Proteomic technologies to identify stress and welfare markers in livestock

Anna Marco-Ramell, Laura Arroyo, Daniel Valent, M. Carmen Olivan, Antonio Velarde and Anna Bassols

OUR QUESTIONS:

1) How is the environment affecting animal physiology?

2) Can useful biomarkers be identified?

3) Proteomic technologies are useful to address these issues?
Two experiments related to housing in bovine and porcine

<table>
<thead>
<tr>
<th>BOVINE</th>
<th>COWS UNDER DIFFERENT PRODUCTION SYSTEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PORCINE</td>
<td>GILTS AFTER SWITCHING TO INDIVIDUAL HOUSING</td>
</tr>
</tbody>
</table>
# RESEARCH OF WELFARE BIOMARKERS BY PROTEOMIC APPROACHES IN COWS UNDER DIFFERENT PRODUCTION SYSTEMS

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Cattle Breed</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Alberes</td>
<td>Albera mountains - Semiferal conditions</td>
</tr>
<tr>
<td>B in A</td>
<td>Bruna dels Pirineus</td>
<td>Albera mountains - Semiferal conditions</td>
</tr>
<tr>
<td>B</td>
<td>Bruna dels Pirineus</td>
<td>Berguedà – Good pastures, human contact</td>
</tr>
</tbody>
</table>

Samples were collected in spring
Proteomic approach: DIGE (Differential Gel Electrophoresis)
Sample: pooled serum enriched in low-abundant proteins with Proteominer™

DIGE: Samples are labeled with Cy dyes and run together in the same gel:

Brunes at Albera (semiferal)
Brunes (control)

22 differential spots were selected and identified by MALDI-MS and MS/MS
12 spots were found upregulated in Bruna cows living in mild conditions, and 10 spots were downregulated. Only 15 of them could be identified.

