Identification and functional characterization of novel antimicrobial proteins using high-throughput, biochemical and microbial approaches

Joël Gautron, Sophie Réhault-Godbert, Nicolas Guyot, Larbi Bedrani, Yves Y. Nys

Identification and functional characterization of novel antimicrobial proteins using high-throughput, biochemical and microbial approaches

Joël GAUTRON (joel.gautron@tours.inra.fr)
« Function and regulation of egg proteins »

S. Réhault-Godbert, N. Guyot, L. Bedrani, Y. Nys
INRA
UR83 Recherches Avicoles
37380 Nouzilly
FRANCE

The Chicken Egg

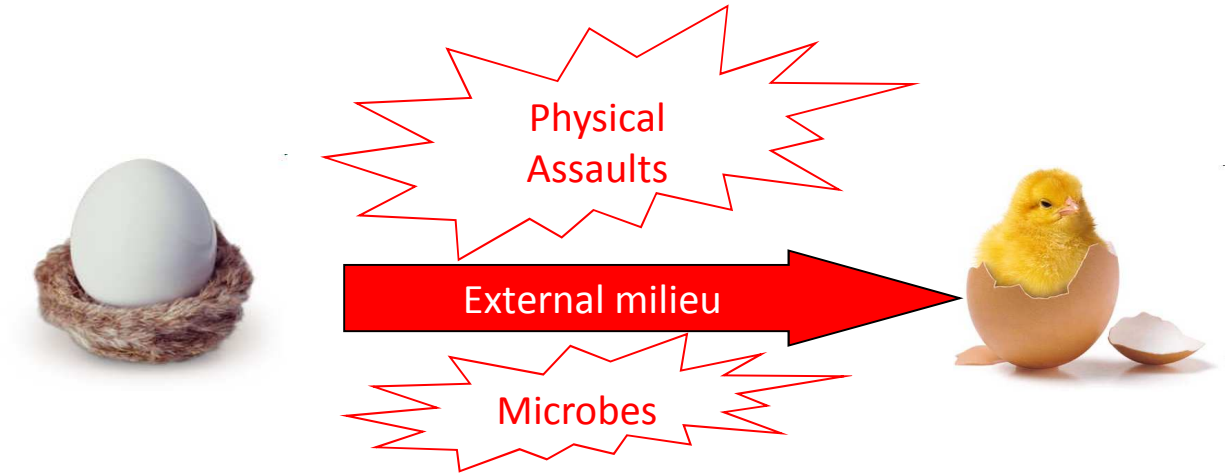
Container for extra-uterine development of the embryo

Must contains the entire components essential for the embryo development

→ **Almost perfect nutritional value**

Basic food for humans all around the world

→ **Major source of compounds with a broad range of biological activities**



→**Protective systems (Egg natural defenses)**

- **Physical (eggshell as a barrier)**
- **Chemical (molecular defense of the egg)**

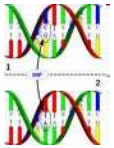
. Antimicrobial proteins

Team objectives

Identification and characterisation of molecular actors (proteins) involved in egg natural defences

Improve egg defences

Genetic variability
(Markers assisted selection)



Variability induced by egg storage, environment, housing systems and embryonic development

I. Identification of egg proteins (global approach)

→ The classical techniques

Fractionation of egg components
- Salt precipitation
- Chromatography
- Electrophoresis
- Molecular biology

→ Identification of the major components
(about 50 egg proteins were identified in 2006)

→ The recent developments

- 2004, Publication of the chicken genome sequence (*Gallus gallus*)
- cDNA and ESTs libraries (Identification of 630 000 functional genes in chickens)
- Availability of « omics » high-throughput techniques and data mining

→ 2012, several hundred egg proteins are described

↓

Functions ?

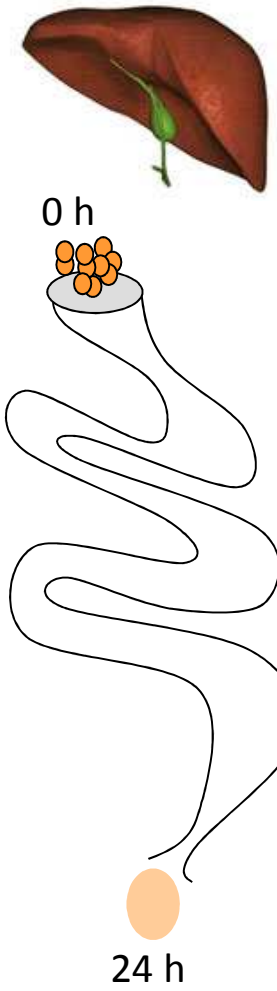
↓

Potential for new egg components with bioactive properties, high potential for industry or/and involved in natural egg defenses

