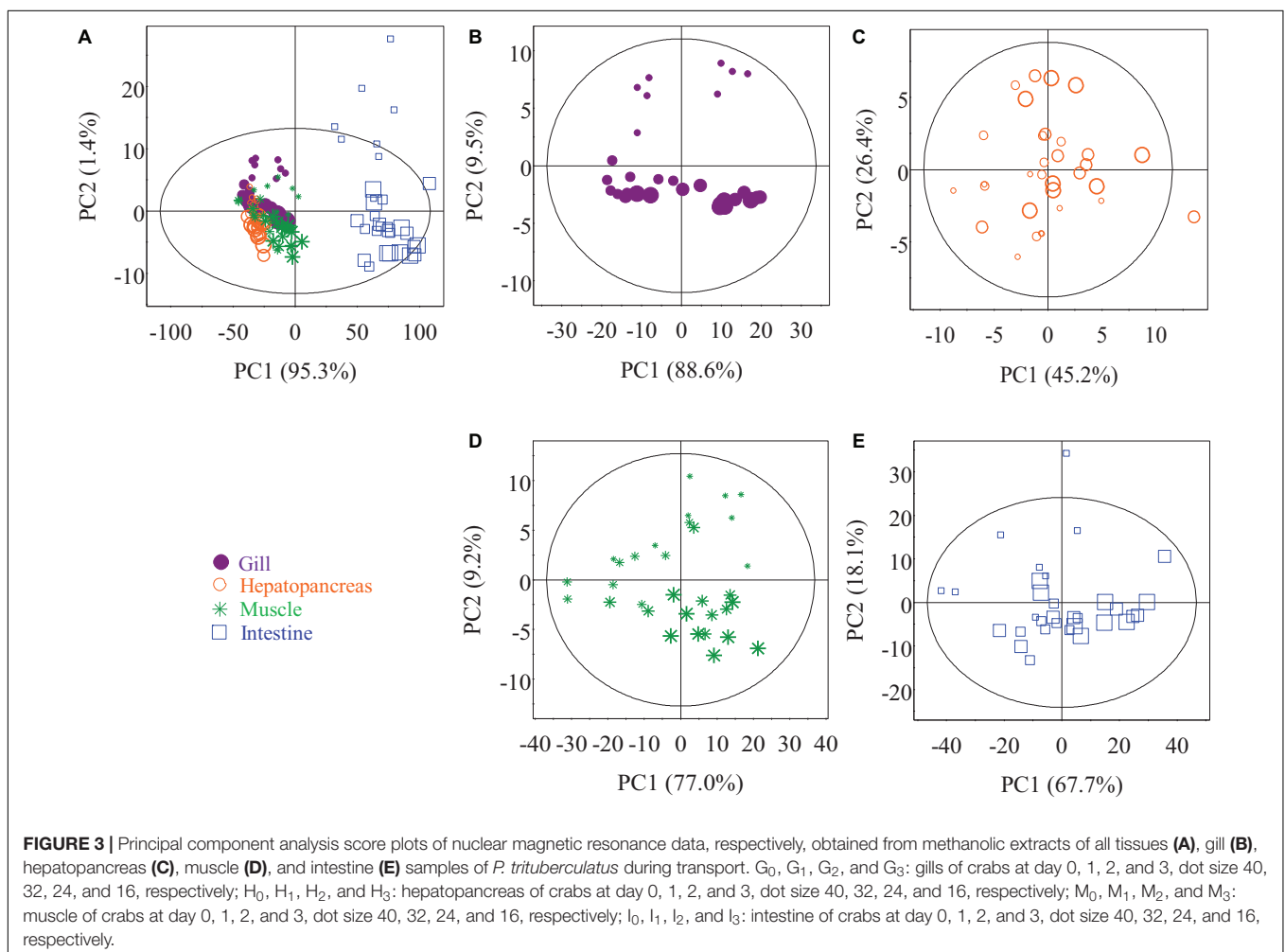


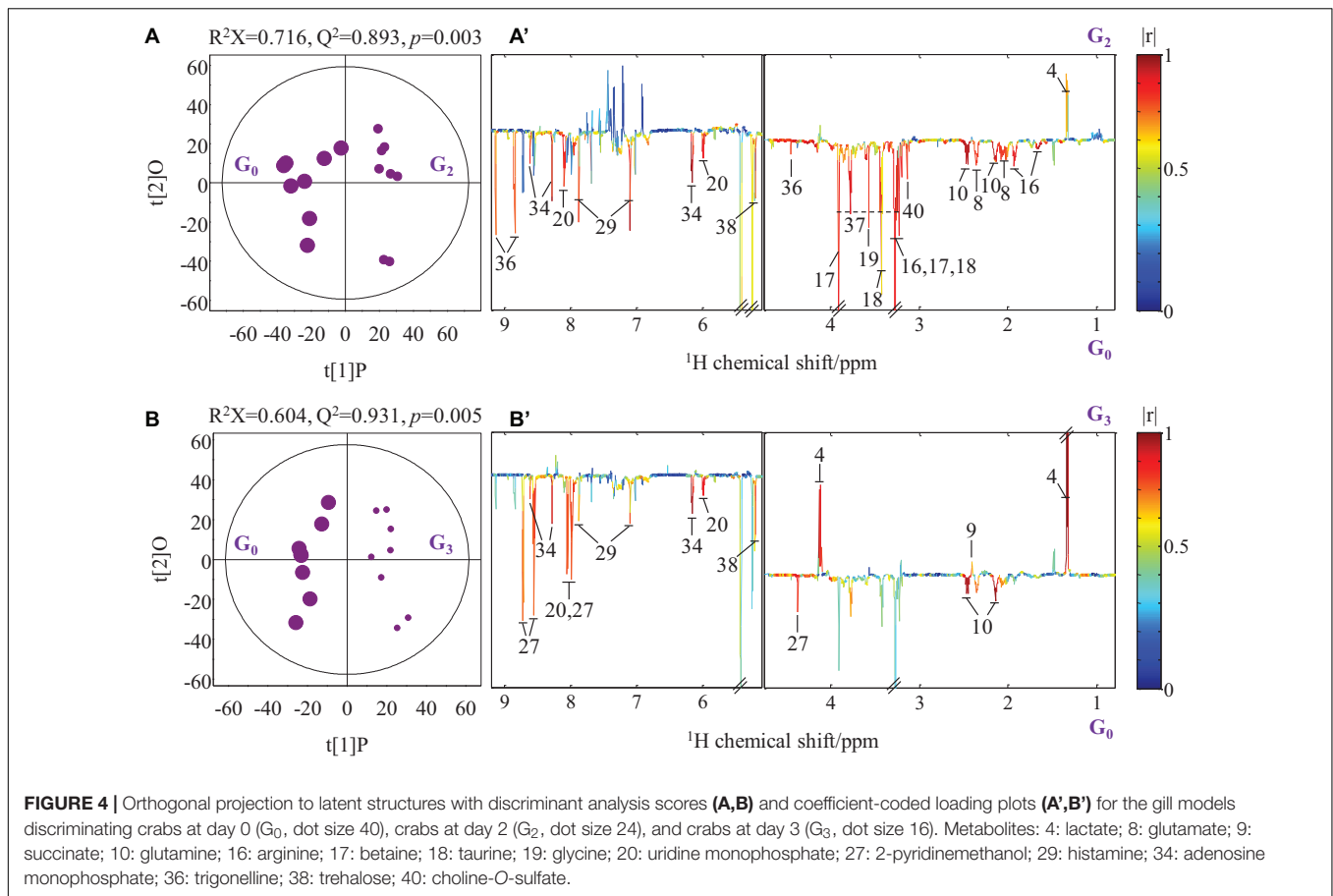
FIGURE 2 | The survival rate of *P. trituberculatus* during transport.