Marco-Ramell et al. (2012) Journal of Proteomics 75; 4399-4411
<table>
<thead>
<tr>
<th># Spot</th>
<th>Protein name</th>
<th>Fold Change (B/BA)</th>
<th>T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1045</td>
<td>Paraoxonase 1</td>
<td>1.46</td>
<td>1.96E-04</td>
</tr>
<tr>
<td>1427</td>
<td>Selenium-Dependent Glutathione Peroxidase</td>
<td>-5.33</td>
<td>4.16E-04</td>
</tr>
<tr>
<td>1430</td>
<td>Glutathione Peroxidase 3</td>
<td>-2.10</td>
<td>4.32E-04</td>
</tr>
<tr>
<td>1297</td>
<td>Protein AMBP precursor</td>
<td>1.59</td>
<td>0.001</td>
</tr>
<tr>
<td>1300</td>
<td>Alpha-2-HS-Glycoprotein precursor</td>
<td>-1.78</td>
<td>1.68E-06</td>
</tr>
<tr>
<td>843</td>
<td>Serum albumin</td>
<td>-1.44</td>
<td>0.005</td>
</tr>
<tr>
<td>691</td>
<td>Complement C3</td>
<td>1.82</td>
<td>0.05</td>
</tr>
<tr>
<td>775</td>
<td>Complement component C9 precursor</td>
<td>-1.90</td>
<td>5.37E-04</td>
</tr>
<tr>
<td>786</td>
<td>Complement C1s subcomponent</td>
<td>-1.7</td>
<td>0.01</td>
</tr>
<tr>
<td>797</td>
<td>Prepro complement component C3</td>
<td>1.4</td>
<td>1.57E-03</td>
</tr>
<tr>
<td>1049</td>
<td>Conglutinin</td>
<td>1.7</td>
<td>3.63E-04</td>
</tr>
<tr>
<td>1357</td>
<td>Immunoglobulin J chain</td>
<td>-1.83</td>
<td>1.68E-06</td>
</tr>
<tr>
<td></td>
<td>Oxidative stress</td>
<td></td>
<td>Acute phase proteins</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------</td>
<td>--------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td></td>
<td>RSD^A CV^B (%)</td>
<td>B (Bruna) BA (Bruna at Alberes)</td>
<td>A (Alberes)</td>
</tr>
<tr>
<td><strong>GPx (U/L)</strong></td>
<td>389.21</td>
<td>68.87a 197.59b 910.09c</td>
<td></td>
</tr>
<tr>
<td><strong>PON-1 (KU/L)</strong></td>
<td>2.012</td>
<td>9.256a 6.379b 7.333b</td>
<td></td>
</tr>
<tr>
<td><strong>GR (U/L)</strong></td>
<td>33.22</td>
<td>200.16a 185.39a 159.29b</td>
<td></td>
</tr>
<tr>
<td><strong>SOD (U/L)</strong></td>
<td>61.48</td>
<td>284.28a 285.48a 336.62b</td>
<td></td>
</tr>
<tr>
<td><strong>Carbonyl groups (AU)</strong></td>
<td>0.207</td>
<td>0.990a 1.159b 1.232b</td>
<td></td>
</tr>
<tr>
<td><strong>α2HSG (mg/mL)</strong></td>
<td>0.39</td>
<td>1.99a 1.17b 0.50c</td>
<td></td>
</tr>
<tr>
<td><strong>Haptoglobin (mg/mL)</strong></td>
<td>0.18</td>
<td>0.16a 0.24a,b 0.27b</td>
<td></td>
</tr>
<tr>
<td><strong>SAA (μg/mL)</strong></td>
<td>23.80</td>
<td>13.06a 20.12a 17.12a</td>
<td></td>
</tr>
<tr>
<td><strong>Cortisol (ng/mL)</strong></td>
<td>325.80</td>
<td>45.40a 155.37a,b 306.85b</td>
<td></td>
</tr>
<tr>
<td><strong>Faecal Corticosterone (ng/g)</strong></td>
<td>38.86</td>
<td>36.18a 49.05b 38.99a</td>
<td></td>
</tr>
<tr>
<td><strong>Cholesterol (mg/dL)</strong></td>
<td>38.93</td>
<td>125.47a 120.30a 162.21b</td>
<td></td>
</tr>
</tbody>
</table>
Recordar posar lo del piedrafita

Cortisol
CorticoMS
CorticoMF
Colesterol
LDL
HDL
TG
Hbut
NEFA
Hp
SAA
HSGa
GPx
Gred
CO
PON1
CK

-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5

-2
-1
0
1
2
3

Discriminant Plot

Funct 2 (13,4%)
Funct 1 (86,6%)

Alberes
Brunes
Brunes at Alberes

65th Annual Meeting of the EAAP 2014
A combination of antioxidant enzymes, such as glutathione peroxidase (GPx), together with a negative acute phase protein (\(\alpha_2\)-HSG) and cholesterol.

"Unwelfare" ratio = (GPx + Chol) / \(\alpha_2\)-HSG
LIVING IN HARD ENVIRONMENTS LEADS TO COMPLEX CHANGES:

OXIDATIVE STRESS

IMMUNE SYSTEM, LOW GRADE INFLAMMATION

FECAL CORTICOSTERONE and CORTISOL

LIPID MOBILIZATION (CHOLESTEROL)
CHANGES OF HOUSING FROM PEN TO INDIVIDUAL STALLS IN REPRODUCTIVE GILTS

Day 1

Day 3

Day 4

Day 5

Quarantine facility

Groups of 10-15 gilts

Mating room

Individual boxes of reduced dimension
First quantitative proteomic analysis: **DIGE**
Sample: serum