I. Identification of egg proteins (global approach)

Egg formation

Spatial and temporal regulation



Liver:
Egg yolk proteins (several weeks)

Ovary and infundibulum:
Vitelline membranes (less 1 h)

Magnum:
egg white proteins (1 to 4 h 30)

Isthmus:
eggshell membranes (4h30 to 6 h)

Uterus:
eggshell calcification (6 to 24 h)

Different tissues or organs
Egg components are deposited at different times
Different physiological stages

↓

Comparison of gene expression in the various segment of the reproductive tract

↓

Use of cDNA microarrays to compare global gene expression between tissues

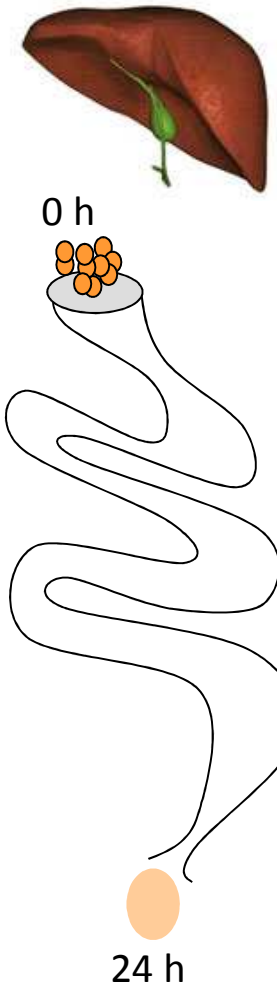
↓

Identification of genes specifically related to the egg yolk, the vitelline membranes, the egg white, the eggshell membranes and the eggshell calcification process

I. Identification of egg proteins (global approach)

Egg formation

Spatial and temporal regulation



Liver:
Egg yolk proteins (several weeks)

Ovary and infundibulum:
Vitelline membranes (less 1 h)

Magnum:
egg white proteins (1 to 4 h 30)

Isthmus:
eggshell membranes (4h30 to 6 h)

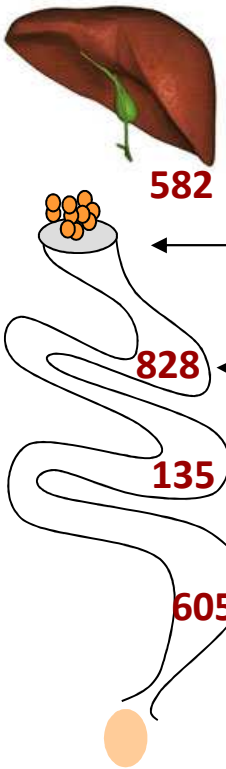
Uterus:
eggshell calcification (6 to 24 h)

Chemical defense

Physical defense

I. Identification of egg proteins (global approach)

→ transcriptomic approach



Liver :
Egg yolk proteins

Ovary and infundibulum :
Vitelline membranes

Magnum :
Egg white proteins

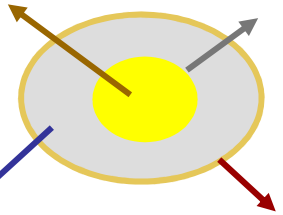
Isthmus :
Eggshell Membrane

Uterus :
Shell calcification

→ Proteomic approach

316 proteins in yolk

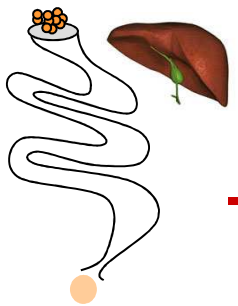
137 proteins in vitelline membranes



148 proteins in egg white

528 proteins in shell

I. Identification of egg proteins (global approach)

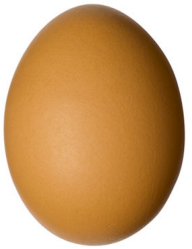


Transcriptomics

Liver
Infundibulum
Magnum
Isthmus
Uterus

Proteomics

Egg proteomes data mining
yolk
Vitelline membranes
White
Shell



Bioinformatics
Biostatistics

Bioinformatic analysis of egg protein sequences
(Mammal homologs, conserved domains, Molecular networks)