when *P. trituberculatus* were kept in the oxygen-filled bag containing seawater at 12°C. The assigned metabolites in the crab tissues are listed in **Supplementary Table 1** with ^1H and ^{13}C NMR data. In multivariate data analysis, PCA score plots based on the first and second principal components (PC1 and PC2) were obtained from all ^1H NMR spectral data obtained from aqueous methanol extracts of the four tissues (**Figure 3**). The PCA results differentiated the intestinal tissue and the other three tissues in PC1 (**Figure 3A**). Notably, six outliers were observed for the intestinal extracts in this PCA plot. However, these intestine samples were still included in subsequent analyses as the distinct metabolomic profile of the intestine compared with that of the other three tissues may result in these outliers. This view was supported by the plot in **Figure 3E**, where only one sample was outside the confidence ellipse. Furthermore, in **Figure 3A**, minor clustering was observed among gill, hepatopancreas, and muscle. Although tissue type played a dominant role in separating crab metabolic profiles, transport time still played an essential role in the metabolite changes in some tissues. The metabolite changes in the gill, muscle, and intestine displayed a time-dependent pattern (**Figures 3B,D,E**),

whereas no apparent clustering was observed among the four hepatopancreas samples at different sampling times (**Figure 3C**).

Pairwise comparison *via* OPLS-DA was conducted with sevenfold cross-validation of the ^1H NMR spectral data from the transported crabs compared with their control counterparts. A total of 12 OPLS-DA models were constructed for the four tissue samples. However, only eight OPLS-DA models were of good quality, as evaluated based on high values of prediction indicators $R^2\text{X}$ and Q^2 but low p -values in CV-ANOVA (**Figures 4, 5** and **Supplementary Figures 1, 2**). No significant difference was observed in the ^1H NMR spectral data for the four tissue types between day 0 and day 1, which indicates that the significant metabolomic differences occurred after one transport day. In comparison with the control crabs (day 0), crabs at day 2 showed a significant elevation in the levels of gill lactate and a significant depletion in the levels of gill glutamate, glutamine, arginine, glycine, taurine, betaine, trehalose, trigonelline, histamine, choline-*O*-sulfate (COS), uridine monophosphate (UMP), and adenosine monophosphate (AMP) (**Figure 4**). Moreover, crabs at day 3 showed a significant elevation in the levels of gill lactate



