Proteomic technologies to identify stress and welfare markers in livestock

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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?

On Farm

Breed Differentiation
Nutrition and Feeding
Animal welfare
Quality

Two experiments related to housing in bovine and porcine

BOVINE	**COWS UNDER DIFFERENT PRODUCTION SYSTEMS**
PORCINE	**GILTS AFTER SWITCHING TO INDIVIDUAL HOUSING**

RESEARCH OF WELFARE BIOMARKERS BY PROTEOMIC APPROACHES IN COWS UNDER DIFFERENT PRODUCTION SYSTEMS

GROUP	Cattle Breed	Habitat	
A	Alberes	Albera mountains - Semiferal conditions	
B in A	Bruna dels Pirineus	Albera mountains - Semiferal conditions	
B	Bruna dels Pirineus	Berguedà – Good pastures, human contact	

Samples were collected in spring

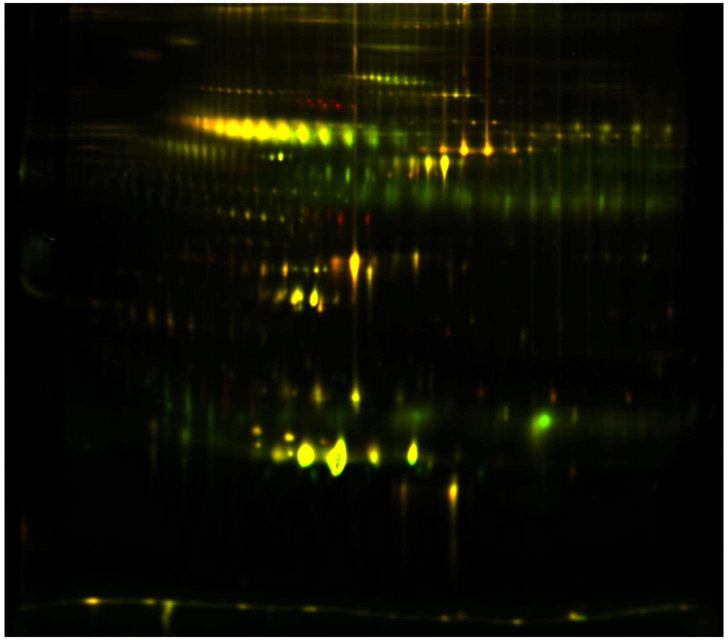
Proteomic approach: DIGE (Differential Gel Electrophoresis)

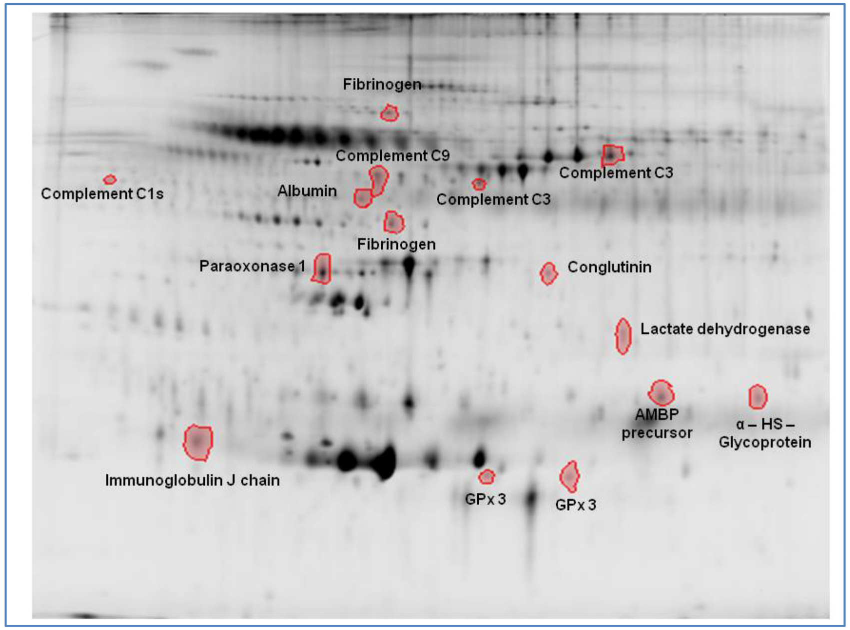
Sample: pooled serum enriched in low-abundant proteins with Proteominer™

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.