- ✓ *Integrative analysis of proteomics and transcriptomics data*
- ✓ *Integrative analysis of egg literature*
- ✓ *Use of databases, bioinformatic and text-mining tools*

→ **Integrated analysis of biological activities of egg components**

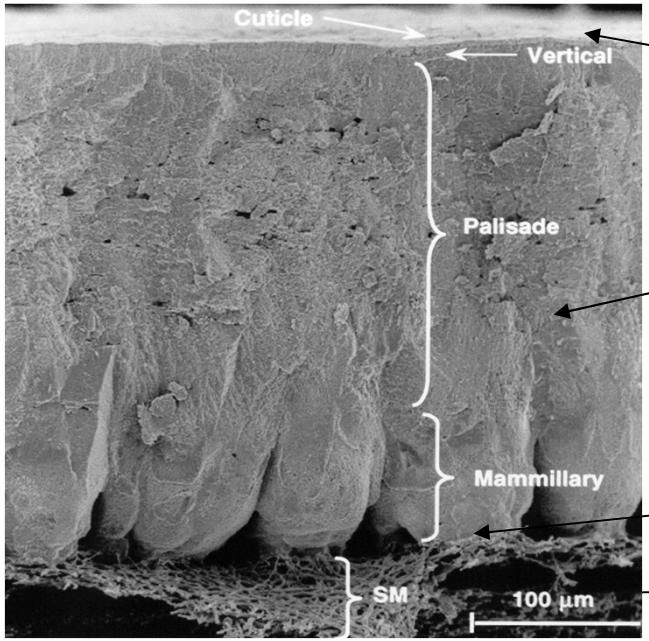
II. Characterization of proteins involved in the egg antimicrobial protection

II.1. Physical defense (eggshell biomineralization)

→ **Eggshell biomineralization in uterus (one of the fastest on earth)**

→ **Into the uterine fluid with the appropriate physico-chemical conditions**

Hypersaturation of calcium and bicarbonates

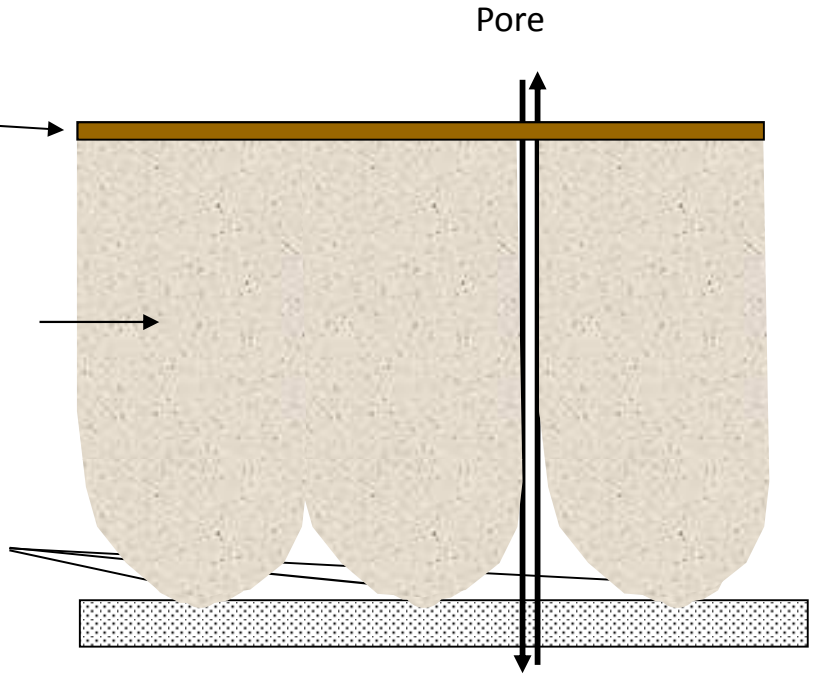


Cuticle

Palissadic layer

Mammilary knobs

Eggshell membranes

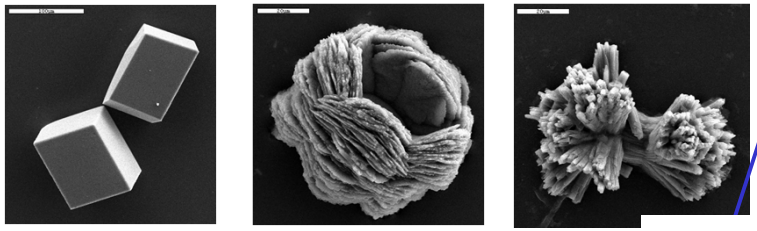


II. Characterization of proteins involved in the egg antimicrobial protection

II.1. Physical defense (eggshell biomineralization)