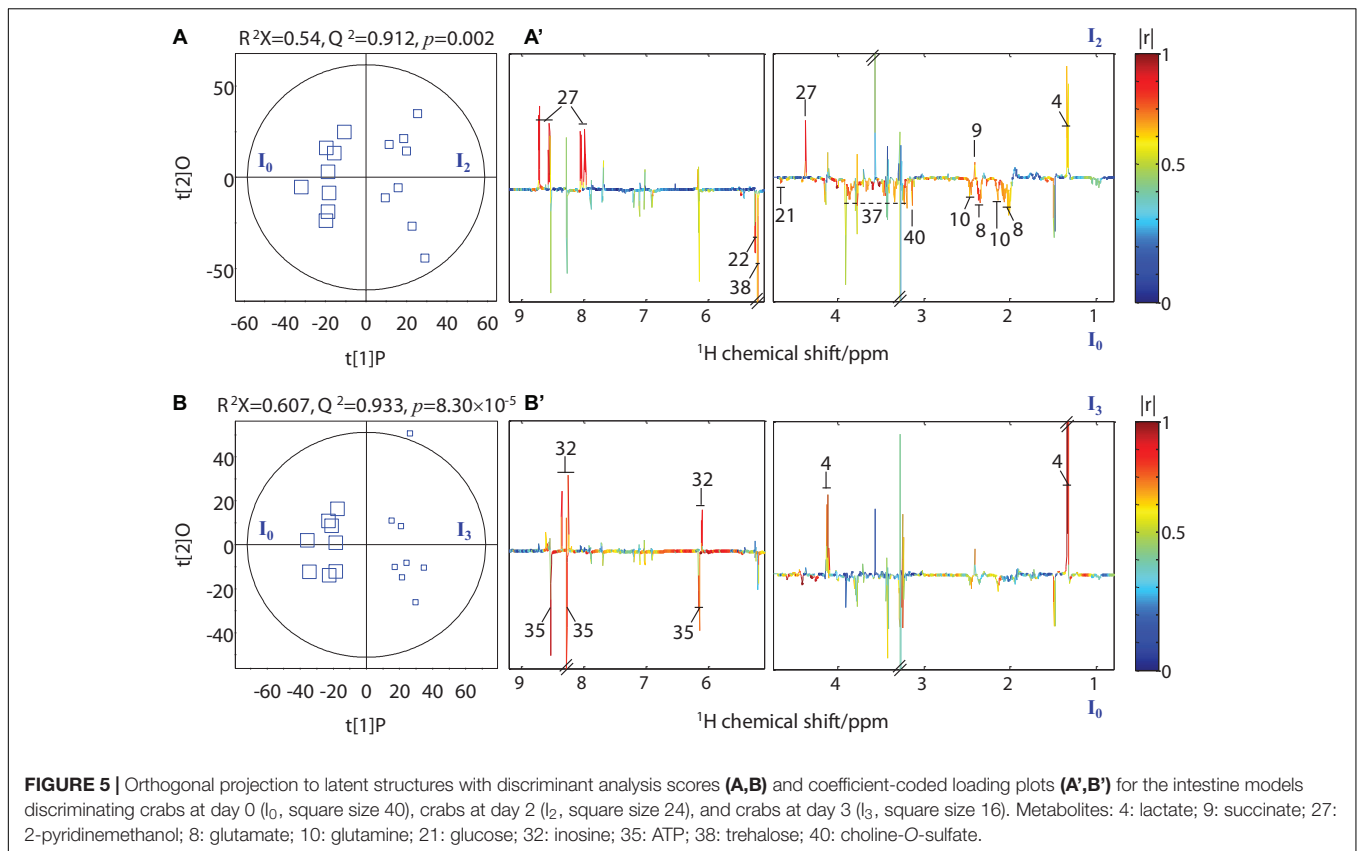


and succinate and a significant depletion in the levels of gill glutamine, UMP, 2-pyridinemethanol, histamine, AMP, and trehalose, when compared with the control crabs. For hepatopancreas and muscle, the number of metabolites with significantly altered levels was considerably lower than that of the gill. In detail, crabs at day 2 showed significant elevation in alanine levels in the hepatopancreas, whereas crabs at day 3 showed a significant elevation in lactate levels and a significant depletion in AMP levels in the hepatopancreas, when compared with the control hepatopancreas samples (**Supplementary Figure 1**). Simultaneously, a significant depletion in the levels of trimethylamine-*N*-oxide (TMAO) and trigonelline in the muscle of crabs at day 2 and a significant elevation in lactate levels and succinate in the muscle of crabs at day 3 was observed when compared with the control muscle samples (**Supplementary Figure 2**). Intestine presented a higher number of metabolites with significantly altered levels than hepatopancreas and muscle. In the crabs at day 2, intestinal lactate, succinate, and 2-pyridinemethanol levels were significantly higher, and glutamate, glutamine, glucose, trehalose, and COS levels were significantly lower in controls (**Figure 5**). For crabs at day 3, intestinal lactate and inosine significantly increased, and ATP significantly decreased. These metabolic changes suggest that tissue-specific metabolic modulation occurs in *P. trituberculatus* during live transport.

The changes in the concentration of this organic acid in the four tissue types with time compared with their control counterparts were calculated to examine the temporal dynamics of lactate concentration. Overall, a gradual elevation in the lactate level was observed in all tissues throughout the experiment (**Figure 6**). The highest accumulation of lactate was found in the gill and muscle tissues at day 3, increased by 1172.4 and 908.8%, respectively, relative to their control counterparts; hepatopancreas and intestine, respectively, showed 427.7 and 273.2% increases.

