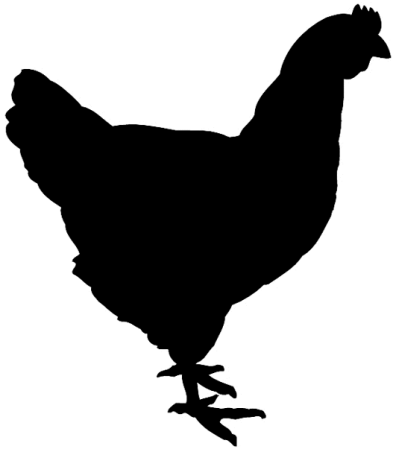


science for people, life & earth

New insights on eggshell mineralization and how they can contribute to maintain shell quality

Joël Gautron
joel.gautron@inrae.fr

Lilian Stapane (INRAe, France), Alejandro Rodriguez-Navarro (University of Granada, Spain), Yves Nys (INRAe, France) and M.T. Hincke (University of Ottawa, Canada)

université de TOURS

UNIVERSIDAD DE GRANADA

uOttawa

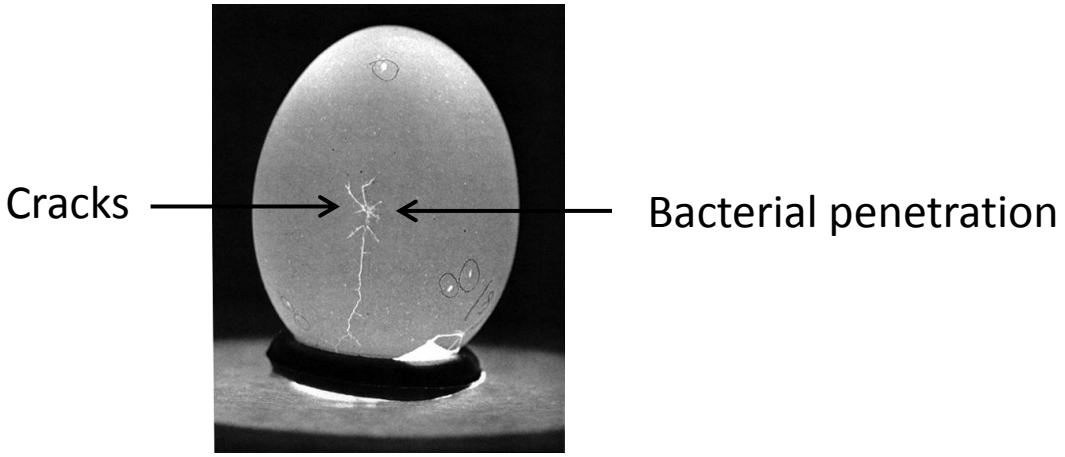
Défenses de l'œuf DOVE Valorisation Evolution

UMRBOA Biologie des Oiseaux & Aviculture

The chicken eggshell

Natural envelope to ensure physical defence of egg

- *Protects the developing embryo*
- *Ensures that table eggs remains free of pathogens*