→ **95% of calcium carbonate on calcitic polymorph**

Calcite Aragonite Vaterite



Quantity

Interaction

Mechanical properties
- about 300 µm
-eggshell breaking strength (about4 kg)

Control of the calcification process

→ **3,5% of organic matter (organic matrix)**
Proteins and proteoglycans

II. Characterization of proteins involved in the egg antimicrobial protection

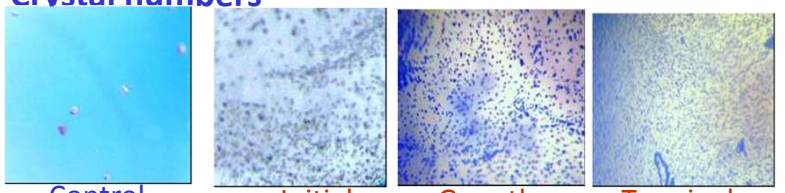
1. Physical defense (eggshell biomineralization)

Kinetic of crystal nucleation

Time to observe first crystals

Control	900 minutes
Uterine fluid	< 10 secondes

Crystal numbers



Control: 40 crystals

Initial

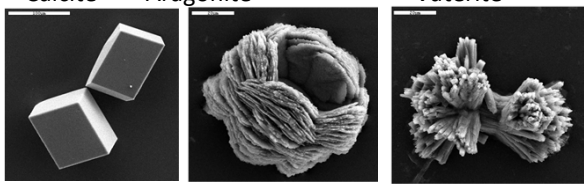
Growth

Terminal

Uterine fluid >10 000

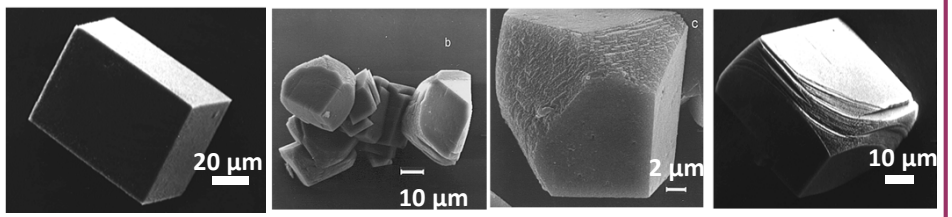
Effect of uterine fluid on in vitro calcite crystal growth

Calcite crystal polymorphs



		Calcite	Aragonite	Vaterite
Control	(%)	55	22.5	22.5
Uterine fluid	(%)	100	0	0

Calcite crystal morphologies



Control — Initial — Growth — Terminal

→ **Identification and characterization of organic matrix proteins**

II. Characterization of proteins involved in the egg antimicrobial protection

1. Physical defense (eggshell biomineralization)

→ Identification and characterization of organic matrix proteins

Classical techniques

→ 6 novel eggshell specific proteins
(ovocalyxins, ovocleidins)

(Gautron, Hincke, Nys et al., 1995, 1997, 2001, 2007, 2010, 2011)

Eggshell proteome

→ 528 different proteins

(Mann et al., 2006-2007)

Uterine transcriptome

→ 469 genes encoding 437 different proteins

(Jonchere, Gautron, Nys et al., 2010)

- Supply of minerals for shell mineralization
- Involved in mineralization
- Interactive with other proteins (chaperone)
- Antimicrobial proteins
- Proteases-antiproteases

↓

Molecular markers for selection assisted markers (SAM)

II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → <u>All compartments</u>

ANTIMICROBIAL COMPONENTS

- Molecules degrading microbial components
- Molecules decreasing bioavailability of iron and vitamins
- Molecules inhibiting the activity of microbial proteases
- Immunoglobulin superfamily