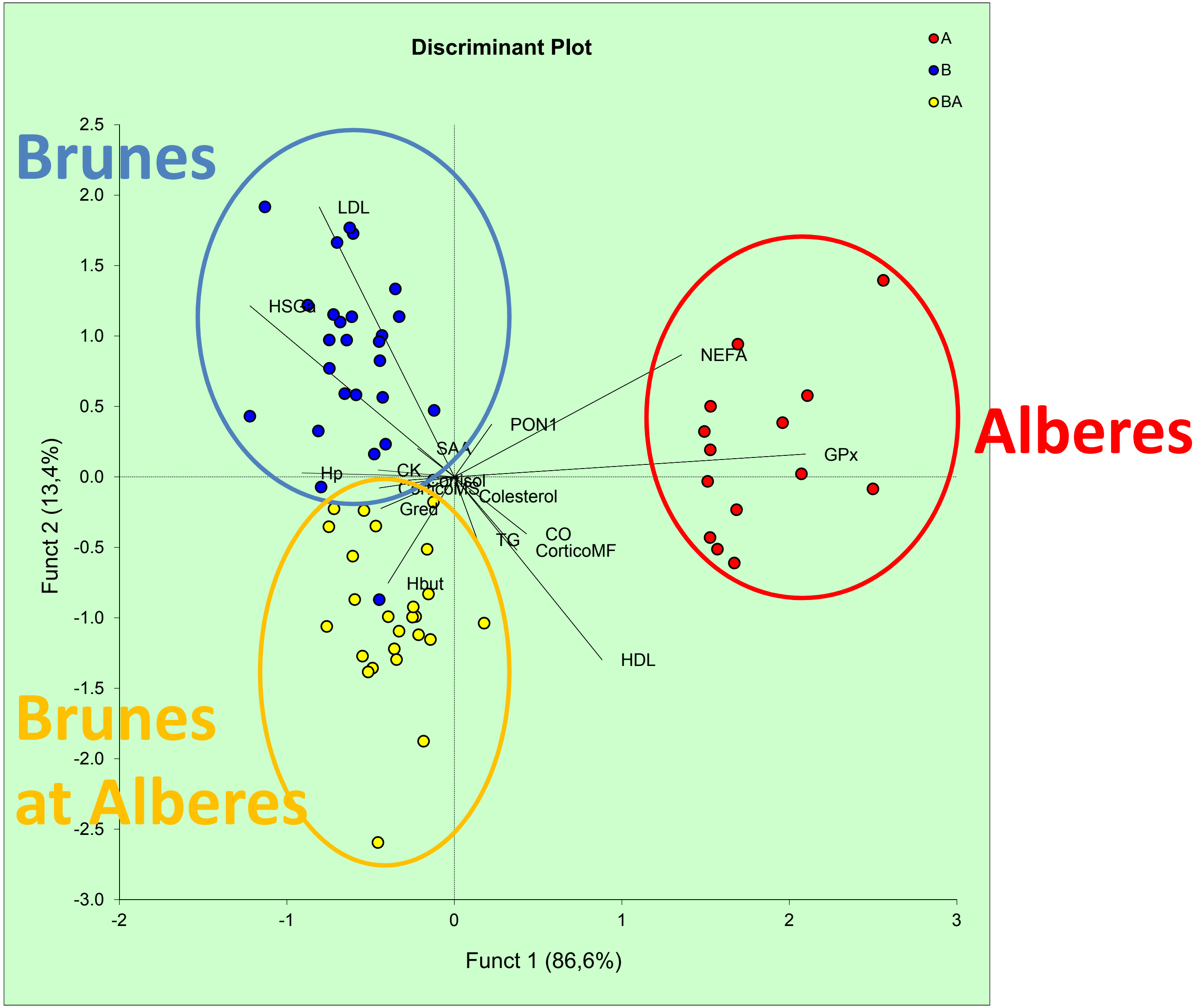


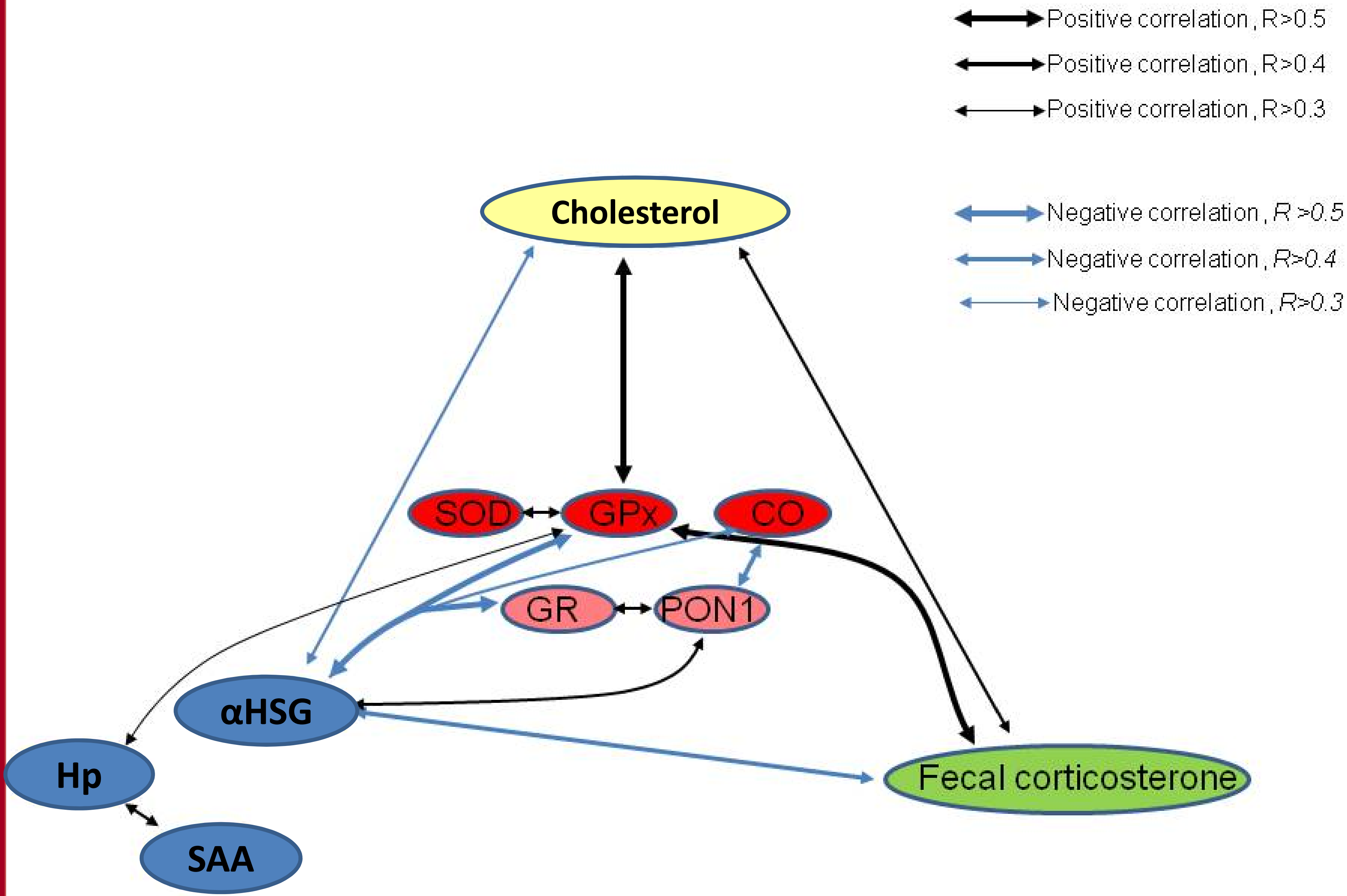
Marco-Ramell et al. (2012) Journal of Proteomics 75; 4399-4411

# Spot	Protein name		Fold Change (B/BA)	T-test
1045	***Paraoxonase 1***	Oxidative stress	**1,46**	1,96E-04
1427	***Selenium-Dependent Glutathione Peroxidase***	Oxidative stress	**-5.33**	4.16E-04
1430	***Glutathione Peroxidase 3***	Oxidative stress	**-2.10**	4.32E-04
1297	***Protein AMBP precursor***	Acute phase protein	**1,59**	0,001
1300	***Alpha-2-HS-Glycoprotein precursor***	Acute phase protein	**-1,78**	1,68E-06
843	***Serum albumin***	Acute phase protein (negative)	**-1,44**	0,005
691	***Complement C3***	Complement system	**1,82**	0,05
775	***Complement component C9 precursor***	Complement system	**-1,90**	5,37E-04
786	***Complement C1s subcomponent***	Complement system	**-1,7**	0,01
797	***Prepro complement component C3***	Complement system	**1,4**	1,57E-03
1049	***Conglutinin***	Complement system	**1,7**	3,63E-04
1357	***Immunoglobulin J chain***	Immune system	**-1,83**	1,68E-06

VALIDATION OF OXIDATIVE STRESS MARKERS, ACUTE PHASE PROTEINS, GLUCOCORTICOIDS AND LIPID MARKERS

		RSD^{A}	CV^{B} (%)	B (Bruna)	BA (Bruna at Alberes)	A (Alberes)
Oxidative stress	*GPx (U/L)*	389.21	99.24	68.87^{a}	197.59^{b}	910.09^{c}
	PON-1 (KU/L)	2.012	26.18	9.256^{a}	6.379^{b}	7.333^{b}
	GR (U/L)	33.22	18.29	200.16^{a}	185.39^{a}	159.29^{b}
	SOD (U/L)	61.48	20.59	284.28^{a}	285.48^{a}	336.62^{b}
	Carbonyl groups (AU)	0.207	18.35	0.990^{a}	1.159^{b}	1.232^{b}
Acute phase proteins	*α2HSG (mg/mL)*	0.39	31.70	1.99^{a}	1.17^{b}	0.50^{c}
	Haptoglobin (mg/mL)	0.18	82.33	0.16^{a}	0.24^{a,b}	0.27^{b}
	SAA (µg/mL)	23.80	142.29	13.06^{a}	20.12^{a}	17.12^{a}
GC	*Cortisol (ng/mL)*	325.80	194.31	45.40^{a}	155.37^{a,b}	306.85^{b}
	Faecal Corticosterone (ng/g)	38.86	16.09	36.18^{a}	49.05^{b}	38.99^{a}
Lipids	*Cholesterol (mg/dL)*	38.93	38.93	125.47^{a}	120.30^{a}	162.21^{b}


Discriminant Plot
● A
● B
○ BA
Brunes
Alberes
Brunes at Alberes
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-2
-1
0
1
2
3
Funct 2 (13,4%)
Funct 1 (86,6%)
LDL
HSGa
NEFA
PON1
SAA
GPx
Hp
CK
Cortisol
CorticoMS
Colesterol
Gred
CO
TG
CorticoMF
Hbut
HDL



