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Hypothermal effects on survival, energy homeostasis and expression of energy-related genes of swimming crabs *Portunus trituberculatus* during air exposure

Yunliang Lu [a,b], Dan Zhang [a,b], Fang Wang [a,b,*], Shuanglin Dong [a,b]

[a] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, Shandong, China
[b] Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, China

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ABSTRACT

Previously, dry or semi-dry approach under the hypothermal condition is proved to be an alternative method in transport of live swimming crabs *Portunus trituberculatus*. However, we wondered whether this method can improve crab survival when temperature is kept as cool as possible. In this study, we hypothesized that there is a thermal threshold below which dry or semi-dry approach (air exposure) could cause crab physiological disruption and therefore aggravate their mortality. To test the above hypothesis, crabs (23 °C) were exposed to air at temperatures ranging from 4 to 16 °C. Results showed that crabs had a worse survival and vigor at temperatures below 12 °C. Then we tested crab energy metabolism to explore the possible reason. It was shown that total adenine nucleotide and adenylate energy charge in gills were remarkably reduced by air exposure of below 12 °C. This increased the need for crabs to re-balance energy metabolism, which was indicated by the upregulation of *AMPKα* and *HIF-1α*. Meanwhile, there was a significant increase of the expression of Na^+/K^+*-ATPase*, *V-type ATPase* and *HSP90* at temperatures below 12 °C, while all treatments shared a similar level of hemocyanin, urate and lactate in hemolymph and expression of cytochrome *c* oxidase and NADH-ubiquinone reductase in gills. These results implied that dry or semi-dry approach below 12 °C could exert detrimental effects on *P. trituberculatus*, and perturbation of energy homeostasis, which is more related with changes of energy-demanding physiological pathways, is a possible reason of crab death and poor vigor.

1. Introduction

Air exposure is a periodic or special event for many aquatic animals in natural environments (e.g. during the ebb tide) or in aquaculture practices (e.g. during the transport). It is noticed that crustaceans are the most hypoxia-sensitive organisms among marine fauna (Vaquer-Sunyer and Duarte, 2008). Thus, an internal hypoxia emerges in most crustaceans species following air exposure or during transportation out of water, as a result of the interruption of branchial water circulation and the resulting collapse of gill filaments (Durand and Regnault, 1998; Urbina et al., 2013). This usually leads to a possible destruction of internal energy homeostasis (Morris and Oliver, 1999; Speed et al., 2001) and therefore an elevated reliance on anaerobic glycolysis for animals to provide more energy (Albalat et al., 2010; Durand and Regnault, 1998; Lorenzon et al., 2007; Simonik and Henry, 2014; Urbina et al., 2013).

Temperature is an important factor influencing animal survival during transport out of water. Several studies have shown that during transport under emersion condition, crustaceans have a lower concentration of glucose, lactate and ammonia at lower temperature (Barrento et al., 2011; Chen and Chen, 1998; Lorenzon et al., 2007; Ridgway et al., 2006). This indicate that in a certain temperature range, lower temperature could decrease the stress that crustaceans have to bear during air exposure. As a result, the energy demand are reduced for these animals (Morris and Oliver, 1999). Actually, crustacean survival during air exposure is closely related with their energy metabolism. This idea is evidenced by our previous work which showed that the swimming crab *Portunus trituberculatus* could maintain energy homeostasis during dry or semi-dry transport at lower temperature and they had a better survival (Lu et al., 2015a). However, we wondered whether crustaceans could balance energy status and improve survival when temperature further decrease.

Energy metabolism is the base for organisms to realize all their organismal, organic, cellular and molecular functions. In organisms, its balance is the synergistic results of ATP production from

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, Shandong, China.
E-mail address: Wangfang249@aliyun.com (F. Wang).

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aerobic and anaerobic pathways and ATP consumption due to all kinds of functional activities such as ionic regulation and protein synthesis. Hypoxia inducible factor 1 (HIF-1) and AMP-activated protein kinase (AMPK) are two important regulators of energy metabolism. They have been widely reported in crustaceans exposed to stresses such as hypoxia (da Silva-Castiglioni et al., 2010; Hardy et al., 2013; Hardy et al., 2012; Holman and Hand, 2009; Kodama et al., 2012; Li and Brouwer, 2007; Soñanez-Organis et al., 2009; Soñanez-Organis et al., 2010) and ambient temperature (Frederich et al., 2006; Frederich et al., 2009; Podolski, 2011).

Herein, two remarkable questions have to be pointed out. (i) There is scarce information centering on what temperature range is suitable for animal survival during air exposure. (ii) Whilst abundant information is available on how aquatic animals respond physiologically to air exposure, we still lack the understanding of how they respond to such a stress at the molecular level. For the latter question, as far as we know, little information is available in crustaceans (Chung and Zmora, 2008), fishes (Hung et al., 2007) and bivalve molluscs (Han et al., 2013; Ivanina et al., 2010; Place et al., 2008). Undoubtedly, this situation not only limits the comprehensive understanding how aquatic organisms respond to air exposure, but also may influence the optimization of transportation method.