II. Characterization of proteins involved in the egg antimicrobial protection

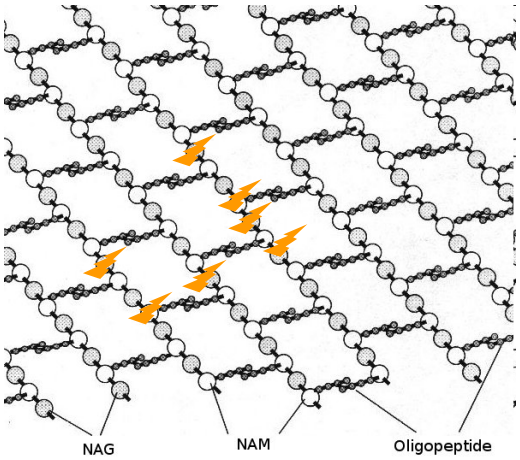
Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (1/10)

Hydrolases

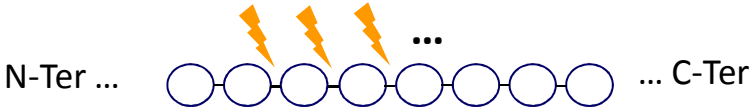
Lysozyme (all compartments)

Hydrolyze bacterial peptidoglycane



Similar to acyloxylhydrolase (Egg white)

Proteases



Several proteases in eggs

Antimicrobial activities already demonstrated in other species. No experimental evidences for chickens

*Direct action (*Degradation of antimicrobial proteins)

Indirect action
(Activation of antimicrobial precursors, production of antimicrobial peptides)

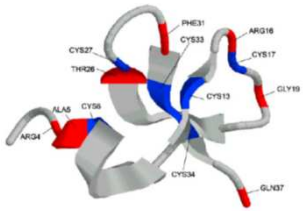
II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (2/10)

Avian beta-défensins

Cationic peptides
Broad spectrum of antimicrobial activity



Histones

Basic proteins from nucleosomes
Agent of innate host defense (Gram + and Gram – strains)

Protein name	Localization
Beta-defensins	
AvBD-11	EW, VM, ES
AvBD-10	ES
Gallin	EW
Cygnin, similar to meleagrin	EW
meleagrin	EW
AvBD-9	EY, Ut
Histones proteins	
Histone H2A.Z	EW, EY, ES
Similar to Histone protein	EW
Histone H1	EW
Histone H2A-III	EW, EY, ES
Histone H2A	EW, EY, ES
Histone H4	EY, ES
Histone H2A-VIII	ES
Histone H2A-IV	EW, EY, ES
Histone H2A.J	EY, ES
Histone H2A.V	EY, ES
Histone H2B 1/2/3/4/6	EY
Histone H2B 5	EY
Histone H2B 8	EY
Histone H4 type VIII	EY, ES

EY: Egg yolk, VM: Vitelline membrane, EW: Egg white, ES: Eggshell, Ut: Uterus

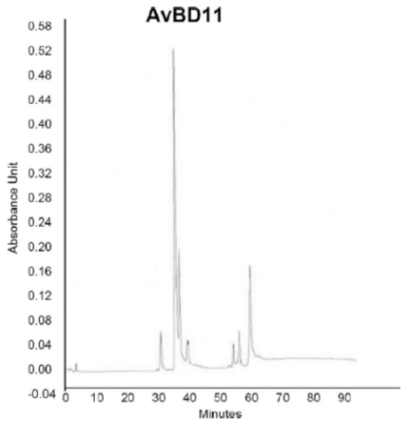
II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (3/10)

Characterization of AvBD-11 (Hervé-Grépinet et al., 2010)

→ Development of scheme of purification, identification using mass spectrometry



→ Antimicrobial tests (Lehrer)

Bacterial group, species	MIC[a] (μM) (95% confidence interval)	
	MSI-94[b]	AvBD11
Gram positive		
S. aureus ATCC 29740	0.33 (0.19–0.48)	0.90 (0.27–1.7)
L. monocytogenes	0.28 (0.13–0.43)	0.18 (0.08–0.27)
Gram negative		
S. Enteritidis ATCC 13076	0.31 (0.25–0.35)	0.35 (0.27–0.46)
S. Enteritidis LA5	0.15 (0.10–0.21)	0.40 (0.29–0.49)
S. Typhimurium ATCC 14028	0.25 (0.11–0.40)	0.32 (0.31–0.32)
E. coli ATCC 25922	0.37 (0.23–0.52)	0.05 (0.04–0.05)