<table>
<thead>
<tr>
<th>Spot</th>
<th>Identification</th>
<th>Fold Study</th>
<th>Fold Study</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>802</td>
<td><em>Haptoglobin precursor</em></td>
<td>1.73</td>
<td>1.34</td>
<td>1.679E-05</td>
</tr>
<tr>
<td>808</td>
<td><em>Haptoglobin precursor</em></td>
<td>2.19</td>
<td>1.63</td>
<td>0.010</td>
</tr>
<tr>
<td>1021</td>
<td><em>Apolipoprotein A-I</em></td>
<td>-1.70</td>
<td>-1.53</td>
<td>7.712E-06</td>
</tr>
<tr>
<td>1213</td>
<td>$\alpha 1$-antichymotrypsin 3</td>
<td>1.20</td>
<td>1.16</td>
<td>0.026</td>
</tr>
<tr>
<td>1029</td>
<td><em>Peroxiredoxin-2</em></td>
<td>5.78</td>
<td>4.86</td>
<td>2.085E-04</td>
</tr>
</tbody>
</table>

Marco-Ramell et al. (2014) submitted to Animal
Second quantitative proteomic analysis: *iTRAQ*

Isobaric tag for relative and absolute quantitation

Sample: serum enriched in low-abundant proteins with Proteominer™

Samples

1. Samples
2. iTRAQ labeling
   - 114 label
   - 115 label
   - 116 label
   - 117 label
3. Combine

Peptide fractionation on a 3100 OFFGEL Fractionator according to its pI

Peptide analysis on a LTQ-Orbitrap Velos coupled to the nano-UPLC ACQUITY system

Quantify
- iTRAQ reporter ions

Identify
- MS/MS fragmentation pattern
122 proteins with a variation ≥ ± 1,2 fold versus Day 1 were identified.
NETWORK ANALYSIS WITH STRING

Antioxidant defenses

Lipoprotein transport

Acute phase

Complement system

Cellular structure
iTRAQ gives a relative quantification of proteins in the sample

Extracellular increasing vs Day 1
Extracellular decreasing vs Day 1
Intracellular increasing vs Day 1
Intracellular decreasing vs Day 1
<table>
<thead>
<tr>
<th></th>
<th>D1</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp, mg/mL</td>
<td>1.50 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33 ± 1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.73 ± 0.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.54 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRP, µg/mL</td>
<td>9.97 ± 6.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.77 ± 9.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.66 ± 6.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.61 ± 8.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pig-MAP, mg/mL</td>
<td>2.45 ± 1.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.84 ± 1.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.27 ± 0.96&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.82 ± 1.04</td>
</tr>
<tr>
<td>Apo A-I, ratio</td>
<td>2.01 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.45 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.68 ± 0.37&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.71 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Superindex indicates significant differences between days (P<0.05)
VALIDATION IN ALL THE INDIVIDUALS AND DAYS

<table>
<thead>
<tr>
<th>Oxidative stress markers</th>
<th>D1</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonyl groups, ratio</td>
<td>1.89 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.10 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.87 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.84 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPx, U/mL</td>
<td>33.24 ± 10.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.70 ± 10.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.27 ± 10.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.01 ± 6.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD, U/L</td>
<td>133 ± 20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>151 ± 18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>125 ± 27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113 ± 35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>tGSH, mmol/L</td>
<td>0.60 ± 0.17</td>
<td>0.66 ± 0.18</td>
<td>0.70 ± 0.10</td>
<td>0.58 ± 0.17</td>
</tr>
</tbody>
</table>

Superindex indicates significant differences between days ($P<0.05$)
VALIDATION IN ALL THE INDIVIDUALS AND DAYS

<table>
<thead>
<tr>
<th>Cortisol</th>
<th>D1</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (serum)</td>
<td>8.47 ± 4.92</td>
<td>7.22 ± 6.08</td>
<td>6.32 ± 4.79</td>
<td>6.49 ± 5.10</td>
</tr>
<tr>
<td>(µg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol (saliva)</td>
<td>6.53 ± 4.69</td>
<td>25.01 ± 42.28</td>
<td>14.54 ± 12.36</td>
<td>12.02 ± 9.88</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cholesterol</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mg/dL</td>
<td>101 ± 18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>138 ± 46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>114 ± 39&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Superindex indicates significant differences between days ($P < 0.05$)
EXPOSITION TO CHALLENGING CONDITIONS LEADS TO COMPLEX CHANGES

OXIDATIVE STRESS

IMMUNE SYSTEM, LOW GRADE INFLAMMATION

CORTISOL

LIPID MOBILIZATION (CHOLESTEROL)

CELL DAMAGE
Glucocorticoids (catecholamines, others,...) 

