

# Functional genomics of pre-slaughter stress responses in chicken muscle

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## Summary

Preslaughter conditions affect meat quality. However, the underlying molecular mechanisms are poorly investigated. In the present study, genomic tools were used to assess in an integrative way the biological response of chicken muscle to a stress situation before slaughter. Thigh muscles (tensor fascia latae and biceps femoris) were sampled immediately after sacrifice in standard broilers which were individually exposed (stressed) or not (control) to a restraint-transport (2h) combination stress. The analysis of transcripts using oligonucleotide microarrays show that the expression of 55 genes was affected by this stress. Proteomic analysis showed differential expression of 35 identified proteins. Integrate analysis of these data using Ingenuity System<sup>©</sup> proposes a significant network corresponding to small molecule biochemistry and cytoskeletal organisation. In addition, differential expressions of genes and proteins consistently indicate an inhibition of muscle glycolysis in response to stress. The analysis of metabolic profiles using metabolomics technique confirms the results obtained on genes and proteins since stressed animals showed lower relative quantities of the first metabolites of glycolysis.

Keywords: genomics, proteomics, stress, muscle

## Introduction

Many studies reported that pre-slaughter conditions, and subsequent stress responses, influence meat qualities. In poultry, several acute stress conditions before slaughter were reported to decrease meat qualities (Debut et al., 2003 and 2005). Most of the studies concerning the effects of pre-slaughter stress on meat quality focused mainly on the mobilization of energetic compounds in muscle (Fernandez and Tornberg, 1991). The aim of the present work is to better understand molecular mechanisms underlying muscle response to stress by analysing muscle transcriptome, proteome and metabolome in chickens.

## Materials and methods

**Animals.** Six-week-old standard broiler chickens (n = 30) were either scarified by decapitation immediately after capture (=non-treated (C)) or after being submitted to a restraint test (consisting in placing individually chickens in a “crush-cage”) combined with transport for 2 hours (TR). The thigh muscles (*tensor fascia latae* and *biceps femoris*) were collected at sacrifice. Blood samples were also collected at sacrifice to measure the plasma corticosterone level using a specific radio immunoassay (Etches, 1976).

**Metabolomic analysis.** The identification and relative quantification of most of the phosphorylated metabolites of key metabolic pathways and of the components of the Krebs cycle, were carried out using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of cell extracts to which labeled substrates were added (Mashego *et al.*, 2004). The relative abundance of targeted metabolites was expressed as the ratio of experimental peak area to the peak area of labeled substrate.

**Transcriptomic analysis.** Total RNA extractions were carried out on muscles samples with TRIzol Reagent (Invitrogen). Following RNA purification and DNA digestion, total RNA was quantified and its integrity was assessed. Gene expression was analyzed by hybridization of the chicken oligonucleotides 20 K microarray (GEO: GPL5480). Five µg of each RNA sample were reverse-transcribed and Cy3 or Cy5 fluorescent-labelled using the ChipShot™ direct labelling kit (Promega). Microarray analysis were realised on six animals of each experimental group selected on the base of their corticosterone levels. Labelling was done according the dye switch method and labelled cDNA from

control and treated animals were both hybridized to the microarray. Hybridization experiments were carried out with an automatic hybridization chamber. The microarray slides were scanned using a laser scanner (Axon Instruments) and then analysed with GenePix Pro 6.1 software. Data filtering, normalisation and statistical analysis were performed using the Bioconductor package Limma (Smyth, 2005) in the R statistical environment. A filter procedure eliminated non informative spots. The ratio Cy5/Cy3 used was expressed as the Log2 of the ratio of median pixel of intensity of the two red and green spots. Log2 median ratio values were then normalized by "Robustspline" procedure (Smyth and Speed, 2003) A linear model and a "moderated" Student test (Smyth, 2004) were used to test the effect of treatment (i.e. TR vs C). False discovery rate (FDR) was determined using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