II. Characterization of proteins involved in the egg antimicrobial protection

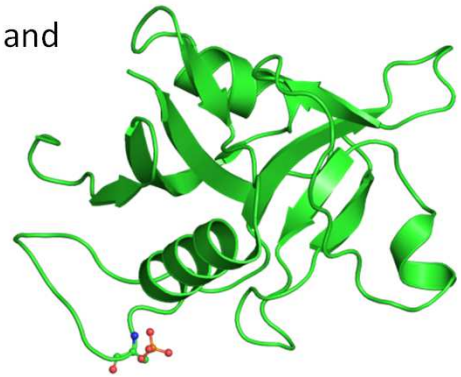
Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (4/10)

C-type lectin-like proteins

Major components of the calcified eggshell of multiple avian species
C-type lectin domain : 110 to 130 residues including four cysteines involved in two disulfide bonds

→ Ovocleidin 17 (eggshell, eggwhite, vitelline membrane)
Bactericidal activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* *(Wellman-Labadie et al. Febs lett, 2008)*



→ Other candidates
Tetranectin (egg yolk)
Collagen XVIII (eggshell)
DEC-205 protein (eggshell)
Mannose binding protein (eggshell, uterus)

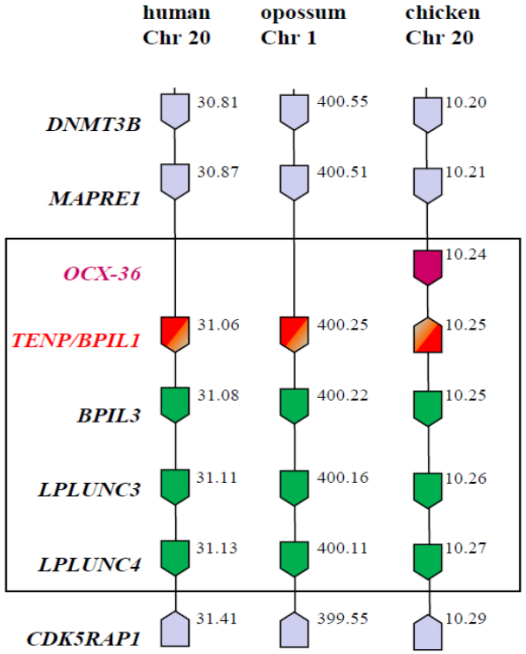
II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → All compartments

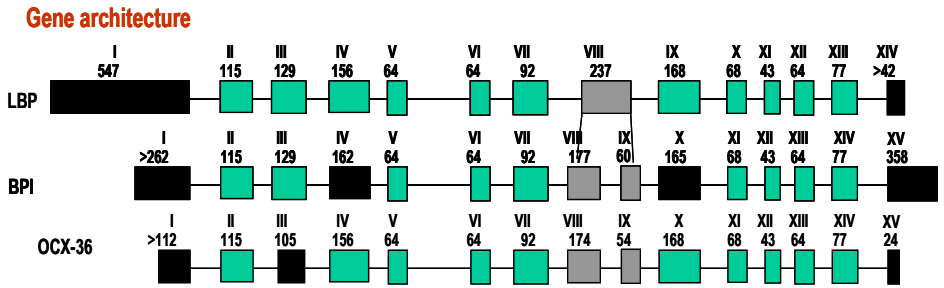
ANTIMICROBIAL COMPONENTS (5/10)

Lipopolysaccharide Binding (LBP) and Bactericidal Permeability Increasing (BPI) proteins

Key components of the innate immune system (permeabilization of LPS, initiation of inflammation upon infection etc.), organized in two loci with the same gene architecture



(Tian et al., Biol Reprod, 2010)



(Gautron et al., JBC, 2007)

II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (6/10)

Lipopolysaccharide Binding (LBP) and Bactericidal Permeability Increasing (BPI) proteins

Key components of the innate immune system (permeabilization of LPS, initiation of inflammation upon infection etc.), organized in two loci with the same gene architecture

→ Ovocalyxin-36 (eggshell and vitelline membranes)

Identity and similarity with LBP/BPI and Plunc families proteins
Binds to the lipolysaccharide (LPS) cell wall of the gram negative bacteria (death of bacteria)
Early recognition of bacterial product
(Gautron et al., JBC, 2007)

OCX-36 binds to E. Coli LPS
Modestly inhibit the bacterial growth of Bacillus subtilis, Staphylococcus aureus, E. Coli, Pseudomonas aeruginosa
(Correiro, Hincke et al., 2010)

→Other candidates

TENP (all compartments)
BPIL2 (Egg White, Vitelline membrane)
Similar to BPI (Egg white)

II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → All compartments

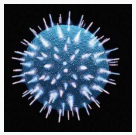
ANTIMICROBIAL COMPONENTS (7/10)

Molecules decreasing bioavailability of irons and vitamins

→Diminish bioavailability for microbes
→Affect growth and survival of bacteria

Name	Localization	Antimicrobial activity
Ovotransferrin	ES, EW, MV, EY	+
RBP	ES, EW, MV, EY	+
Avidin	ES, EW, MV	+
Similar to avidin	EY	?
Vitellogenin 1	EY	?
Vitellogenin 2	EY	?