Positive correlation, R>0.5

Positive correlation, R>0.4

Positive correlation, R>0.3

Negative correlation, *R >0.5*

Negative correlation, *R>0.4*

Negative correlation, *R>0.3*

Cholesterol

SOD

GPx

CO

GR

PON1

αHSG

Hp

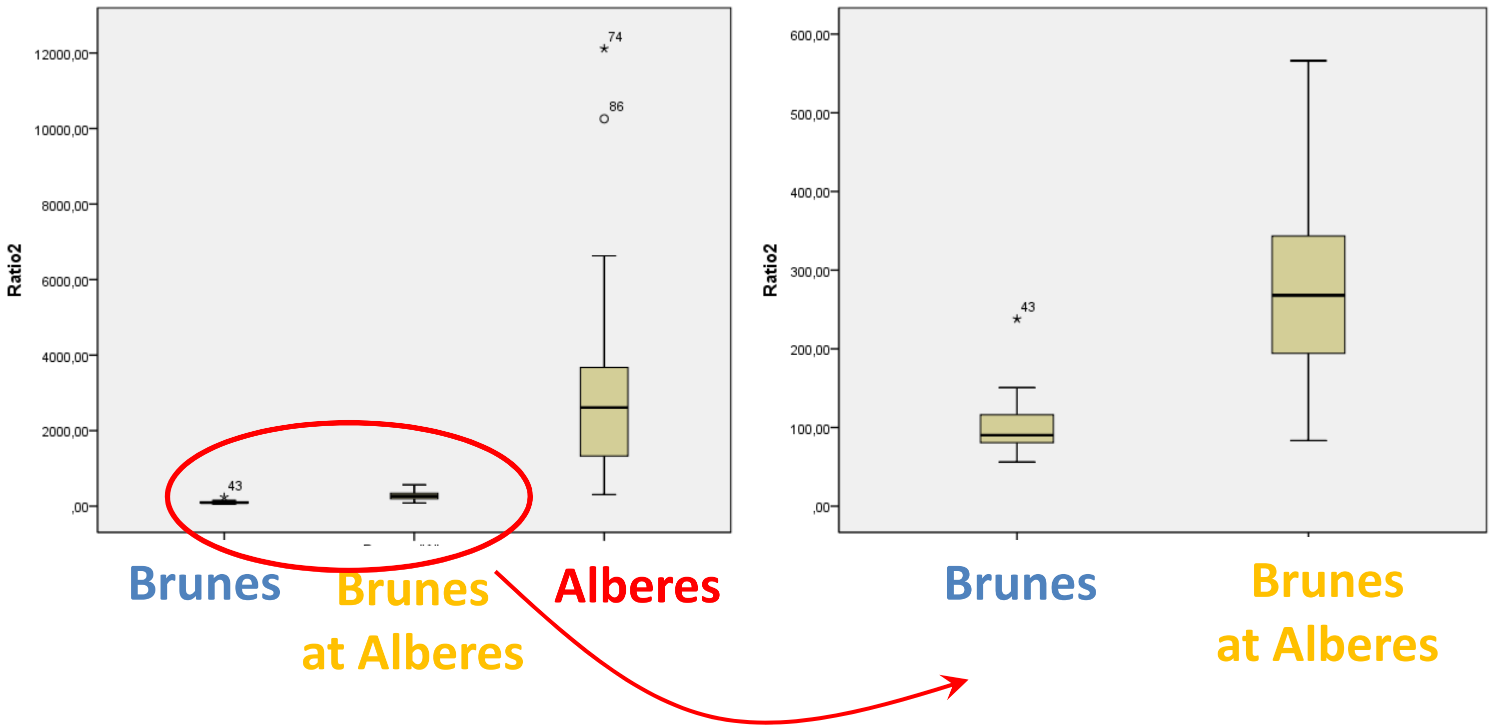
SAA

Fecal corticosterone

BIOMARKER PROFILE TO ASSESS WELFARE IN CHALLENGING LIVING CONDITIONS IN CATTLE

A combination of antioxidant enzymes, such as glutathione peroxidase (GPx), together with a negative acute phase protein (α2-HSG) and cholesterol.

***"Unwelfare"* ratio = (GPx + Chol) / α2-HSG**



LIVING IN HARD ENVIRONMENTS LEADS TO COMPLEX CHANGES:

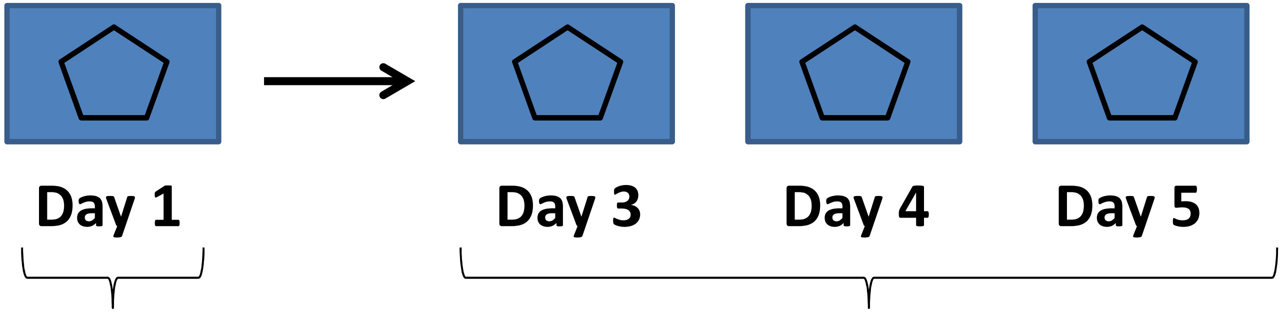
OXIDATIVE STRESS

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

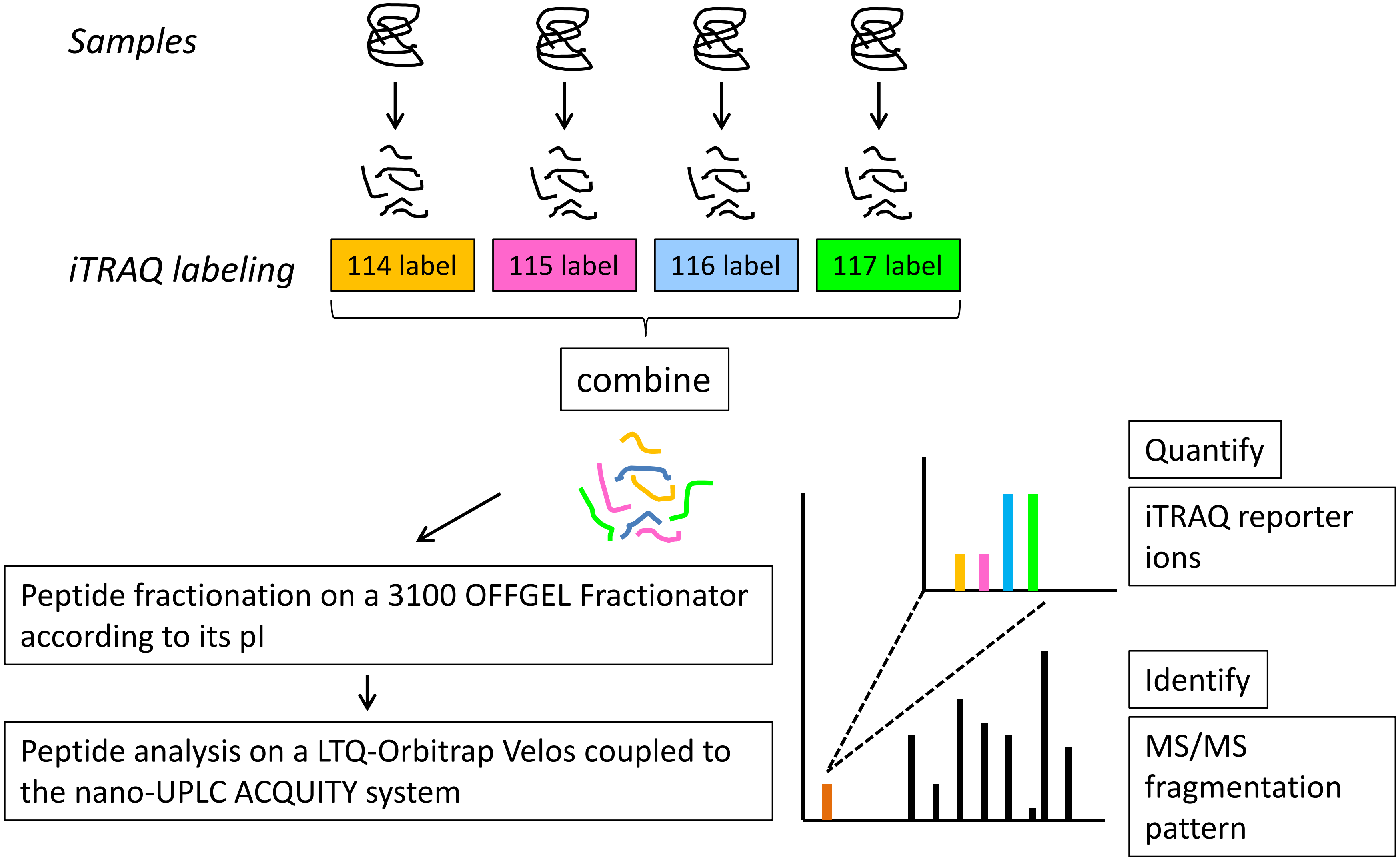
			Day 3	Day 5	
Spot	**Identification**		**Fold Study**	**Fold Study**	***P***
802	***Haptoglobin precursor***	Acute phase	**1,73**	**1,34**	1,679E-05
808	***Haptoglobin precursor***	Acute phase	**2,19**	**1,63**	0,010
1021	***Apolipoprotein A-I***	Acute phase	**-1,70**	**-1,53**	7,712E-06
1213	***α1-antichymotrypsin 3***	Acute phase	**1,20**	**1,16**	0,026
1029	***Peroxiredoxin-2***	Oxidative stress	**5,78**	**4,86**	2,085E-04