**Proteomic analysis.** Soluble proteins were extracted from thigh muscles according to a modified procedure of Laville et al. (2009). Gels were finally stained with Coomassie colloidal blue procedure according to Molloy et al. (1998). All the gels were analyzed with the software Image Master 2D Platinum (GE Healthcare, Uppsala, Sweden) to point out proteins of interest. Prior to matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry analysis, the spots of interest where prepared as described by Laville et al. (2009). Peptide mass fingerprint (PMF) of trypsin-digested spots was determined in positive-ion reflector mode using a Voyager DE Pro MALDI-TOF-MS (PerSeptive Biosystems, Framingham, MA). PMFs were compared to *gallus* nrNCBI (01/2009, 32 977 seq) and *Aves* nrNCBI (12/2008, 102 448 seq) protein sequence databases (<http://www.ncbi.nlm.nih.gov/Database/>) using MASCOT 2.2 software [<http://www.matrixscience.com>].

**Statistical Analysis.** Experimental data were analyzed using the GLM-general factorial ANOVA procedure using the SAS software system for Windows statistical package program, version 9.1 (SAS Institute Inc., Cary, NC, USA).

## Results and discussion

At the end of the restraint test combined with transport (TR), corticosterone levels in TR chickens were six fold higher than basal corticosterone levels in C chickens ( $30.8 \pm 1.9$  and  $5.9 \pm 1.1$  ng/ml respectively,  $p < 0.0001$ ). This result

indicates that TR treatment induced activation of hypothalamic-pituitary-adrenal axis which is involved in stress response, as it has been previously described in birds (Hazard et al., 2008).

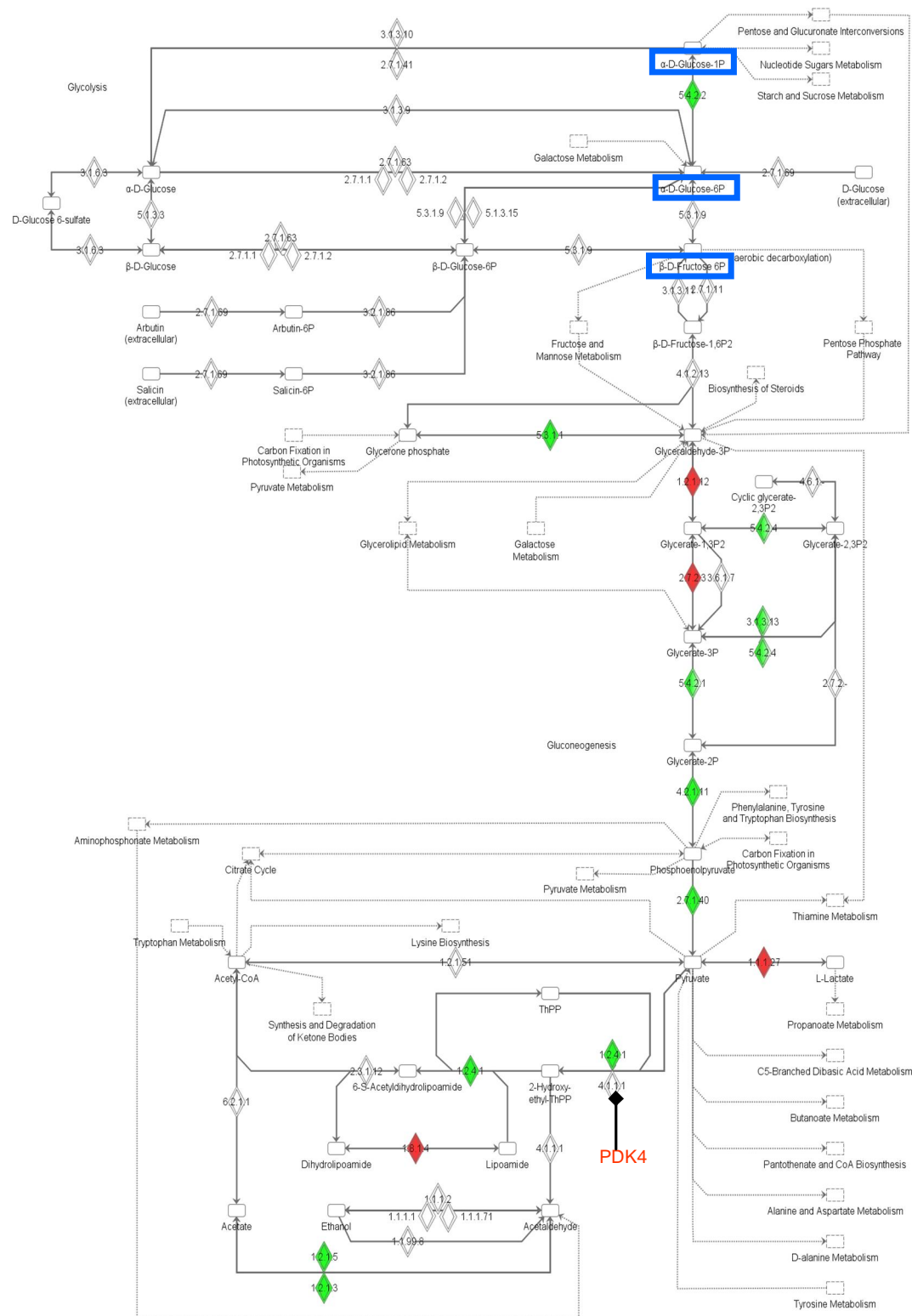
In the present study, 55 transcripts were identified to be significantly up- or down-regulated by treatment. Sixteen transcripts were unknown. Among the remaining 39 transcripts, 12 were significantly up-regulated and 27 down-regulated by TR treatment. The major functional categories for these genes concern cellular transport (proteins, phospholipids...), transcription factors, glycolysis, actin cytoskeleton organization, transduction signal, proteolysis and cell growth/maintenance. On 2-DE gels, out of 265 matched spots, 45 spots were differentially expressed between control and TR muscles. Thirty-five were successfully identified by mass spectrometry and corresponded to 29 individual proteins. Among these, 11 protein spots were over-expressed in TR group and 14 under-expressed compared to C group. Moreover, 20 spots were also not present on the gels of TR group.