DISCUSSION

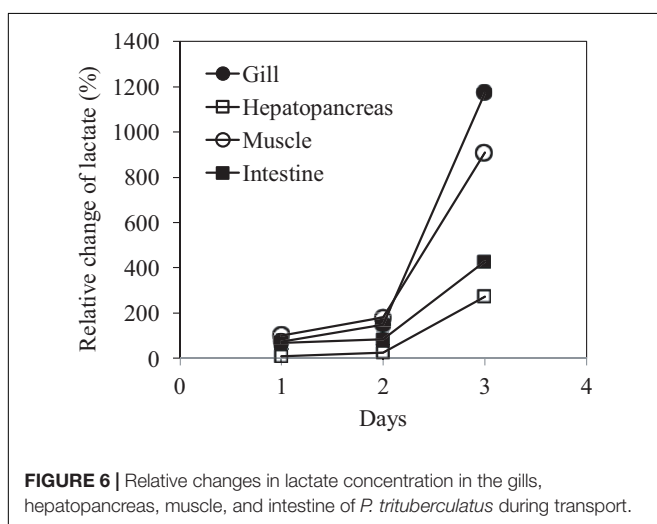
Freshness plays a crucial role in crab quality, reducing rapidly after crab death (Wang et al., 2018). In the present study, 80% of the crabs kept in the transport bag were able to survive for 24 h, which is considerably longer than those under air exposure even at the optimal temperature (10°C; <12 h) (Lu et al., 2015, 2016; Dong et al., 2019). Our results confirm that air exposure is a detrimental stressor for *P. trituberculatus* post-capture. Although an array of evidence has indicated the adverse effects of air exposure on *P. trituberculatus* (Lu et al., 2015; Dong et al., 2019) and other crab species such as porcelain crabs (Stillman and Somero, 1996), *Carcinus maenas*, *Necora*



puber (Durand and Regnault, 1998), *Cancer pagurus* (Barrento et al., 2011, 2012), *Neohelice granulata* (Geihs et al., 2013, 2014), *L. santolla* (Urbina et al., 2013), and *C. maenas* (Simonik and Henry, 2014), a dry or semi-dry approach remains prevalent, for economic reasons, in the transport of live crabs. Our study shows that water-bathed *P. trituberculatus* survive much longer than air-exposed individuals, offering the possibility of long-distance transport of *P. trituberculatus*.

Although most swimming crabs could survive 1 day in the transport bag, mortality rose sharply on day 2. Previous studies have suggested that loss of energy homeostasis results in crab mortality (Lu et al., 2015, 2016). Our results showed that gills exhibited the most significant metabolic change among the four tissue types studied, which is predictable, as the gills are the gas/solute exchange organs of *P. trituberculatus*; they also play an essential role in osmoregulation, acid-base regulation, waste excretion, and immune and antioxidative responses (Burnett et al., 2006; Lorenzon et al., 2008; Romero et al., 2011; Dong et al., 2019). A significant elevation in lactate levels and succinate together with a significant depletion in the levels of 12 metabolites suggests that gill metabolism changed profoundly after crab storage in the transport bag for 2 days.

Notably, we observed a >10-fold increase in gill lactate levels relative to controls. As a well-known end-product of anaerobic respiration in crustaceans (Spicer et al., 1990), lactate is often observed to accumulate in stressed crabs. For example, lactate accumulation has been reported in the hemolymph of *C. pagurus* during air exposure or water transport (Lorenzon et al., 2008; Barrento et al., 2011) and in *P. trituberculatus* under temperature fluctuation or air exposure (Lu et al., 2015, 2016). The gills of crustaceans engage anaerobic pathways under hypoxic stress (McMahon, 2001; Racotta et al., 2002). In light of these observations, severe hypoxia in the gills may occur after 3-day transport, as indicated by the significantly elevated gill succinate level, an essential metabolite in the tricarboxylic acid



cycle (TCA). Succinate accumulation may be associated with a branched, non-cyclic TCA cycle where succinate is produced reductively under hypoxia (Rosenqvist et al., 1973). However, lactate accumulation could be an adaptive response to a stressful situation (Barton, 2002), as lactate can act as a positive effector in increasing the oxygen affinity of hemocyanin (Morris et al., 1986; Bridges, 2001). Moreover, lactate also acts as the predominant gluconeogenic precursor (Jenssen et al., 1993). In this study, it is not surprising that no significant changes were observed in gill glucose and ATP levels. In addition, the gill AMP level decreased markedly from day 2 in the transport bag. AMP is a direct agonist of AMP-activated protein kinase, which is involved in energy-sensing (Hardie, 2003), and AMP depletion further confirms that the ATP levels in the gills were not low. Taken together, energy homeostasis seems to be maintained in the gills of *P. trituberculatus* after 3-day transport, although the pathway of energy metabolism has been modified.