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

iTRAQ labeling

114 label

115 label

116 label

117 label

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

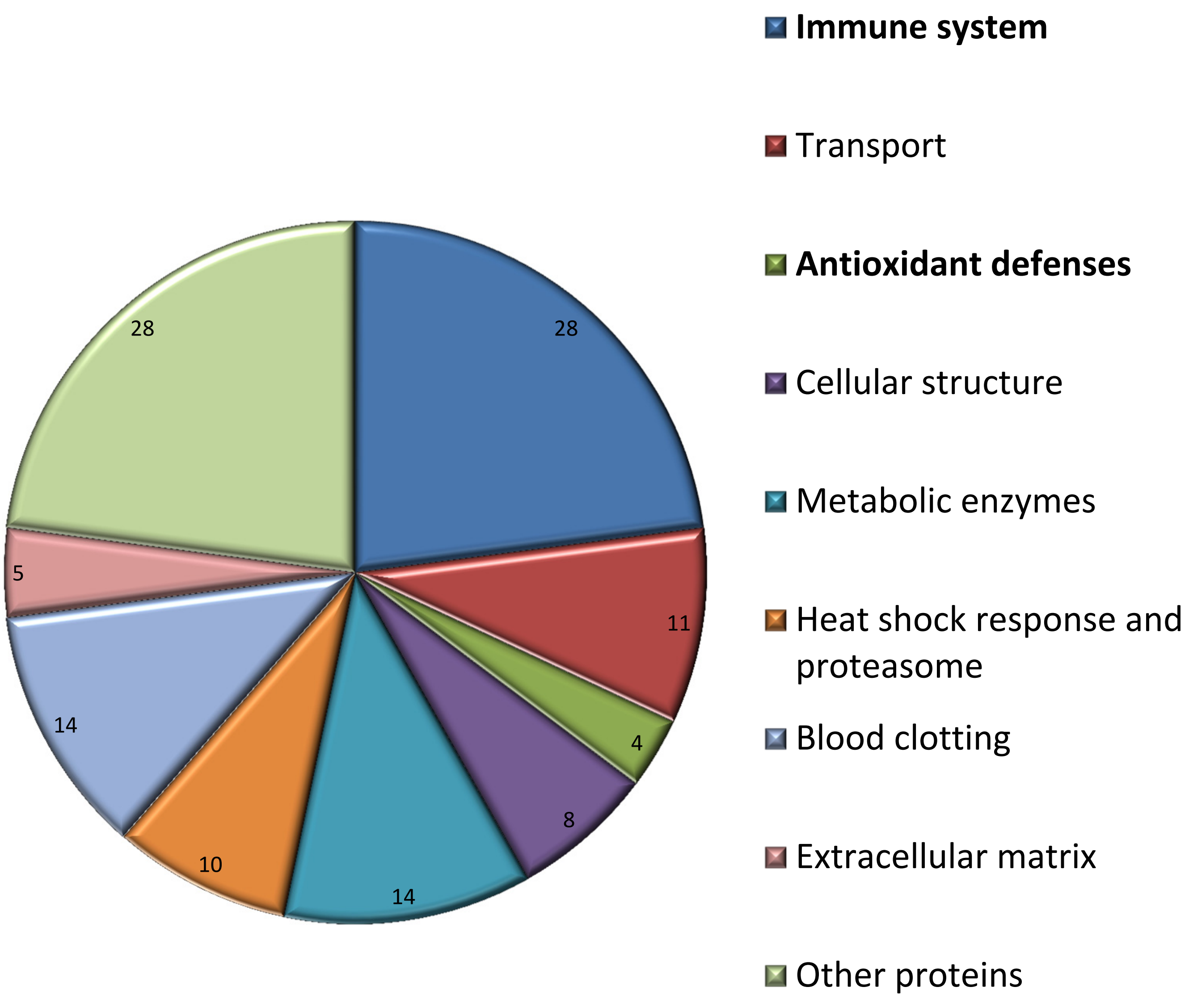
Quantify

iTRAQ reporter ions

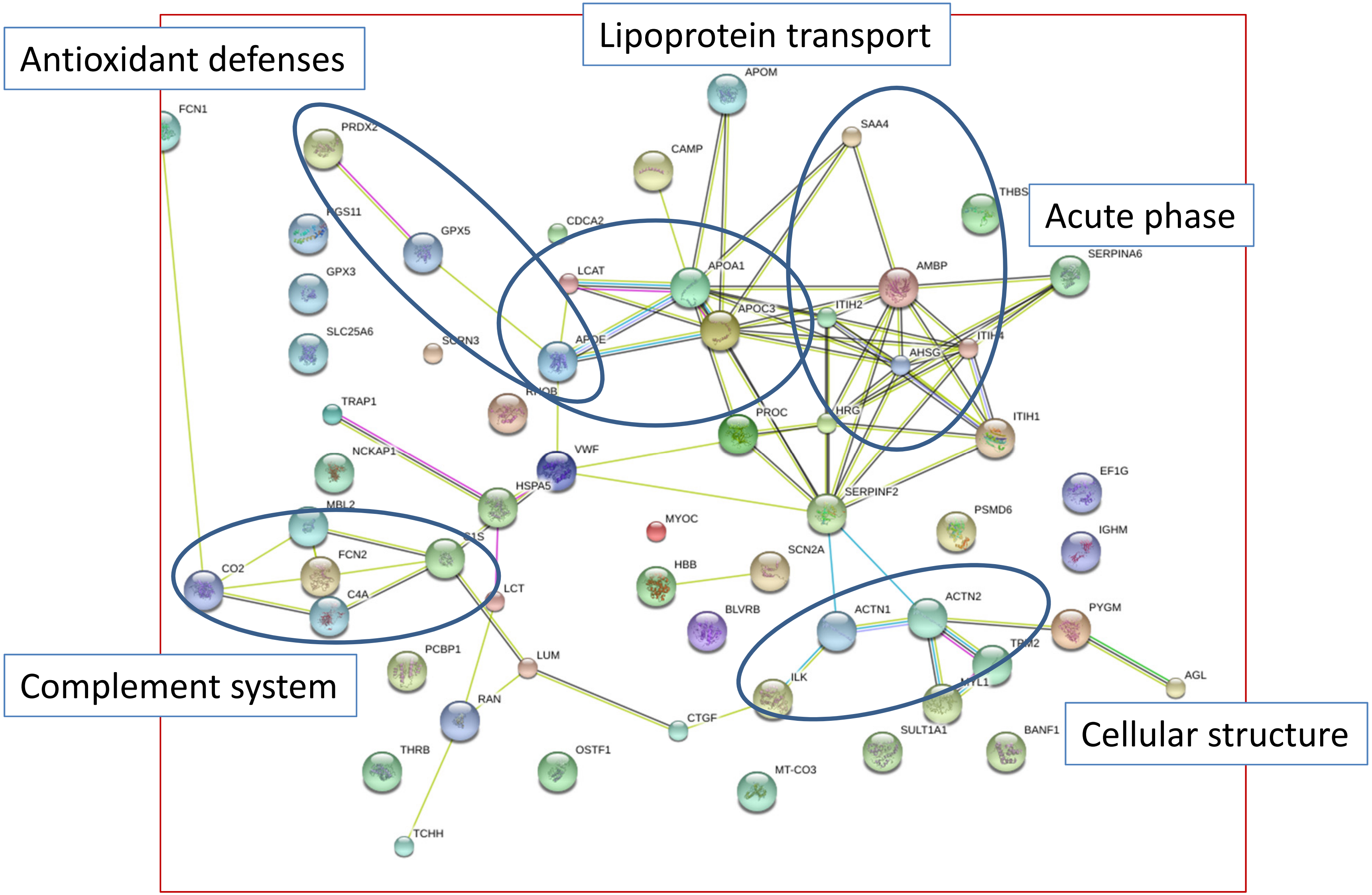
Identify

MS/MS fragmentation pattern

122 proteins with a variation ≥ ± 1,2 fold versus Day 1 were identified



NETWORK ANALYSIS WITH *STRING*

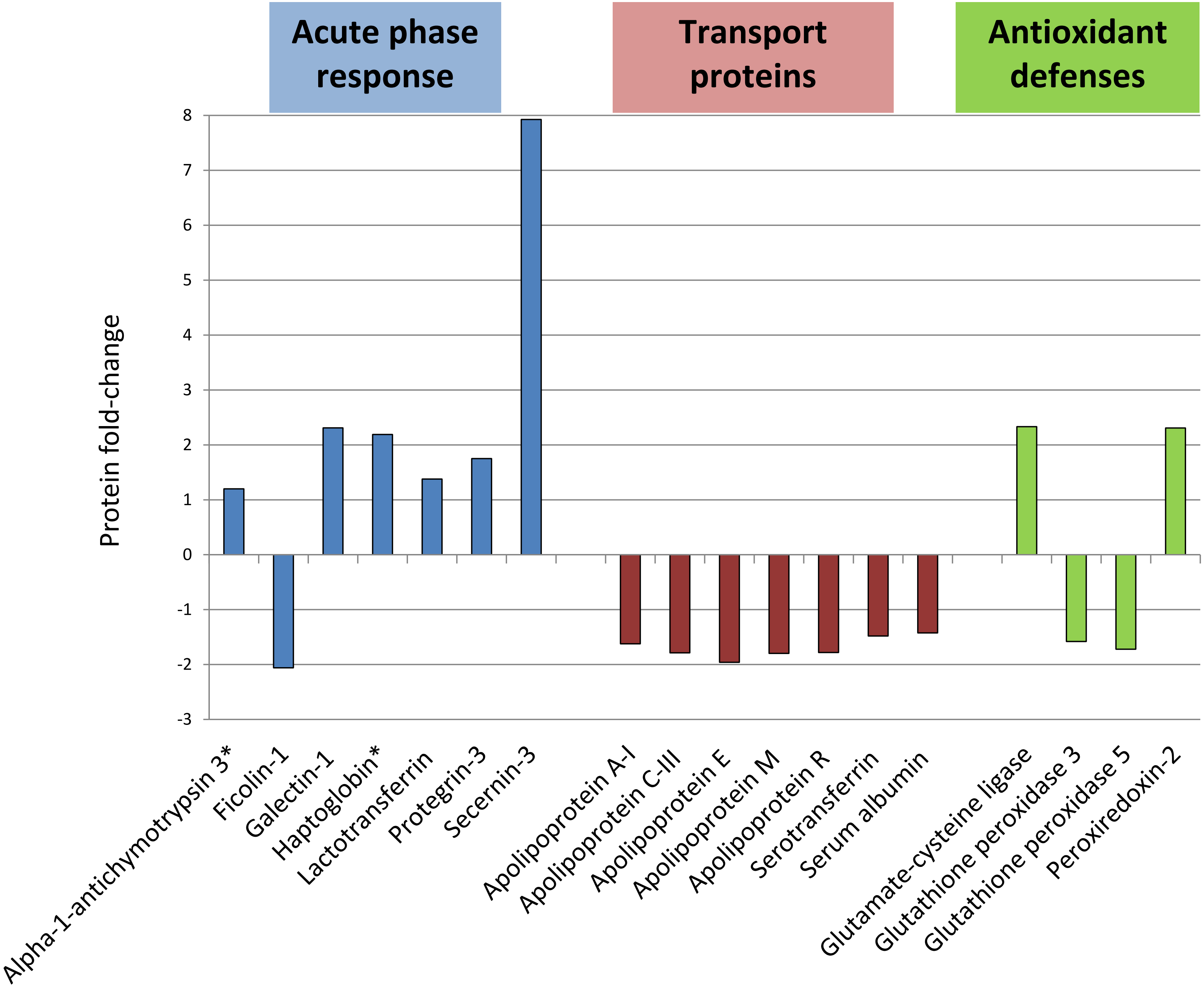

Antioxidant defenses

Lipoprotein transport

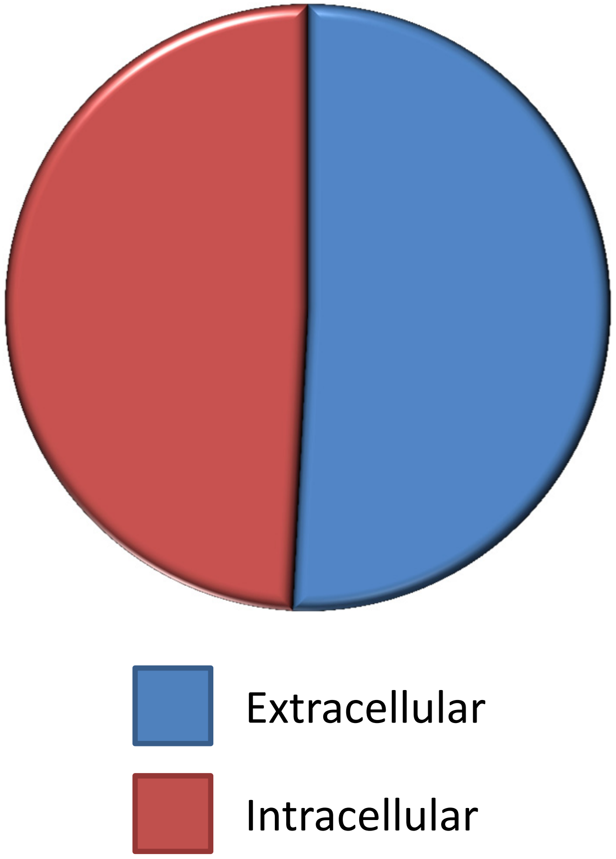
Acute phase

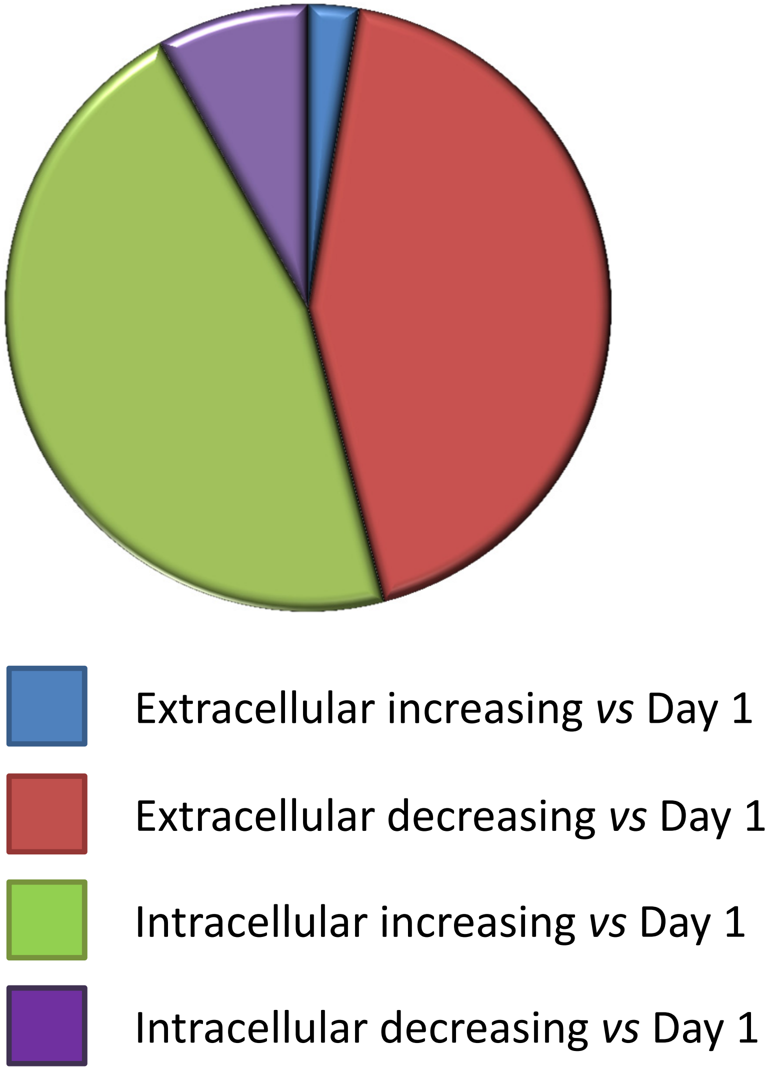
Complement system

Cellular structure


Acute phase response
Transport proteins
Antioxidant defenses
Protein fold-change
8
7
6
5
4
3
2
1
0
-1
-2
-3
Alpha-1-antichymotrypsin 3*
Ficolin-1
Galectin-1
Haptoglobin*
Lactotransferrin
Protegrin-3
Secernin-3
Apolipoprotein A-I
Apolipoprotein C-III