LIVER ADIPOSE TISSUE 

LIPID MOBILIZATION 

ROS, OTHER SOURCES OF OXIDATIVE STRESS 

Oxidation markers 
Antioxidant enzymes 

Innate immune system involvement 

Acute phase proteins 

Cell damage 

STRESS
PRE-SLAUGHTER STRESS MARKERS IN PIGS LIVING IN STANDARD CONDITIONS OR WITH GOOD HUMAN INTERACTION

Sample: peripheral blood mononuclear cells (PBMCs)

Uncharacterized protein OS=Sus scrofa
Filamin-A (Fragment) OS=Sus scrofa
Coronin (Fragment) OS=Sus scrofa
Fibrinogen beta chain OS=Sus scrofa
Uncharacterized protein OS=Sus scrofa
Testin OS=Sus scrofa
Pleckstrin OS=Sus scrofa
Elongation factor 1-alpha OS=Sus scrofa
Annexin OS=Sus scrofa GN=ANXA1 PE=2 SV=2
PDZ and LIM domain protein 1 OS=Sus scrofa
Monoglyceride lipase OS=Sus scrofa
Malate dehydrogenase, cytoplasmic OS=Sus scrofa
Tropomyosin alpha-1 chain OS=Sus scrofa
Uncharacterized protein OS=Sus scrofa
Proteasome subunit alpha type OS=Sus scrofa
High mobility group protein B1 OS=Sus scrofa
Proteasome activator complex subunit 1 OS=Sus scrofa
Myosin regulatory light polypeptide 9 OS=Sus scrofa
Uncharacterized protein OS=Sus scrofa
Calcium-binding protein A9 OS=Sus scrofa
Peptidyl-prolyl cis-trans isomerase A OS=Sus scrofa
Uncharacterized protein (Fragment) OS=Sus scrofa
Uncharacterized protein OS=Sus scrofa
Histone H2B OS=Sus scrofa

Preliminary results shows the involvement of structural proteins and metabolic enzymes
ACKNOWLEDGEMENTS

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SERIDA. **M. Carmen Oliván**

Department of Molecular Biology. Aarhus University (Denmark). **Emoke Bendixen**

Proteomics Platform. Institut de Recerca Vall d’Hebron. Barcelona. **Francesc Canals.**

Proteomics Platform. Institut de Recerca Biomèdica. Barcelona. **Eliandre de Oliveira.**
SAINT ANTHONY’S DAY. CALDES DE MONTBUI. CATALONIA.
The treatment with ProteoMiner™ of serum pools from groups B and BA was performed by triplicate in three different days to allow for technical replicates of the enrichment procedure.

Three gels were run, each of them containing one ProteoMiner™ replicate of each group of cows and one pool of all samples as internal standard (Cy2).

Dye swap was performed in order to avoid any possible bias.
Reproducibility of the Proteominer™ treatment

After 2D-electrophoresis and image analysis, the standard normalized volume of the fifteen differentially expressed spots for each of the three gels is represented.
**DIGE**

For the proteomic approach, pools of serum samples at days 1, 3 and 5 were formed. They were labeled with Cy2, Cy3 or Cy5 fluorochromes and run in 4 different gels.

The goal was to find a stress biomarker associated to change in housing and, not to the sampling procedure.

Serum proteomes were analyzed and compared between days. 27 differential spots were selected for identification ($p < 0.05$, ±1.2 fold variation between days).