In addition to energy-related metabolites, we also detected a significant decrease in glutamine, taurine, betaine, and UMP levels in gills. Taurine and betaine are well recognized as osmoregulators in aquatic animals under stress (Carr et al., 1996; Avella et al., 2009; Ye et al., 2013). Therefore, the significantly decreased levels of these four metabolites strongly indicate a change in amino acid metabolism, osmoregulation, and nucleic acid metabolism in the gill. It seems like a positive metabolic modulation in the gill for crabs to survive longer.

Significantly altered lactate levels and AMP were also observed in the hepatopancreas of *P. trituberculatus*, indicating an adaptive switch from aerobic to anaerobic metabolism for energy generation. The hepatopancreas is an essential organ in crabs, with multiple functions, including digestion, metabolism, and stress resistance (Wang et al., 2014; Huang et al., 2015; Sun et al., 2015). However, the number of metabolites with significantly altered levels was considerably lower in the hepatopancreas than in the gills, indicating a less severe metabolic change in this organ during transport.

Muscle tissues showed a significant alteration in levels of only four metabolites during the experiment. Among them, two energy-related metabolites – lactate and succinate – were significantly elevated after 3-day transport, indicating an adaptive modulation in the strategy for energy generation. Concomitantly, levels of two compatible osmolytes – TMAO (Yancey et al., 2002) and trigonelline (Hammer et al., 2012) – significantly decreased at day 2. Accumulation of TMAO and trigonelline was observed in the muscle of *C. maenas* exposed to a high seawater CO₂ level (Hammer et al., 2012) and *P. trituberculatus* exposed to low salinity (Ye et al., 2014). Therefore, we speculate that osmotic balance is altered in the muscle during transport.

Aside from the gills, the intestine presented the most significant metabolic change in the studied *P. trituberculatus* tissues. The significantly altered levels of lactate and succinate in the intestine suggest an adaptive regulation of energy generation. However, the significantly decreased intestine ATP level at day 3, which was not found in other tissues, may imply an energy imbalance in the intestine. The intestine of *P. trituberculatus* harbors vast bacteria (Zeng et al., 2016; Shi et al., 2019). Consequently, the energy demand of the intestine may be

higher than that of the other three tissues tested here. Although proteins and lipids can be an energy source for crustaceans (Barclay et al., 1983; Pascual et al., 2006), starvation before and during transport likely leads to the energy deficiency in the intestine of *P. trituberculatus*. In addition, we also observed increased 2-pyridinemethanol levels and decreased glutamate, glutamine, trehalose, and COS levels at day 2 and increased inosine levels in the intestine at day 3. Trehalose and COS are involved in antioxidation and osmoregulation (Hanson et al., 1991; Benaroudj et al., 2001). These observations indicate the perturbed amino acid metabolism, nucleic acid metabolism, and osmoregulation in *P. trituberculatus* intestine during the transport process.

CONCLUSION

The transport bag could effectively extend the post-capture survival time for *P. trituberculatus* at the laboratory scale. Their ability to survive may be attributed to adaptive metabolism, as suggested by the switch from an aerobic to an anaerobic pathway for energy generation, and a decline in amino acid metabolism, nucleic acid metabolism, and osmoregulation. Our study provides a promising method for live transport of *P. trituberculatus*. Further research should be carried out under commercial transport conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CS and FP performed the research. YY and CW designed the protocol. FP analyzed the data. CS and YY wrote the manuscript. All authors participated in the revision of this manuscript by providing comments and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.724156/full#supplementary-material>

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