Apolipoprotein E
Apolipoprotein M
Apolipoprotein R
Serotransferrin
Serum albumin
Glutamate-cysteine ligase
Glutathione peroxidase 3
Glutathione peroxidase 5
Peroxiredoxin-2

iTRAQ gives a relative quantification of proteins in the sample





VALIDATION IN ALL THE INDIVIDUALS AND DAYS

Acute phase proteins

	D1	D3	D4	D5
Hp, mg/mL	1.50 ± 0.61^{a}	2.33 ± 1.05^{b}	1.73 ± 0.73^{ab}	1.54 ± 0.64^{a}
CRP, µg/mL	9.97 ± 6.32^{a}	26.77 ± 9.20^{b}	21.66 ± 6.84^{b}	23.61 ± 8.48^{b}
Pig-MAP, mg/mL	2.45 ± 1.12^{ab}	2.84 ± 1.20^{b}	2.27 ± 0.96^{ab}	1.82 ± 1.04
Apo A-I, ratio	2.01 ± 0.37^{c}	1.45 ± 0.23^{a}	1.68 ± 0.37^{ab}	1.71 ± 0.30^{b}

Superindex indicates significant differences between days (P< 0,05)

VALIDATION IN ALL THE INDIVIDUALS AND DAYS

Oxidative stress markers

	D1	D3	D4	D5
Carbonyl groups, ratio	1.89 ± 0.16^{a}	2.10 ± 0.21^{b}	1.87 ± 0.23^{a}	1.84 ± 0.21^{a}
