Gene array and real time PCR analysis of the adrenal sensitivity to adrenocorticotropic hormone in pig

Dominique HAZARD (1), Laurence LIAUBET (2),
Magali SANCRISTOBAL (2), Pierre MORMEDE (1)

(1) INRA, UMR1286, Laboratoire PsyNuGen,
Université Victor Segalen Bordeaux 2, F-33076, Bordeaux
(2) INRA, UMR444, Laboratoire de Génétique Cellulaire,
F-31326, Castanet-Tolosan, France

European integrated project SABRE
Food Quality and Safety

Context

- Cortisol is produced by adrenal gland cortices under ACTH control; it plays an important role in numerous physiological processes (metabolisms, immune system, central nervous system).
- In porcine production, fonctional variability of HPA axis influences important traits such as growth rate, feed efficiency, carcass composition, meat quality, newborn survival.
- Genetic selection for leaner carcasses has also reduced cortisol production, the consequences of which have still to be analysed.
- Our research objective is the study of genetic mechanisms responsible for individual variation in HPA axis activity and its functional consequences.

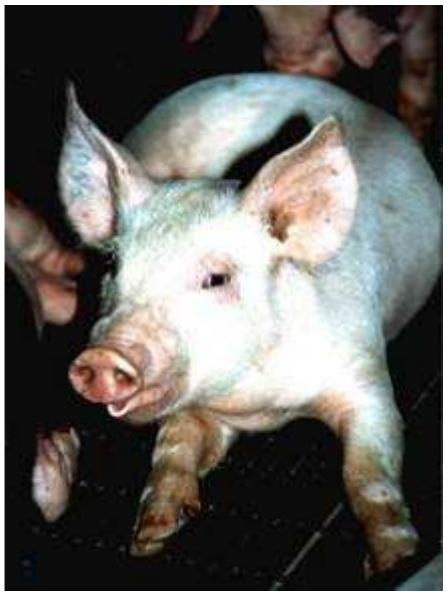
Sensitivity of the adrenal cortex to ACTH

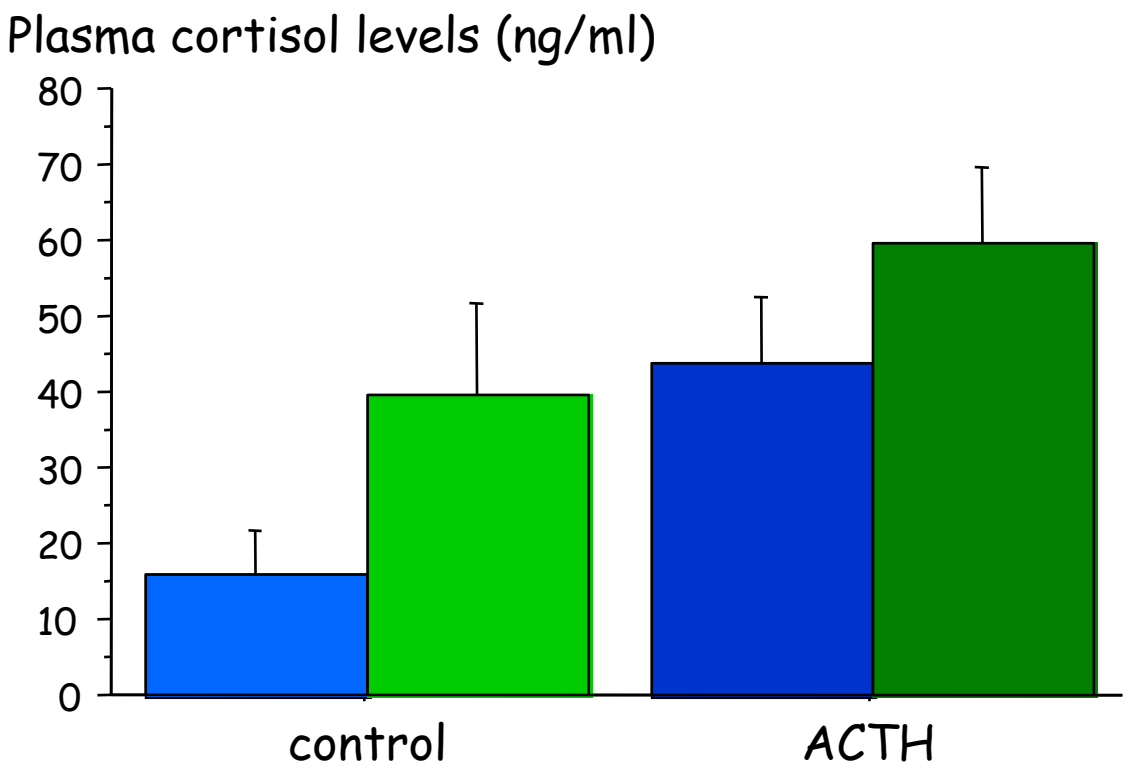
- Is the main source of genetic variation in cortisol production
- Is an individual trait
  - Pigs - Hennessy *et al.* 1988
  - Humans 'Adrenal phenotype'
    Bertagna *et al.* 1994 ; Coste *et al.* 1994
- Is heritable
  - $h^2 = 0.26$ half-sib analysis in 357 litters from 24 boars (D.P. Hennessy)
  - Divergent selection
    - In chicken, response to ACTH (Edens et Siegel, 1975)
    - In trout, confinement stress (Pottinger et Carrick, 1999)
- The objective of the present work is to search for molecular mechanisms responsible for genetic variation in adrenal cortex responses to ACTH