Protease inhibitors



Proteases
= Virulence factors

Inactivation/hydrolysis of host proteins

- Highly represented in egg (>40)
- Unknown function

II. Characterization of proteins involved in the egg antimicrobial protection

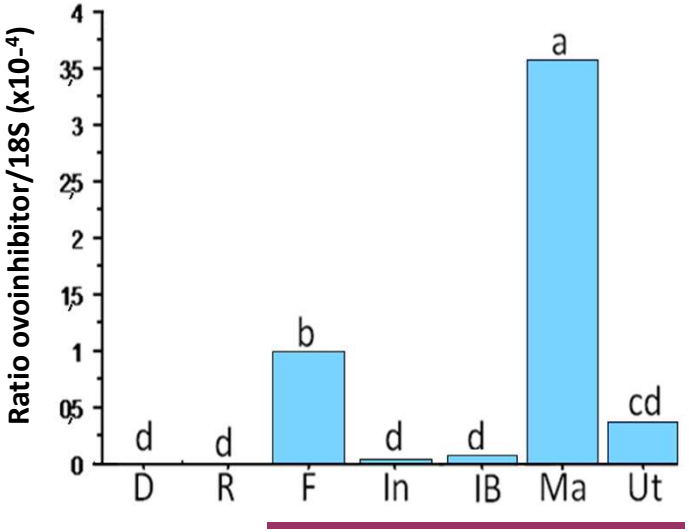
Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (8/10)

Protease inhibitors

Characterization of Ovoinhibitor

Tissue distribution



Tissues involved in egg formation

Antimicrobial activity

Tested Bacteria : strains secreting proteases

Gram + bacteria :

- ✓*Staphylococcus aureus*

Gram - bacteria :

- ✓*Pseudomonas aeruginosa*
- ✓*Bacillus cereus*
- ✓*Bacillus subtilis*
- ✓*Bacillus thuringiensis*

→ **Activity against *Bacillus thuringiensis***

II. Characterization of proteins involved in the egg antimicrobial protection

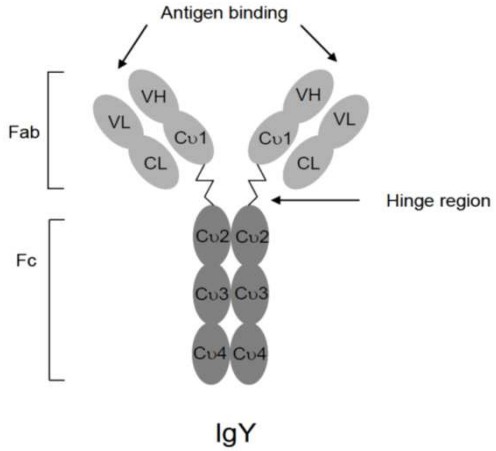
Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (9/10)

Immunoglobulins superfamily

Immunoglobulins

Operators of the adaptative humoral immune response (antigen presentation, toxins and pathogens neutralization and elimination)
Localization: egg yolk >> egg white
Ig Y >>> Ig M=Ig A (Ig D, Ig E)



IgY

Protection from early infection

Proteins with Ig-like Domains

Widespread domain. Ig fold : antiparallel b-strands arranged into two sheets linked by a disulfide bond
Various tissue distribution, amino-acid composition and biological roles

Protein name	Localization
CEPU-Se alpha 2 isoform	EW, VM, ES
CEPU-1	EW, VM, ES
Protein CEPU-1	ES
Neogenin	ES
Basement membrane-specific heparan sulfate proteoglycan core protein	ES
Neuroplastin	Ut
Pro-neuregulin-1, membrane-bound isoform	EW
Semaphorin-3C	VM, ES
Muscle, skeletal receptor tyrosine protein kinase	EW
Butyrophilin subfamily 1 member A1	Ut
Basigin	ES
VH1 protein	ES
ICOS ligand	Ut
Beta-2-microglobulin (IR)	ES, Ut
T-cell surface glycoprotein CD1b4	EW