GPx, U/mL	33.24 ± 10.41^{b}	35.70 ± 10.65^{b}	33.27 ± 10.12^{b}	24.01 ± 6.81^{a}
SOD, U/L	133 ± 20^{ab}	151 ± 18^{b}	125 ± 27^{a}	113 ± 35^{a}
tGSH, mmol/L	0.60 ± 0.17	0.66 ± 0.18	0.70 ± 0.10	0.58 ± 0.17

Superindex indicates significant differences between days (*P*< 0,05)

VALIDATION IN ALL THE INDIVIDUALS AND DAYS

Cortisol	D1	D3	D4	D5
Cortisol (serum) (µg/dL)	8.47 ± 4.92	7.22 ± 6.08	6.32 ± 4.79	6.49 ± 5.10
Cortisol (saliva) (ng/mL)	6.53 ± 4.69	25.01 ± 42.28	14.54 ± 12.36	12.02 ± 9.88

Cholesterol				
Cholesterol, mg/dL	101 ± 18^{a}	138 ± 46^{b}	94 ± 17^{a}	114 ± 39^{ab}

Superindex indicates significant differences between days (*P*< 0,05)

EXPOSITION TO CHALLENGING CONDITIONS LEADS TO COMPLEX CHANGES

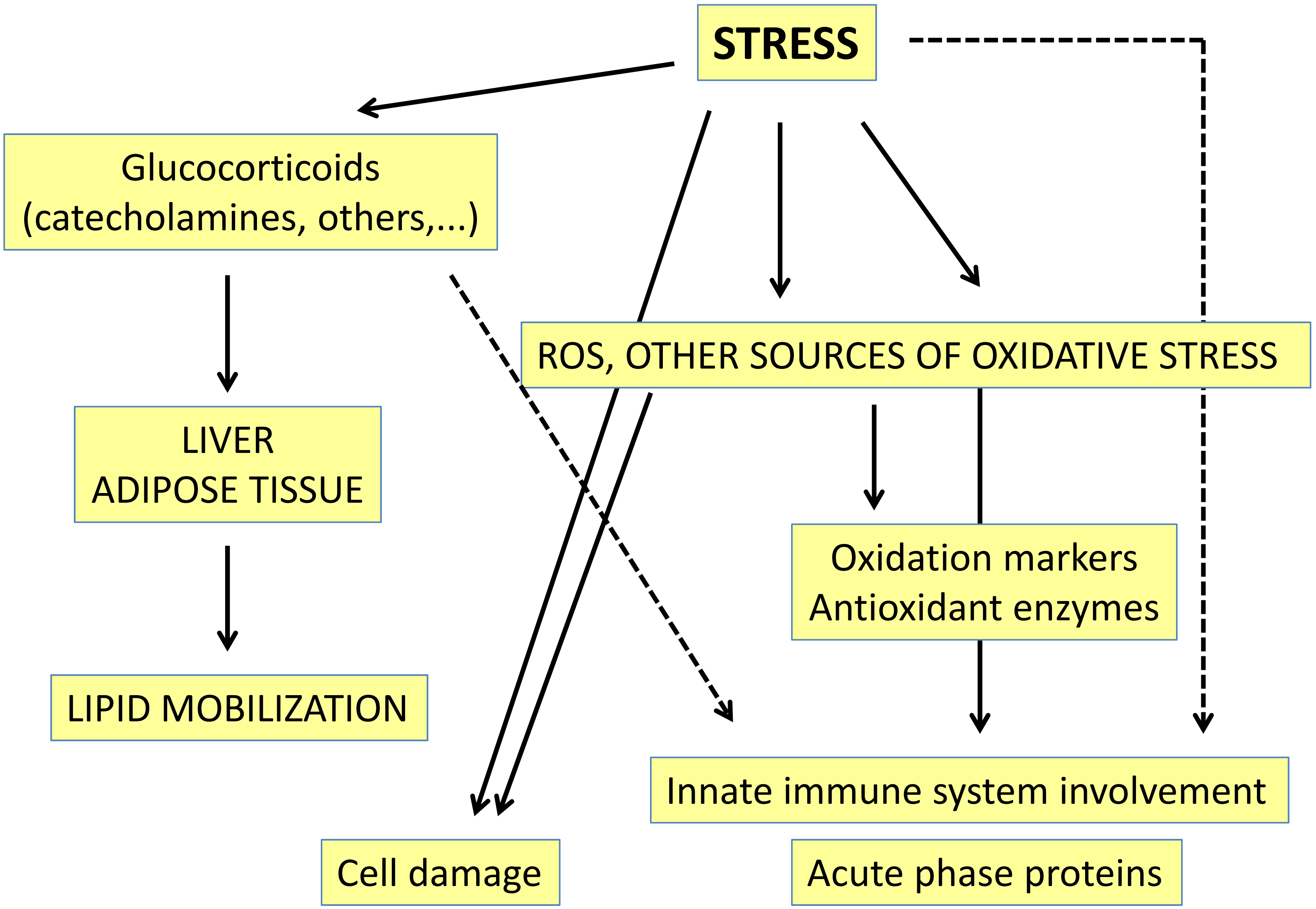
OXIDATIVE STRESS

IMMUNE SYSTEM, LOW GRADE INFLAMMATION

CORTISOL

LIPID MOBILIZATION (CHOLESTEROL)

CELL DAMAGE



STRESS

Glucocorticoids
(catecholamines, others,...)

