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Energy response and modulation of AMPK pathway of the olive flounder *Paralichthys olivaceus* in low-temperature challenged

Yunliang Lu[a,b], Miaomiao Nie[a,b], Ling Wang[a,b], Yinghuai Xiong[c,d], Fang Wang[c,d], Lijuan Wang[a,b], Peng Xiao[a,b], Zhihao Wu[a,b], Ying Liu[e], Feng You[a,b,*]

[a] Key Laboratory of Experimental Marine Biology, National & Local Joint Engineering Laboratory of Ecological Mariculture, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, Shandong, PR China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266200, Shandong, PR China
[c] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, Shandong, PR China
[d] Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266200, Shandong, PR China
[e] School of Marine Science and Environment Engineering, Dalian Ocean University, Dalian 116023, Liaoning, PR China

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ABSTRACT

Cold resistance is important for fish in natural and aquaculture environments. Modulation of energy metabolism has been proved to be vital for fish in cold. However, the interaction of energy response and the known AMPK pathway remains vague in fish coping with cold stress. In the present study, the olive flounders *Paralichthys olivaceus* were subject to low-temperature condition (0.2 ± 0.2 °C). The energy metabolism and modulation of AMPK pathway were analyzed. Results showed that flounders increased ATP-coupled ion transport, as indicated by Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities. Nevertheless, higher levels of aerobic and anaerobic metabolism, respectively indicated by the elevation of COX and LDH activities, contributed to the stable energy homeostasis during cold exposure. The three subunits of AMPK responded differently to cold, and *AMPKα* and *AMPKβ* might be more sensitive in the early stage while *AMPK*γ might be more sensitive in the later stage. The transcriptional response of *LKB1/CaMKKβ-AMPK-NRF1* axis might play an important role in helping flounders against the early cold stress. However, flounders differentiated in cold resistance with the stress time elapsed. Concomitantly a greater reliance on *LKB1-AMPK-NRF1* axis for cold-tolerant flounders compared to those sensitive ones to cold was observed, and this axis was speculated to be related with flounder cold resistance. To our knowledge, this is the first time to explore the possible response of AMPK pathway to cold in fish. Our data showed that the flounder relied on metabolic regulation and modulation of AMPK pathway including not only higher expression levels of genes in this pathway but also changes in its related function axes to maintain energy homeostasis.

1. Introduction

Numerous studies have shown that suitable thermal range is essential for aquatic fish performance such as growth, development and reproduction, while temperature anomalies or extremes will impose adverse effects on them. Fishes have to suffer low temperature condition in winter, leading to a significantly reduced growth (McCarthy et al., 1998; Weber and Bosworth, 2005; Murphy et al., 2012) and an increased mortality (Siikavuopio et al., 2010). This will increase aquaculture cost and decrease commercial profit. Therefore, improving fish cold resistance ability is important to fish aquaculture.

Under low temperature condition, biological functions at all levels will be induced to maintain fish performance. Kapila (2009) found that the cold water fish *Barilius bendelisis* could elevate the heat shock protein 70 level to adapt to cold condition. Shin et al. (2012) showed that Antarctic notothenioid fishes could express more ubiquitin proteins-conjugated proteins to adapt to cold environment. Meanwhile, transcriptomics study showed a higher response of various cellular signaling pathways like FoxO signaling could be induced when warm water fish, zebrafish *Danio rerio*, was exposed to low temperature (8 °C) (Hu et al., 2016). These activities, ranging from molecular to organismal response, are mostly related with energy metabolism because this process provides them with needed power-ATP. The enhanced oxidative capacities, e.g. oxidative production of ATP, could be linked with cold

* Corresponding author at: Key Laboratory of Experimental Marine Biology, National & Local Joint Engineering Laboratory of Ecological Mariculture, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China.
E-mail address: youfeng@qdio.ac.cn (F. You).

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temperatures in fish tissues, in particular the skeletal muscle (Guderley, 2004). For energy metabolism, the ATPase, pyruvate kinase (PK), lactate dehydrogenase (LDH) and cytochrome oxidase (COX) are key regulators. The elevation of these enzymatic values was considered as an important response to cold in *B. bendelisi* (Kapila, 2009). The fish *Channa punctata* also enhanced the activity of Na^+/K^+-ATPase, a major ATP-coupled ion exchanger, in cold (Haque et al., 2011).

Metabolic response is a common phenomenon for animals at low temperature. When they were faced with cold, the internal glucose level could be increased (Sun et al., 1992; Lee et al., 2001) to guarantee sufficient energy fuel during cold adaption. The induced energy metabolism is indeed under the regulation of several regulators, one of which is AMP-activated protein kinase (AMPK). This kinase was composed of a catalytic subunit (AMPKα), a scaffolding subunit (AMPKβ) and a regulatory subunit (AMPKγ). It was evidenced that AMPK was potentially important in stimulating glucose uptake and utilization in the skeletal muscle of fish (Magnoni et al., 2012). This indicated a possible role of AMPK in regulation of cold-induced energy response of fish, whereas direct evidence remains missing. It is recognized that when cells are starved, the increased AMP/ATP ratio will activate the phosphorylation of AMPK and therefore rebalance energy status (Richter and Ruderman, 2009). Besides, AMPK is also regulated by two upstream kinases, the tumor suppressor liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ) (Gormand et al., 2011). However, the cold-induced response of AMPK and its up- and down-stream proteins is still unclear in fish, confining the understanding of fish cold adaption mechanism.