Experimental design

- Large White / Meishan (males, 7 weeks)
- Control / ACTH (Synacthen, 250 µg/animal) – 1 hr before slaughter

ANOVA	
Genotype	<0.001
Treatment	<0.001
Interaction	NS



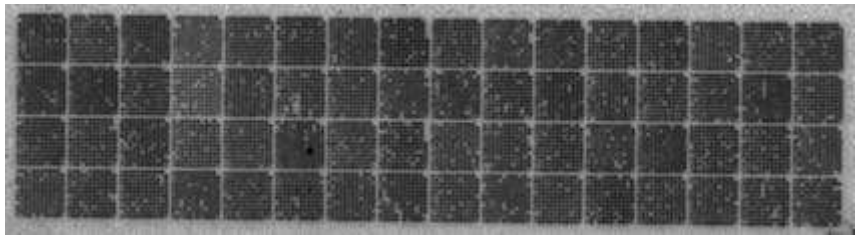


- Harvest of adrenal glands (1h), extraction of total RNA, retrotranscription and ^{33}P labelling
- Hybridization on nylon membranes (CRB GADIE, INRA) spotted with 8959 multi-tissue cDNA clones (Bonnet et al. BMC Genomics 2008, **9**:17)

Two successive hybridizations :

1- Oligonucleotidic probe

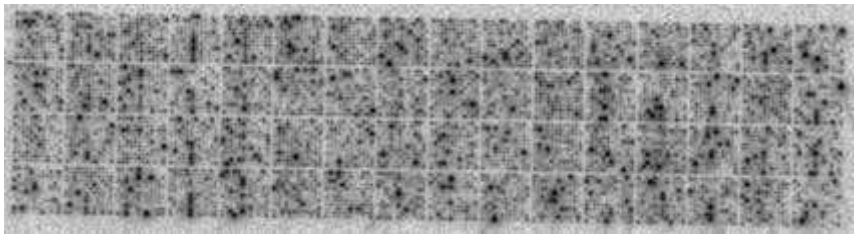
- Plasmid vector (cDNA libraries): pT7T3D (2.9 kb)
- Oligo T7 (kinase) ^{33}P labeling



➔ Measure of the amount of cDNA at each spot

2- Complex probe

- Retrotranscription of total tissue RNA
- ^{33}P labelling (reverse transcriptase)