LIVER
ADIPOSE TISSUE

LIPID MOBILIZATION

ROS, OTHER SOURCES OF OXIDATIVE STRESS

Oxidation markers
Antioxidant enzymes

Innate immune system involvement

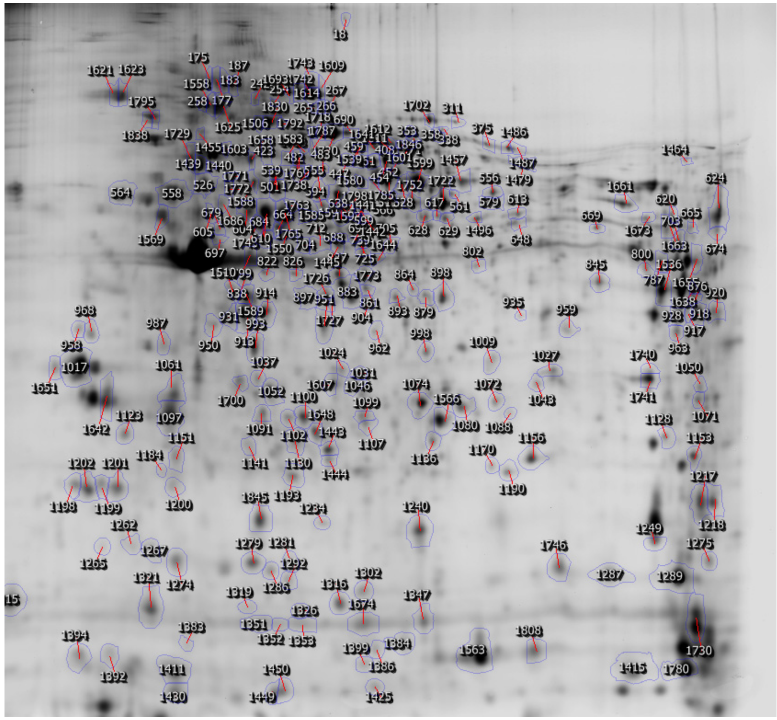
Cell damage

Acute phase proteins

65th Annual Meeting of the EAAP 2014

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

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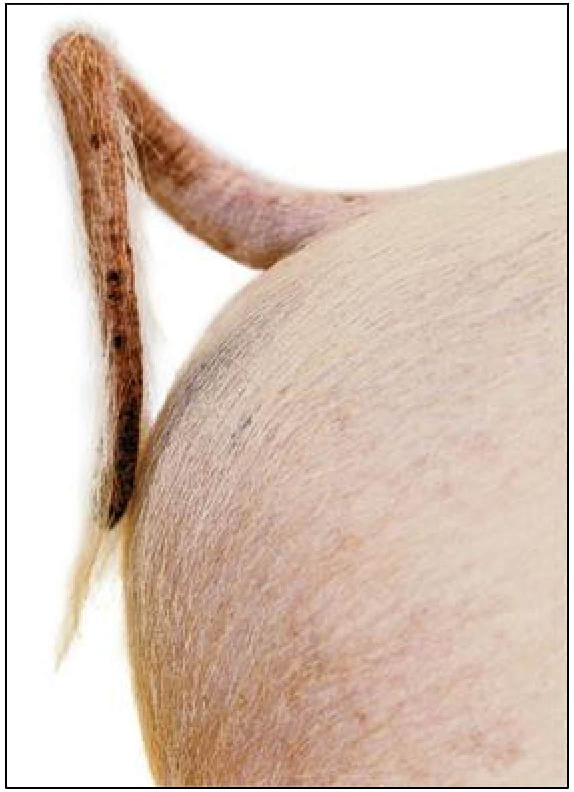
ACKNOWLEDGEMENTS



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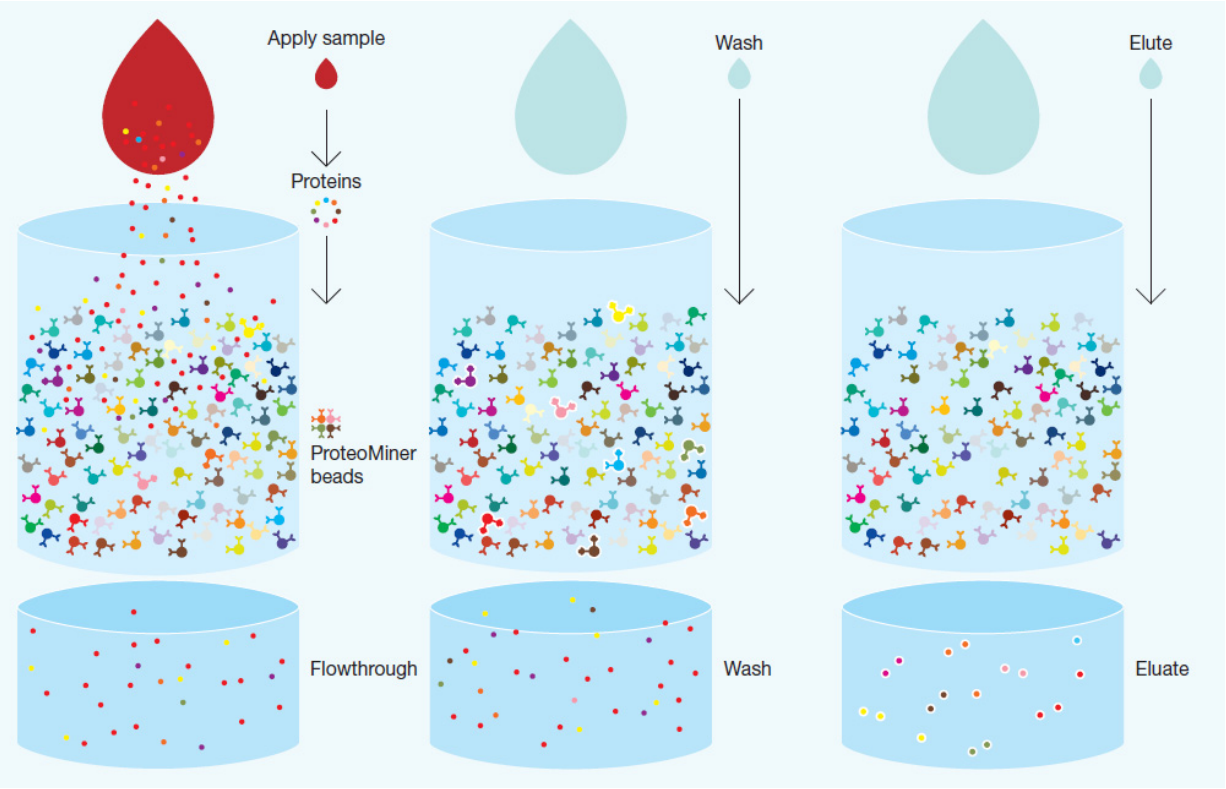
Proteomics Platform. Institut de Recerca Biomèdica. Barcelona. **Eliandre de Oliveira.**