The olive flounder *Paralichthys olivaceus* is commercially important in China, Korea and Japan. This cold-temperate fish is observed to decrease or stagnate in growth rate when temperature falls below 10 °C, reducing the aquaculture yield. Our previous work on flounders showed that, similar to zebrafish (Wang et al., 2014), energy metabolism might be an important response to cold via transcriptomics analysis (Hu et al., 2014). However, this study did not answer the question how flounder regulates its energy metabolism during cold environment. In this study, flounders were exposed to cold extreme (0.2 ± 0.2 °C). Then the muscle energy response was analyzed via the correlation among enzyme activity, adenylate level and modulation of AMPK pathway. For the first time, we aim at exploring the possible energy response and modulation of AMPK pathway of fish under cold condition. Our study could provide an important reference for understanding the cold-tolerant mechanism of fish in the viewpoint of energy metabolism and therefore will be beneficial for overwintering aquaculture.

2. Materials and methods

2.1. Experimental fish and acclimation

This study was conducted in the Institute of Oceanology, Chinese Academy of Science. All juvenile flounders (15–19 cm in total length, TL) were purchased from a local fish farm in Jiaonan district (Qingdao, Shandong, China), transported to the laboratory and acclimated in seawater at 11 °C and 30 salinity for 2 weeks with a photoperiod of 14: 10 h light: dark. During acclimation, the seawater was refreshed periodically two to three times every day. Each tank was continuously aerated. All fish were fed twice a day with formula feed.

2.2. Experimental protocol

After acclimation, 180 healthy individuals were randomly divided into three groups (60 fish in a group), with each in a separate container of 0.5 ton. The three groups were subject to the same cold condition respectively, and thus the experiment was triplicated. According to our pretest results, the target temperature was set to 0.2 ± 0.2 °C and the flounders could reach a mortality of approximate 50% after 28 h of cold exposure at this temperature. All chosen fish were starved for 24 h and then subjected to cold stress in a circulating system. The seawater temperature was lowered to 0.2 °C at a rate of 1 °C/h with the help of a compression refrigerating machine and then kept stable. The control flounders were maintained at 11 °C. After reaching the test temperature, three fish in each container were randomly chosen for sampling at 0, 7 and 14 h, respectively. After 28 h, the fish that were still able to swim were defined as the cold-tolerant (CT) group, while those that could not swim and only had week breath were defined as the cold-sensitive (CS) group. Three individuals in each container were then sampled for the CT and CS groups, respectively.

2.3. Sample collection

For each sampling, total 9 individuals from three replicates were randomly chosen and anesthetized in 40 mg/L MS-222 solution. After measuring fish body traits, including total length (cm), body length (cm), head length (cm) and body width (cm), sampling was immediately performed after severing the fish spinal cord. Muscle tissue was then dissected out, flash-frozen in liquid nitrogen and stored at − 80 °C for further analysis. The fish that were not subjected to cold stress were sampled as the control (n = 9). In this study, the flounder body traits were similar between time intervals (Table 1). All animal work and animal protocols were approved by the Institute of Oceanology, Chinese Academy of Science.

2.4. Determination of enzymes activity

Muscle tissue was weighed and homogenized in 9 volumes of cold saline (0.75% NaCl, pH = 7.0). Homogenates were immediately centrifuged for 10 min at 4 °C and 800 × g. The supernatants were then collected and used for the determination of activities of pyruvate kinase (PK, EC 2.7.1.40), cytochrome c oxidase (COX, EC 1.9.3.1), LDH (EC 1.1.1.27), Na^+/K^+-ATPase (EC 3.6. 1.37) and Ca^{2+}/Mg^{2+}-ATPase (EC 3.6.1.38), and levels of glucose and lactate. All preparation procedures were carried out at 4 °C.

The NADH-linked methods were employed to determine the activity of PK and LDH by recording the absorbance decrease at 340 nm (Speed et al., 2001). The final assay system (pH = 7.5) of PK included 50 mmol·L^{-1} imidazole/HCl buffer, 10 mmol·L^{-1} $MgCl_2$, 75 mmol·L^{-1} KCl, 2 mmol·L^{-1} ADP, 120 μmol·L^{-1} NADH, 2 U lactate dehydrogenase and 1.0 mmol·L^{-1} phosphoenolpyruvate. The final assay system (pH = 7.5) of LDH included 5 mmol·L^{-1} pyruvic acid, 0.1 mmol·L^{-1}

Table 1
Body traits of sampled flounders.

	Total length (cm)	Body length (cm)	Head length (cm)	Body width (cm)
Control	17.24 ± 0.83 [a]	15.59 ± 0.70 [a]	4.35 ± 0.34 [a]	5.79 ± 0.26 [a]
0 h	17.39 ± 1.04 [a]	15.75 ± 0.94 [a]	4.28 ± 0.35 [a]	5.81 ± 0.38 [a]
7 h	17.88 ± 0.91 [a]	16.21 ± 0.97 [a]	4.76 ± 0.19 [a]	5.98 ± 0.48 [a]
14 h	17.12 ± 0.70 [a]	15.50 ± 0.65 [a]	4.29 ± 0.33 [a]	5.72 ± 0.24 [a]
28 h–CS	17.04 ± 0.77 [a]	15.36 ± 0.78 [a]	4.48 ± 0.39 [a]	5.69 ± 0.65 [a]
28 h–CT	17.72 ± 1.05 [a]	16.03 ± 1.05 [a]	4.39 ± 0.44 [a]	5.87 ± 0.31 [a]

Note: Different upper lowercase letters in the same column indicate significant difference between time intervals (P < 0.05).