➔ Measure of individual adrenal gene expression

Quantification of hybridization signal

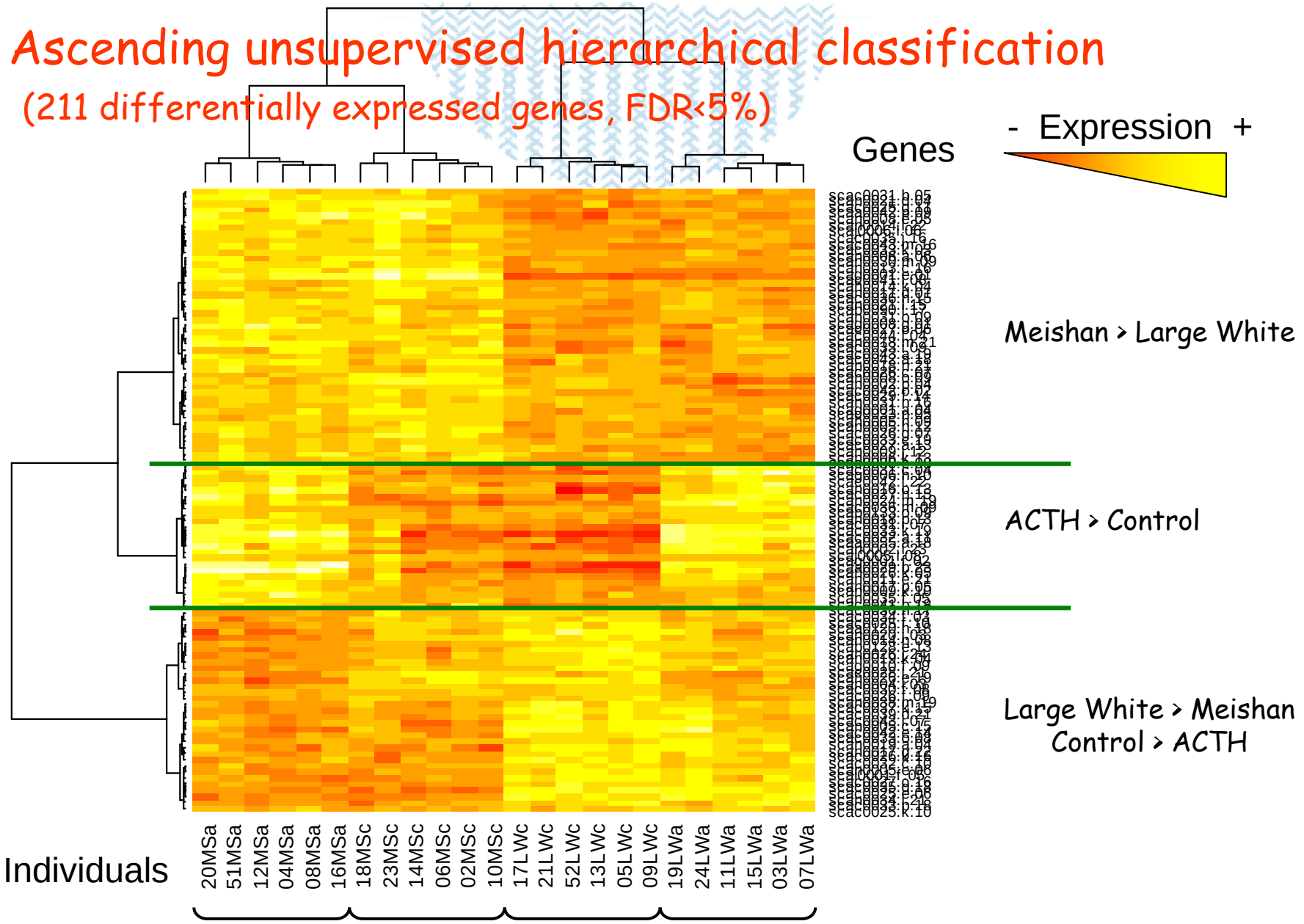
- Exposition of membranes to radiosensitive screens
- Detection with phosphoimager (resolution < 25 µm)
- Quantification by AGScan software (SIGENAE, INRA)
  (Cathelin R. *et al.* Bioinformatics 2007 **23**:247)