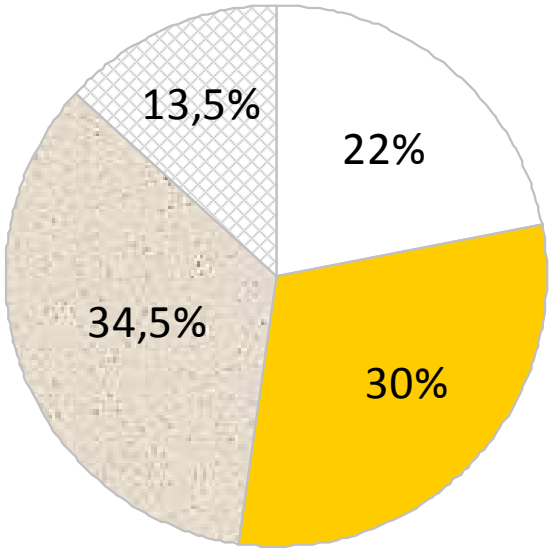
II. Characterization of proteins involved in the egg antimicrobial protection

Chemical defense (molecular protection of the egg) → All compartments

ANTIMICROBIAL COMPONENTS (10/10)

Summary

142 molecules which could potentially degrade microbial components

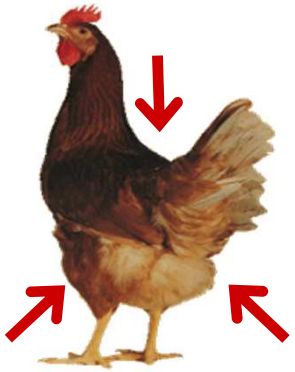


III. Modulation of egg defenses

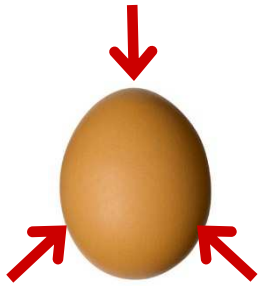
1. Housing systems
Environnement

2. Egg storage environment

Degree of Contamination



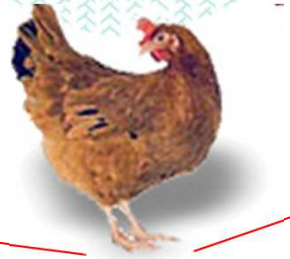
Temperature, atmosphere



? — **Modulation Of antimicrobial activities** — **?**

Impact on hygienic quality of eggs ?

III. Modulation of egg defenses



Microbial challenge

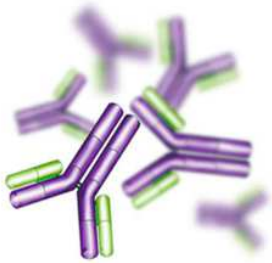
Adaptative response

Immune response

Innate immune response

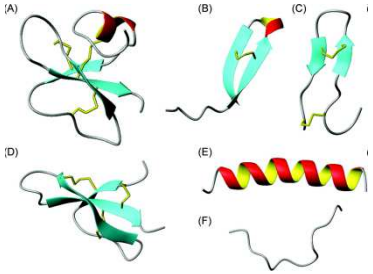
Antibodies production

Proteins and Antimicrobial Peptides



Exportation

Modulation???



Modulation of egg innate immunity ?

III. Modulation of egg defenses

Embryonic development :
Egg white assimilation
Solubilization of eggshell constituents

→

Weakening of egg defences
→ Alternative mechanisms ?
→ Activation of molecular precursors by embryo ?

→ **Differential Proteomic of egg yolk proteins**
(identification of antimicrobial candidates)

→ **Antimicrobial activities of yolk and white**

Function and regulation of egg proteins (FRPO)

INRA, UR83 Recherches Avicoles, 37380 Nouzilly, France

Researchers

Yves NYS

Joël GAUTRON
Head

Sophie REHAULT -GODBERT

Nicolas GUYOT

PhD Students

Larbi BEDRANI

Pauline Marie

Research technicians

Aurélien BRIONNE

Maryse MILLS

Jean-Claude POIRIER

Magali BERGES

SIGENAE (bioinformatic)

ITAVI

Cédric CABAU

Angélique TRAVEL

ALIMENTATION
AGRICULTURE
ENVIRONNEMENT

INRA