Variance analysis of metabolomic data showed that stress significantly affected the content of some of the sugar phosphates (results not shown): the relative abundances of glucose-1-P, glucose-6-P, fructose-6-P and mannose-6-P were significantly reduced in stressed birds (ratios TR/C: 0.63, 0.48, 0.51 and 0.48 respectively). All other metabolites did not show significant variation in response to stress. These results were consistent with previous studies reporting that catecholamines and corticosteroids increased the use of energy stores during the stress response (Sapolsky et al., 2000). Indeed, catecholamines have been reported to increase the use of energy stores by activating hepatic and muscular glycogenolysis, glucocorticoids have been reported to inhibit glycogen synthase in muscle. Interestingly in our study, glycogen phosphorylase was under expressed in TR group compared to C group.

The use of Ingenuity Pathways Analysis has allowed the integration of the data coming from the 3 global analyses conducted in this study and showed that many transcripts and proteins differentially expressed were involved in glycolysis (Figure 1) From this, it is noticeable that almost all the steps of this pathway are affected by TR treatment either at transcripts (4), proteins (10) or metabolites (3) levels. The most surprising effect of the treatment is that almost all the transcripts and proteins were under expressed in TR muscles compared to C ones. On the other hand, gene of the key enzyme of the glycolytic pathway: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (Pfkfb) was up-regulated in TR group. Interestingly, this gene Pfkfb has been reported to be the most highly up-regulated gene in human muscle after adrenaline infusion (Viguerie et al., 2004).

Figure 1. Glycolysis metabolic pathway showing the differentially expressed proteins/metabolites/transcripts. Green nodes represent proteins that are identified to be differentially expressed TR group compared to control group. Red nodes represent transcripts that are identified to be differentially expressed TR group compared to control group. Blue boxes represent metabolites which relative amounts differ between TR group compared to control group. Numbers indicate the expression ratio TR/C. C and TR indicate that the protein was only found on the gel of TR and C birds respectively.

Glycolysis/Gluconeogenesis



## **Conclusion**

In conclusion, we report differential gene and protein expression in muscles in chickens submitted to TR treatment. Some of the identified genes or proteins have been already reported to be regulated by neuroendocrine mediators of stress response, but the majority has not been documented as directly involved in muscle response to stress. Although their contributions in meat quality merit further investigation, the genes and proteins described in this report are excellent potential candidates to explain effects of stress on biochemical and structural characteristics of muscles.

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