Data analysis

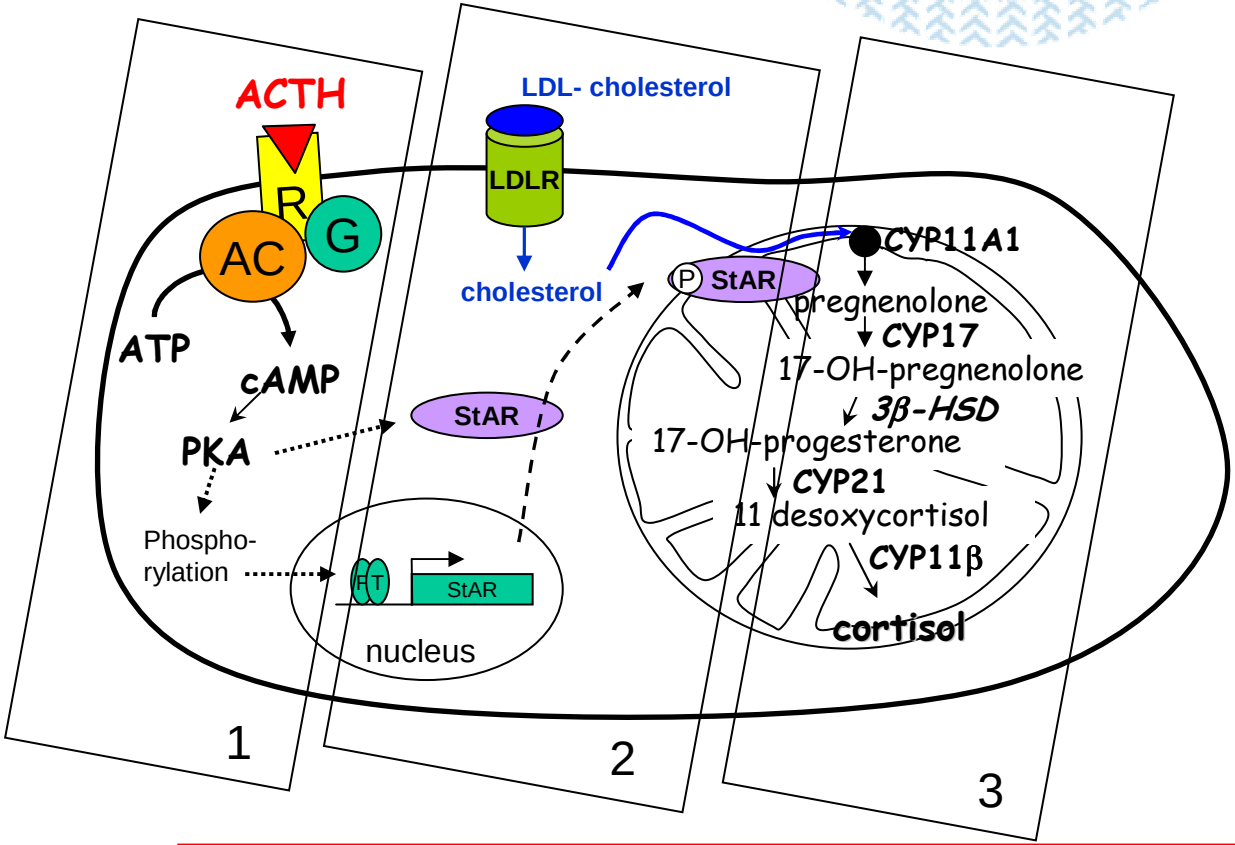
- Data transformation into logarithmic scores and normalization
- Filtering to keep only transcripts with a signal more intense than background level (3686 out of 8959 clones ~40%)
- Analysis with a linear model (R software)
  y= Xoligo + genotype + treatment + genotype X treatment
  (Xoligo = hybridization signal with a vector probe to normalize for the amount of cDNA)
- Correction for multiple comparisons by Benjamini-Hochberg procedure
  - FDR 5% : 241 transcripts (211 genes)
  - FDR 2.5% : 161 transcripts
  - FDR 1% : 102 transcripts

Ascending unsupervised hierarchical classification

(211 differentially expressed genes, FDR<5%)



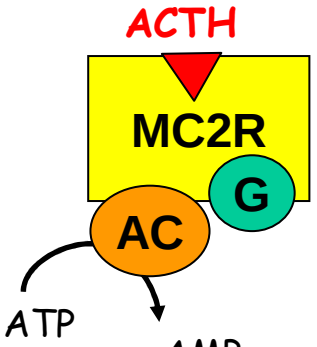
Functional analysis
Mecanisms involved in the response of adrenal cortex to ACTH



ACTH	adrenocorticotropic hormone
R	ACTH receptor (MC2R)
G	G protein
AC	adenylyl cyclase
PKA	protein kinase A
StAR	steroidogenic acute regulatory protein
LDL	low density lipoprotein
LDLR	LDL receptor
CYP11a1	cholesterol side chain cleavage (P450scc)
CYP17	17α-hydroxylase
3β-HSD	3 beta-hydroxysteroid dehydrogenase
CYP21	21 hydroxylase
CYP11β	11β- hydroxylase

1- ACTH receptor signalisation pathway
2- Synthesis and mobilisation of steroid precursor, cholesterol
3- Steroidogenesis

1- ACTH receptor signalisation pathway



PKA **ERK1, ERK2** (MAPK)