P. trituberculatus is an important commercial crab in China. For their live transport, dry or semi-dry approach under the hypothermal condition is usually adopted. Our previous work has preliminarily demonstrated that lower temperature could favor for *P. trituberculatus* survival in air by ameliorating their energy response (Lu et al., 2015a). However, there are still several issues waiting to be addressed: (i) What is the most suitable hypothermal range for emersed crab survival? (ii) How does energy metabolism respond to cold stress? (iii) Whether the idea that AMPK is a better indicator of heat stress than HSPs (Frederich et al., 2009; Han et al., 2013) is applied for the case of cold stress? In this context, we conducted this experiment by placing crabs into air environments with temperatures ranging from 4 to 16 °C. Crab survival, energy status and energy metabolism were then determined. Our study may provide reference in optimizing conditions of the transport of live *P. trituberculatus*.

2. Materials and methods

2.1. Animal collection and maintenance

Healthy subadults of *P. trituberculatus* obtained from a local farm in Jiaonan district (Qingdao, Shandong, China) were reared in glass aquaria (50 cm × 40 cm × 30 cm) in Qingdao National Marine Science Research in July–August 2014, with each crab in a separate chamber to avoid cannibalism. All crabs were acclimated for two weeks in seawater ($T=23\pm1$ °C, salinity 30‰) at the photoperiod of 14: 10 h (light: dark). Crabs were fed once every day with fresh shellfish *Ruditapes philippinarum* and unconsumed food and feces were removed after 3 h of feeding. Seawater of 1/3 in each tank was exchanged daily with fully aerated seawater at 23 °C. Each tank was continuously aerated with air stones.

2.2. Experimental protocol

In practices, a method of packing between layers of moistened wood shavings in ventilated boxes has been applied for the transport of *P. trituberculatus* out of water. In our previous work (Lu et al., 2015b), we observed that temperature ranged from 15 °C to 17 °C at the bottom. Thus, we set 16 °C as the upper limit of air temperature in this study. After acclimation, 80 healthy crabs of legal size (110–130 g wet weight) in the intermolt stage were randomly divided into four groups (20 crabs in each group). Each group was then subdivided into two equal parts (10 crabs for each), with one for sample collection after air exposure of 3 h and another for monitoring changes of crab survival during air exposure.

All experimental specimens were starved for 24 h prior to experiment initiation. Crabs of four groups (10 crabs for each) were then directly transferred from seawater (23 °C) into air environments where temperature was controlled at 4, 8, 12 and 16 °C respectively. After 3 h of air exposure, five crabs in each group were randomly chosen for sampling. The sampling time was established according to our previous work (Lu et al., 2015a). Five crabs immersed in seawater (23 °C, without any treatment) were sampled as controls.

In this study, air exposure was performed in a light growth incubator to simulate the condition of transport out of water. During experiment, crabs were placed into individual hollow plastic boxes (15 cm × 15 cm × 5 cm) and then immediately placed into the incubator. Air conditions were controlled in accord with the ambient environment of control group with humidity of 70–80% and illumination of 50–100 Lx.

2.3. Sample collection

When sampling, chilled disposable syringes of 1 ml were used to extract hemolymph from the ventral sinus via the arthrodial membrane at the base of the last pereiopod. Then, animals were sacrificed and posterior gills were removed, flash-frozen in liquid nitrogen and stored at −80 °C for further analyses.

2.4. Animal survival

According to experiment protocols, another 10 crabs for each group were incorporated into each treatment group and their survival were recorded every 1 h during air exposure until they all died. In the present study, animal vigor was visually delimitated according to Barrento et al. (2011) with slight modification (Table 1).

2.5. Determination of hemolymph parameters

Hemolymph samples were centrifuged for 10 min at 9000 g and 4 °C and then supernatants were collected for analyses of hemocyanin, urate, glucose and lactate. Concentrations of hemocyanin (mmol L^{-1}) and lactate (mmol L^{-1}) were analyzed and calculated with the method described in our previous work (Lu et al., 2015a). Commercial kits from Nanjing Jiancheng Bioengineering Institute (China) were used to analyze concentrations of urate (μmol L^{-1}) and glucose (mmol L^{-1}) in the hemolymph.

2.6. Determination of gill adenine nucleotides

According to our previous work (Lu et al., 2015a), frozen gills were processed to obtain extracts used for the analysis of ATP, ADP and AMP in an Agilent 1100 high-performance liquid chromatography (HPLC, Agilent Corp., USA) system. Adenylate concentration was calculated against respective standard curve and expressed as μmol nucleotide per gram wet weight tissue (μmol gww^{-1}). Total adenine nucleotide (TAN) and adenylate energy charge (AEC) were calculated using following equations.

$$\begin{aligned} TAN &= [ATP] + [ADP] + [AMP] \\ AEC &= ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]) \end{aligned}$$