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Acclimation to cold and warm temperatures is associated with differential expression of male carp blood proteins involved in acute phase and stress responses, and lipid metabolism

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ABSTRACT

The environmental temperature affects plasma biochemical indicators, antioxidant status and hematological and immunological parameters in fish. So far, only single blood proteins have been identified in response to temperature changes. The aim of this study was to compare the proteome of carp blood plasma from males acclimated to warm (30 °C) and cold (10 °C) temperatures by two-dimensional differential gel electrophoresis followed by MALDI-TOF/TOF mass spectrometry. A total of 47 spots were found to be differentially regulated by temperature (> 1.2-fold change, $p < 0.05$): 25 protein spots were more abundant in warm-acclimated males and 22 were enriched in cold-acclimated males. The majority of differentially regulated proteins were associated with acute phase response signalling involved in: i) activation of the complement system (complement C3-H1), ii) neutralization of proteolytic enzymes (inter-alpha inhibitor H3, fetuin, serpinA1, antithrombin, alpha2-macroglobulin), iii) scavenging of free hemoglobin and radicals (haptoglobin, Wap65 kDa), iv) clot-formation (fibrinogen beta and alpha chain, T-kininogen) and v) the host's immune response modulation (ApoA1 and ApoA2). However, quite different sets of these proteins or proteoforms were involved in response to cold and warm temperatures. In addition, cold acclimation seems to be related to the proteins involved in lipid metabolism (apolipoproteins A and 14 kDa) and stress response (corticosteroid binding globulin). We discovered a strongly regulated protein *Cap31* upon cold acclimation, which can serve as a potential blood biomarker of cold response in carp. These studies significantly extend our knowledge concerning mechanisms underlying thermal adaptation in poikilotherms.

1. Introduction

Water temperature is one of the most important environmental factors that affect physiology and behavior in poikilotherms, including fish. The carp (*Cyprinus carpio* L) is an eurythermal fish, which can survive under a wide range of temperatures ranging from near 0 °C to over 30 °C [1]. The optimum temperature for carp growth is between 20 °C and 25 °C [2]. Fluctuations in water temperature affect enzyme reactions, growth efficiency, reproduction, oxidative capacity, membrane composition and immune functionality [3]. The physiological reorganization compensating for such changes is often referred as the acclimatory response (i.e., reversible physiological plasticity).

Eurythermal fish cope with temperature fluctuations by developing suitable compensatory species-specific strategies, such as restructuring of cellular membrane lipid composition (e.g., increasing unsaturated fatty acids to restore the fluidity of cold-rigidified membranes), activation of immune responses, changes in gene expression, protein synthesis and energy production (e.g., increasing expression of ATPase in mitochondria after cold acclimation), and modulation of enzyme catalytic activity (e.g., increasing desaturase activity after cooling) [4–6]. Another adaptation strategy is to express different protein isoforms in a temperature-dependent manner or to produce proteins that are relatively temperature insensitive. In the case of muscle tissues, for example, temperature acclimation has led to the evolution of distinct inter-specific isoforms of myofibrillar proteins such as distinct myosin isoforms expressed in carp at different acclimation temperatures and kinetically distinct forms of enzymes involved in energy metabolism (myofibrillar ATPase) [7–10].

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Environmental temperature affects plasma biochemical indicators, antioxidant status and hematological and immunological parameters. So far, only single blood proteins have been found to adapt to temperature changes, such as heat shock protein (HSP70), complement C3, C4, IgM and warm-temperature acclimation-associated protein 65 kDa (Wap65) [4–6,11,12]. Wap65 is recognized as an essential component for warm acclimation in eurythermal fish species including carp [13,14]. At present, the exact number of blood proteins related to thermal adaptation of fish is unknown due to limitations of the methods applied in previous studies. Recently, methodology for comprehensive analysis of proteins such as transcriptomics and proteomics have been introduced to support complex studies of hundreds or thousands of proteins [15]. Such approaches may contribute to significantly extend our knowledge regarding mechanisms of thermal acclimation.

Recently, transcriptome analysis of carp coping with cold revealed both common stress responses in different tissues as well as distinct tissue-specific transcriptional responses to changing temperature [16]. It is speculated that the ability to adapt to fluctuating environmental temperature by common carp has resulted from tetraploidisation of its genome occurring during evolution, which provides increasing genetic flexibility to introduce genes for temperature adaptation. We hypothesized that thermal acclimation differentially affects the proteome of carp blood. Here, using a proteomic approach we show involvement of multiple blood plasma proteins/isoforms/proteoforms that are differentially expressed in warm- and cold-acclimated carp. These results are important for better understanding the mechanism of thermal adaptation in poikilotherms.