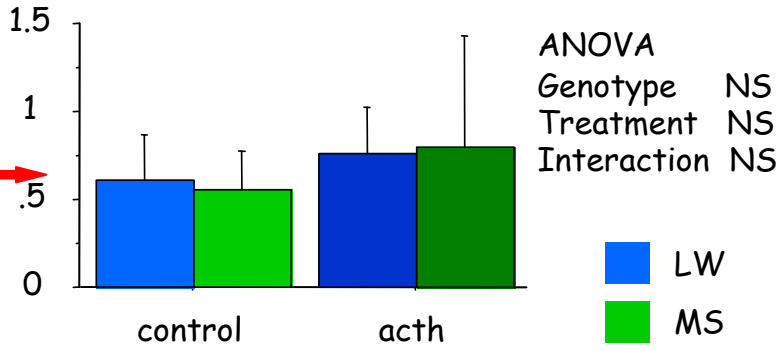
Phosphorylation

Activation of proteins
(StAR, Cholesteryl ester hydrolase…)

↗ Expression of genes involved in cortisol production

	arrays		
Gene	Present	Expressed	Differential
Mc2r	NO		
Gnas (G)	YES	YES	NO
Adcy7 (AC)	YES	YES	NO
Prkaca (PKA)	YES	YES	NO
Mapk1	YES	YES	NO

real-time PCR



ACTH	adrenocorticotropic hormone
MC2R	melanocortin receptor 2 (ACTH-R)
GNAS	guanine nucleotide binding protein (G-protein) alpha subunit
ADCY7	adenylyl cyclase
PKA	protein kinase A
MAPK ERK1/2	mitogen-activated protein kinase