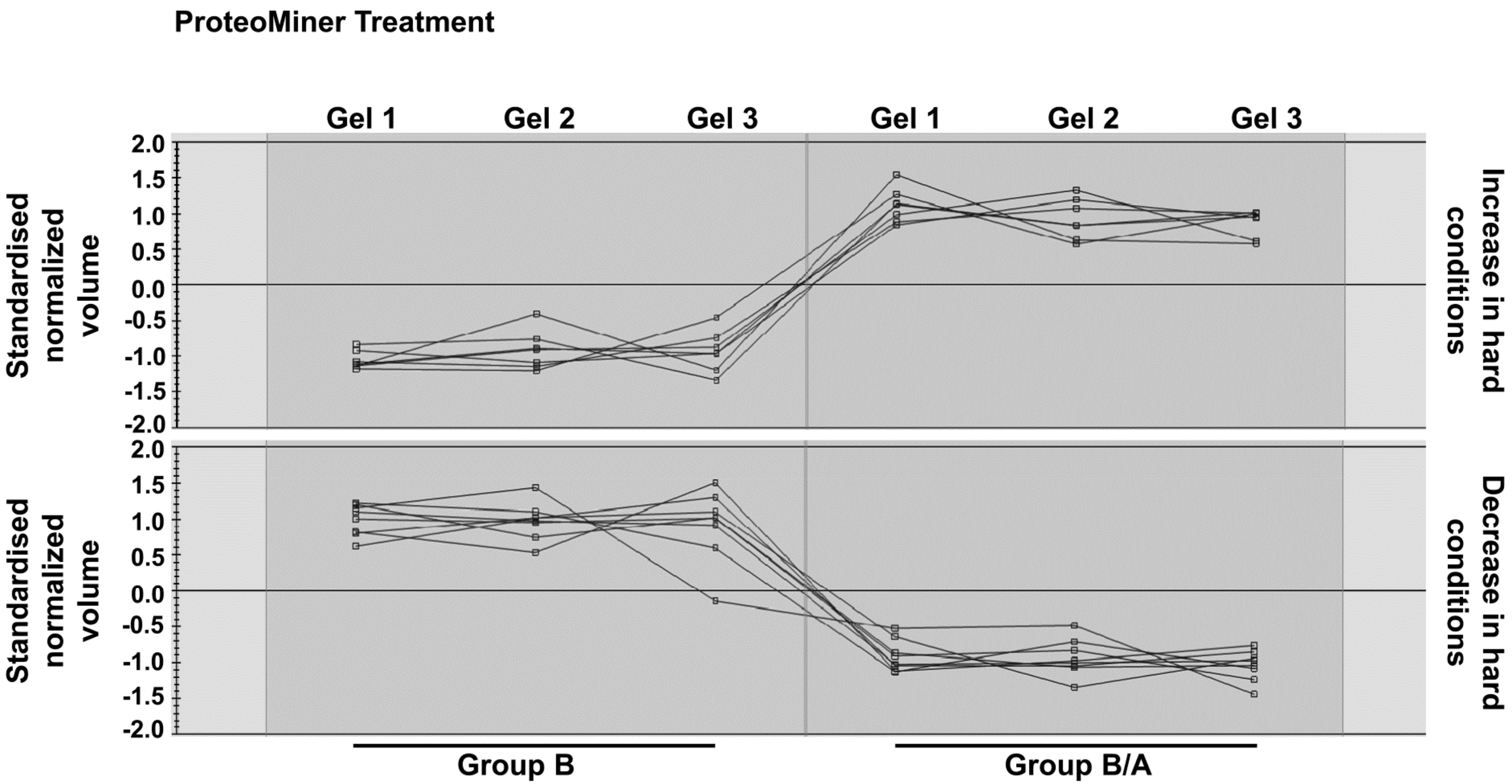
SAINT ANTHONY’S DAY. CALDES DE MONTBUI. CATALONIA.

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.



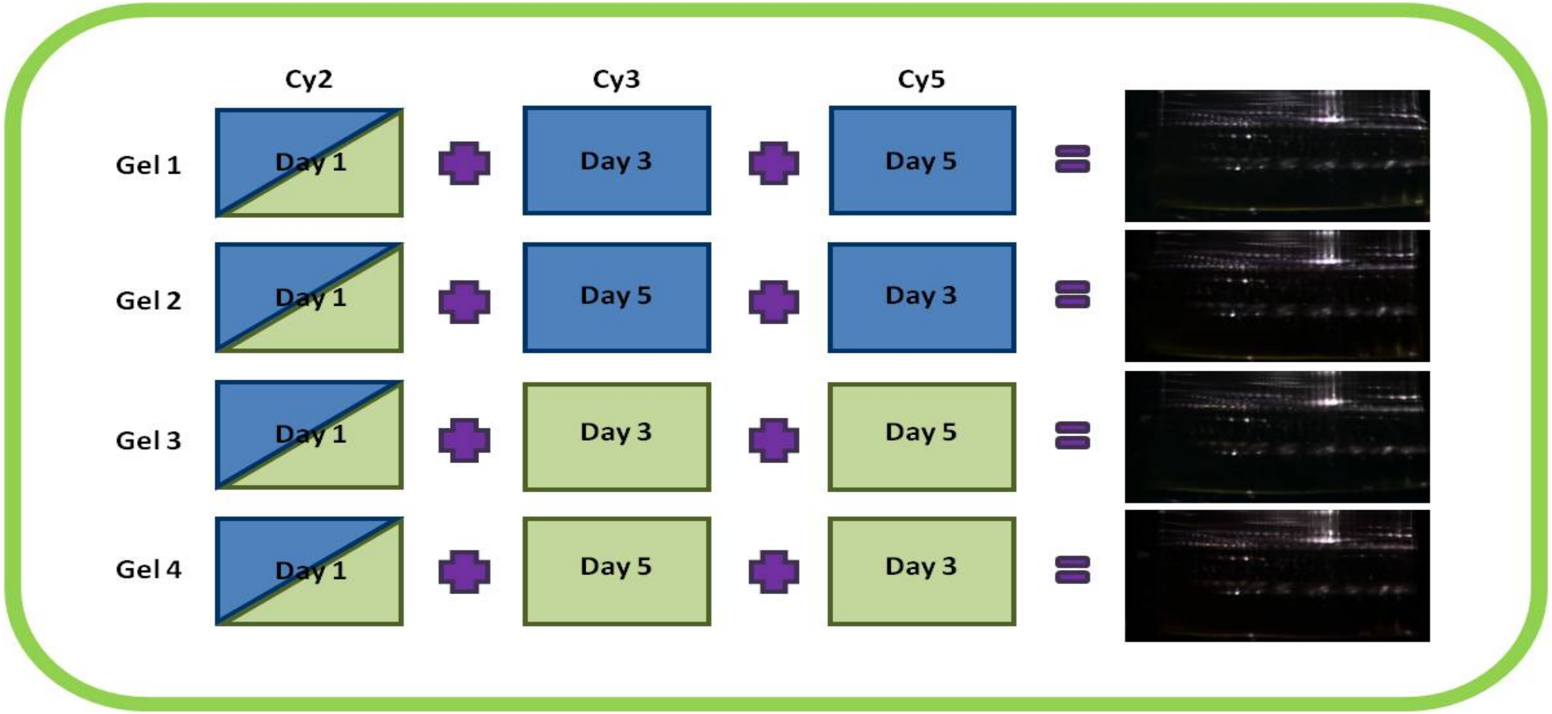


Reproducibility of the Proteominer™ treatment

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.



	Cy2	Cy3	Cy5
Gel 1	Day 1	Day 3	Day 5
Gel 2	Day 1	Day 5	Day 3
Gel 3	Day 1	Day 3	Day 5
Gel 4	Day 1	Day 5	Day 3

The goal was to find a stress biomarker associated to change in housing and, <u>not</u> 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).