2. Material and methods

2.1. Fish origin and broodstock management

Common carp males (weight 1324 ± 511 g, age 3+) were obtained from Gosławice Fishery Farm in June 2015 and transported to the Department of Ichthyology, UWM in Olsztyn. After transport, fish were placed in tanks (1200 L; pH 7.2–7.8; oxygen content: > 90%) at 18 °C. After a week, fish were randomly separated into two groups: one group (n = 6) was acclimated to 10 °C while the second (n = 6) to 30 °C for 5 weeks for both groups in laboratory tanks. The acclimation period and experimental design was determined in reference to Watabe et al. [13,17] and Kinoshita et al. [5]. No food was given during the experimental period, similarly to Choi et al. [18]. After acclimation, blood and liver was collected for proteomic and mRNA analysis, respectively.

Approval from the Animal Experiments Committee in Olsztyn, Poland (no. 93/2011) was received prior to experimentation.

2.2. Collection of carp blood and liver and preparation of blood plasma

Blood samples were taken from the caudal vein of carp by syringe containing 3.8% sodium citrate in 0.8% sodium chloride in a ratio of solution to blood of 1:9. Blood plasma was obtained by centrifugation at 3500 × g for 10 min and stored at −80 °C for future analysis. For liver collection fish were killed with an overdose (400 ppm) of tricaine methane sulphonate (MS-222, Pharmaq Ltd., UK). The collected tissue were immediately covered with RNA later (Sigma-Aldrich, St. Louis, MO, USA).

For two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), aliquots containing approximately 800 µg of blood plasma proteins were precipitated using a Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Samples were resuspended in 80 µL of DIGE labelling buffer consisting of 30 mM Tris, 7 M urea, 2 M thiourea and 4% CHAPS to a protein concentration of 5–10 mg/mL. Additionally we used blood plasma without precipitation for comparative proteomic analysis. The protein concentration prior to and after the cleaning procedure was measured by a Coomassie Plus Kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as the standard.

2.3. 2D-DIGE analysis of blood plasma from warm- and cold-acclimated carp

Protein labelling with CyDye DIGE fluor and 2D electrophoresis was performed as previously described by Dietrich et al. [19]. Briefly, 50 µg of each sample were minimally labeled by incubation with 400 pmol of amine-reactive cyanine dye (Cy3 or Cy5) for 30 min. The internal standard was generated by combining equal amounts of proteins from each of 12 samples and was labeled with Cy2. A dye swap (Cy3/Cy5) was performed between samples of blood plasma from warm and cold acclimated fish (Table S1) to exclude dye bias. Differentially labeled samples were mixed together (Table S1). In each gel therefore blood plasma from cold (Cy3 or Cy5) and warm acclimated fish (Cy5 or Cy3) and internal standard (Cy2) were separated. The samples were loaded onto immobiline DryStrip gels strips (24 cm, pH 3 to 10 non-linear; GE Healthcare). Isoelectric focusing was performed with an IPGphor isoelectric focusing unit (GE Healthcare) and SDS-PAGE was run using the ETTAN Dalt six electrophoresis unit (GE Healthcare) as described by Dietrich et al. [19].

2.4. Image acquisition and quantitative analysis

The CyDye-labeled gels were analyzed by postrun fluorescence imaging with the use of the Typhoon FLA 9500 (GE Healthcare). After the multiplexed images were acquired, image analysis was performed with the use of DeCyder Differential Analysis Software (version 5.0; GE Healthcare). DeCyder software consists of a fully automated image analysis software suite that enables the detection, quantitation, matching and analysis of 2D-DIGE gels using several modules, such as the Differential In-gel Analysis module and Biological Variation Analysis module. Differential In-gel Analysis module was used to calculate protein abundance variations between samples on the same gel. The resulting spot maps were then analyzed by Biological Variation Analysis (BVA) module to provide statistical data on the differential protein expression between blood plasma proteomes of cold and warm acclimated fish. Abundance alterations of proteins were considered to be relevant if i) the corresponding spots were detected in all gels, and ii) Student's *t*-test reached levels of significance with $P < 0.05$ (including false discovery rate correction, FDR). After analysis, gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad). Spots presenting significant differences between cold and warm acclimated blood plasma proteomes were manually excised, trypsin digested, and identified with matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI TOF/TOF, Bruker Daltonics, Bremen, Germany).

2.5. MALDI TOF/TOF protein identification

Spots of interests were cut from the gel and prepared for identification as previously described by Dietrich et al. [20]. MS peptide mass fingerprint and fragment spectra from each individual spot were combined and used to search against the NCBI database (searching 25.10.2016) using the Mascot (version 2.4; Matrix Science Ltd, London, UK) with the following criteria: enzyme, trypsin; fixed modification, carbamidomethylation (C); and variable modifications, oxidation (M) peptide mass tolerance of 100 ppm, fragment mass tolerance of 0.9 Da, and 1 missed cleavage allowed. The search results were filtered with a significant threshold of $p < 0.05$ and a Mascot ion score cutoff of ≥ 30 for at least two peptides.

2.6. Ingenuity pathway and STRING analyses

To understand the biological context of the identified proteins and