➔ No difference in expression of the main actors of ACTH signalisation pathway

3- Steroidogenesis

Cholesterol

Cholesterol side chain cleavage P450scc — **Cyp11a1**

pregnenolone

3 βHSD — 17α-hydroxylase — **Cyp17**

progesterone — 17-OH-pregnenolone

3β-hydroxysteroid dehydrogenase — **Hsd3b4**

17-OH-progesterone

21-hydroxylase — **Cyp21**

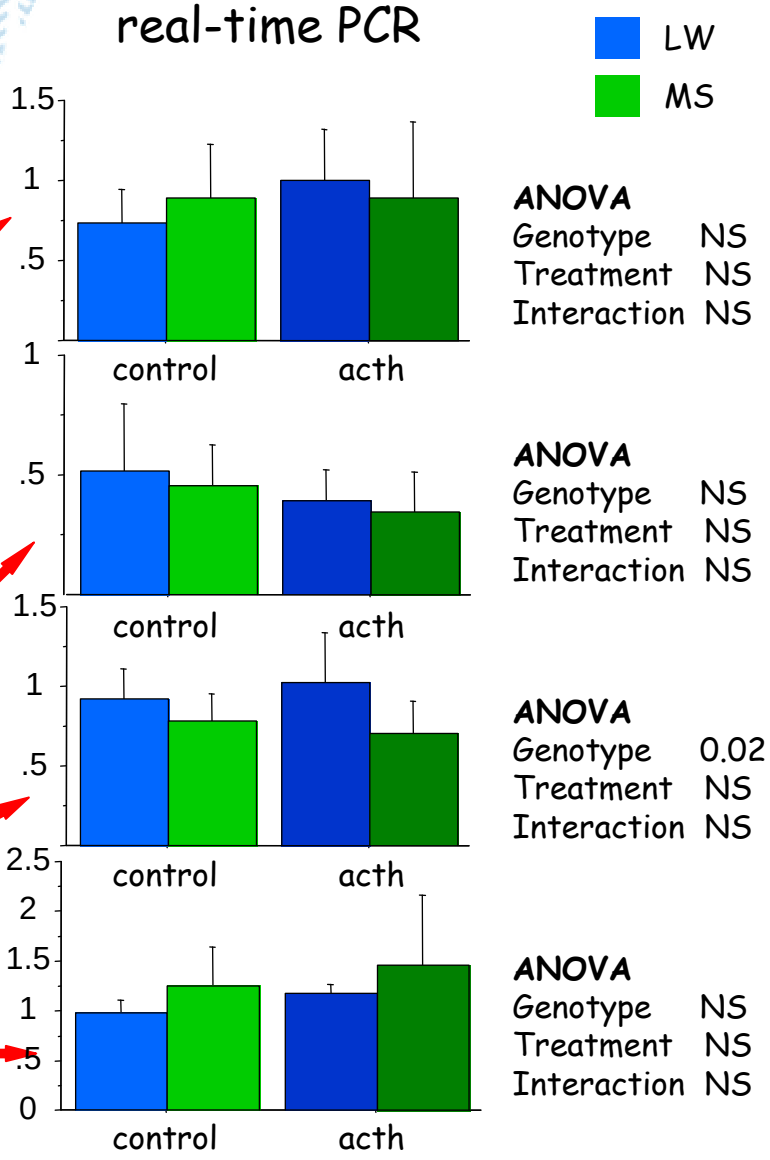
11 desoxycortisol

11β-hydroxylase — **Cyp11β**

cortisol

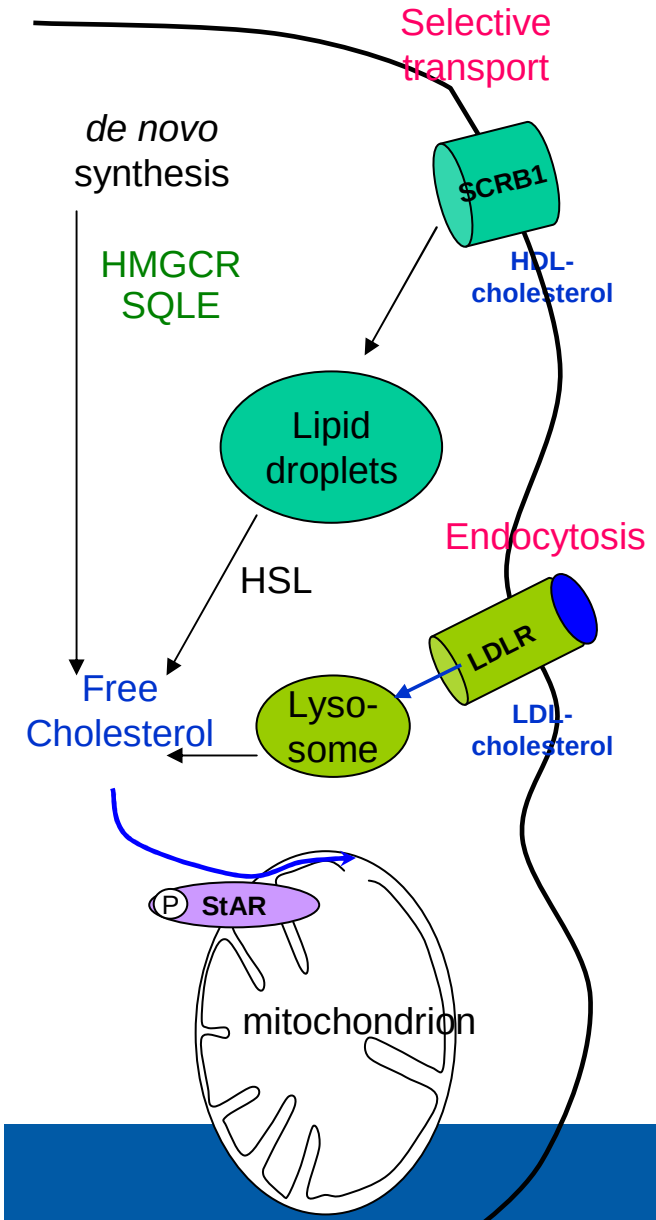
arrays		
Present	Expressed	Differential
YES	YES	NO
YES	YES	NO
YES	YES	NO
YES	YES	NO
YES	YES	NO

real-time PCR



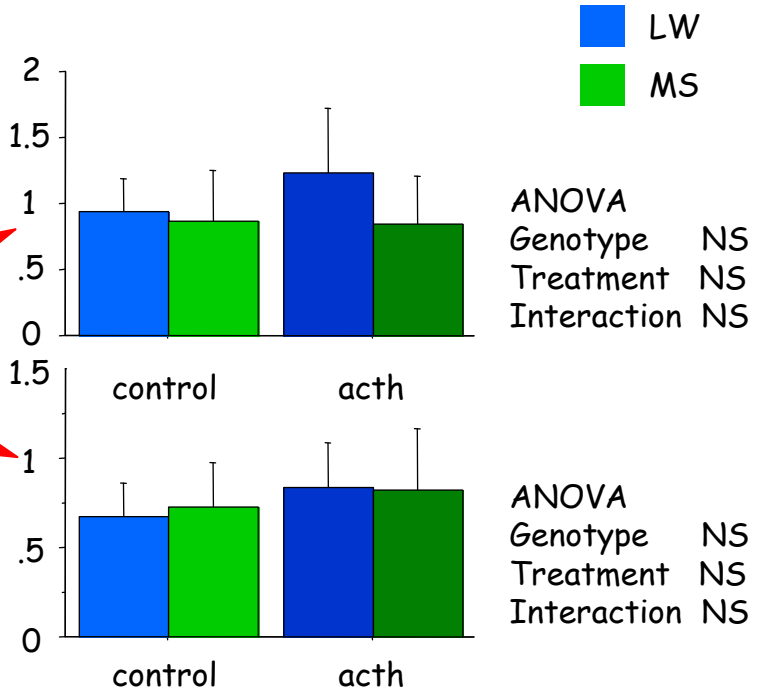
➔ No difference in expression of steroidogenic enzymes

2- Synthesis and mobilisation of steroid precursor, cholesterol (1)



	arrays		
Gene	Present	Expressed	Differential
Hmgcr	YES	YES	NO
Sqle	YES	YES	NO
Scrb1	YES	YES	NO
Hsl	NO		
Ldlr	YES	YES	YES
Star	YES	YES	YES

real-time PCR

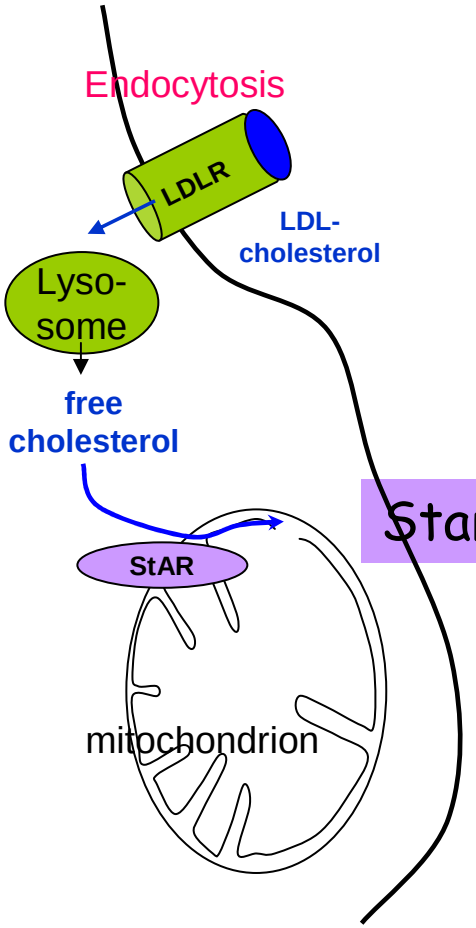


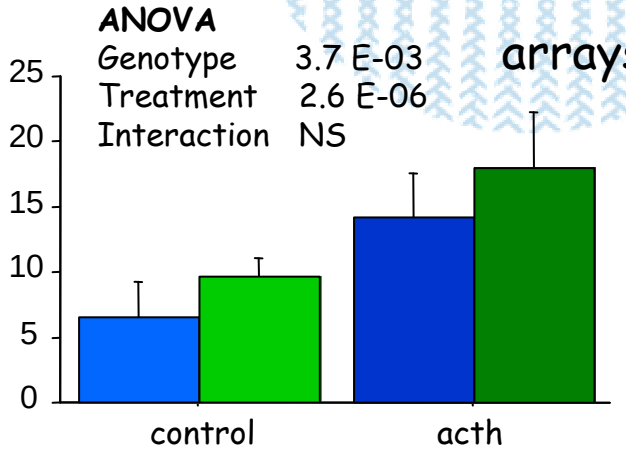
HMGCR hydroxymethylglutaryl coenzymeA reductase

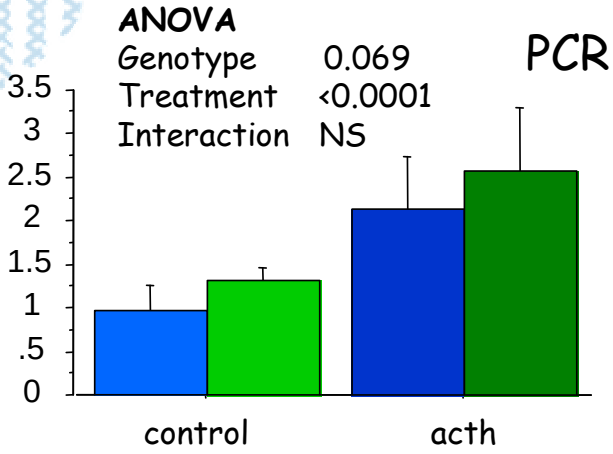
➔ No difference in expression of genes involved
- in cholesterol synthesis
- in selective transport of cholesterol via SCRB1

2- Synthesis and mobilisation of steroid precursor, cholesterol (2)

Ldlr

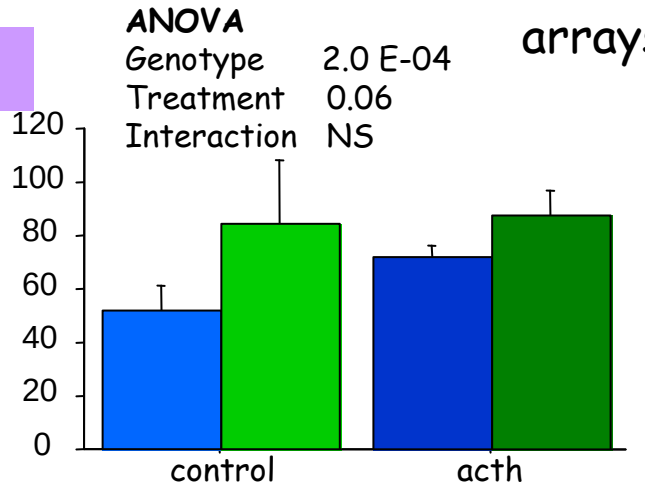


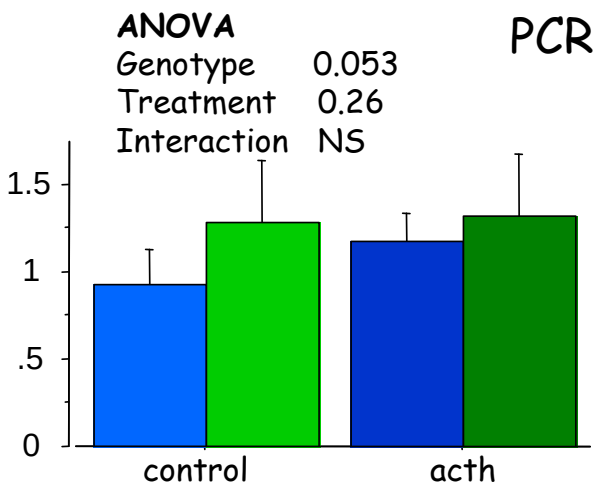




➔ **Ldlr** - **overexpressed in Meishan** (x 1.3 to 1.6)
- **induced by ACTH** (x 1.9 to 2.3)

Star





➔ **Star** - **overexpressed in Meishan** (x 1.4 to 1.6)
- **no response to ACTH**

Conclusion

- Comparison of gene expression in the adrenal glands of Meishan (high cortisol production) and Large White (low production) pigs in response to ACTH shows that the expression of numerous genes differ according to breed and/or treatment.
- Several of these genes are involved in cortisol synthesis
  - No difference in the ACTH signalling pathway and in steroidogenic enzymes
  - Cholesterol transport (*Star, Ldlr*) and several kinases involved in phosphorylation of StAR (*Snf1lk*)
  - Tricarboxylic acid cycle (*Mdh2, Sdha and Suclg2*) involved in heme biosyntheis (*Alas1*)
- Still to be characterized transcripts / genes
- Current studies aim at finding molecular polymorphisms underlying these genetic differences. Further genetic selection will allow the study of functional consequences of different adrenal sensitivity to ACTH and cortisol production.

Hazard D. *et al.* BMC Genomics **9**:101 (2008)

Acknowledgements

All members of the team « Genetic variability in HPA axis activity: molecular mechanisms and functional consequences » (INRA, UMR PsyNuGen, Bordeaux)

Members of the « Experimental Genetics in Animal Production» INRA Le Magneraud (Surgères)

Members of the « Laboratory for Cellular Genetics » INRA Castanet-Tolosan

Members of the « Genopole Toulouse Midi-Pyrénées » (transcriptome)

Members of the group « SIGENAE » (bio-informatic tools and support)