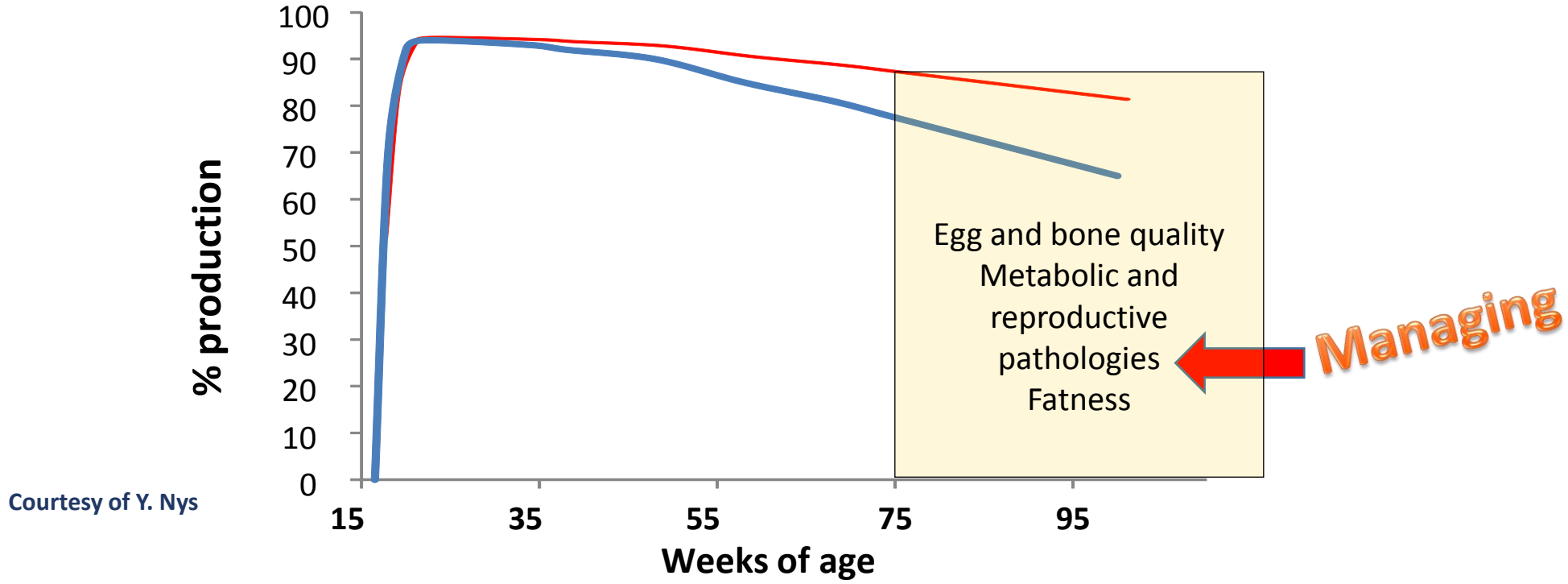
Shell quality depends on Numerous factors

- **Genetic**
- **Hen physiology (age, mold)**
- **Environment of hens (lighting programs, temperature)**
- **Nutrition and management of hens**
- **“Insult”: rearing system, egg transport, egg sorting…**

Genetic, optimal nutrition limit but do not eliminate breakage, notably for elderly birds

Extension of the Laying Period

The current genetic strategy is to improve persistency in lay and to extend the laying cycle of existing flocks (+ 40 days between 2000 and 2011; financial and environmental interest!)



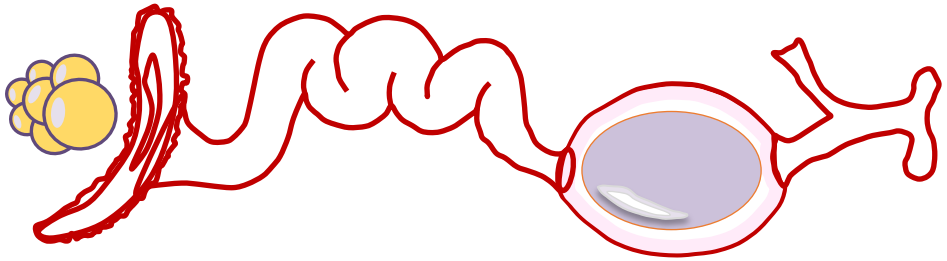
Courtesy of Y. Nys

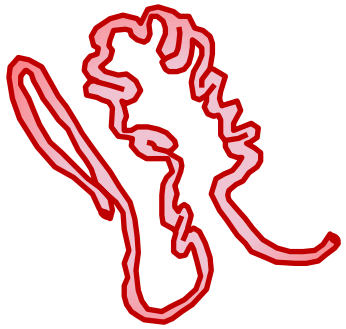
Weekly decreased in egg quality between 70 and 90 weeks of age estimated to be quite linear: - 0.4 haught unit, - 0,02% for egg shell, + 0,05 cm2 egg surface (European data, 2015, practical conditions)

Origin of the calcium deposited in the shell

100 % from feed, but storage in bone

Diet
(calcium)





Calcium

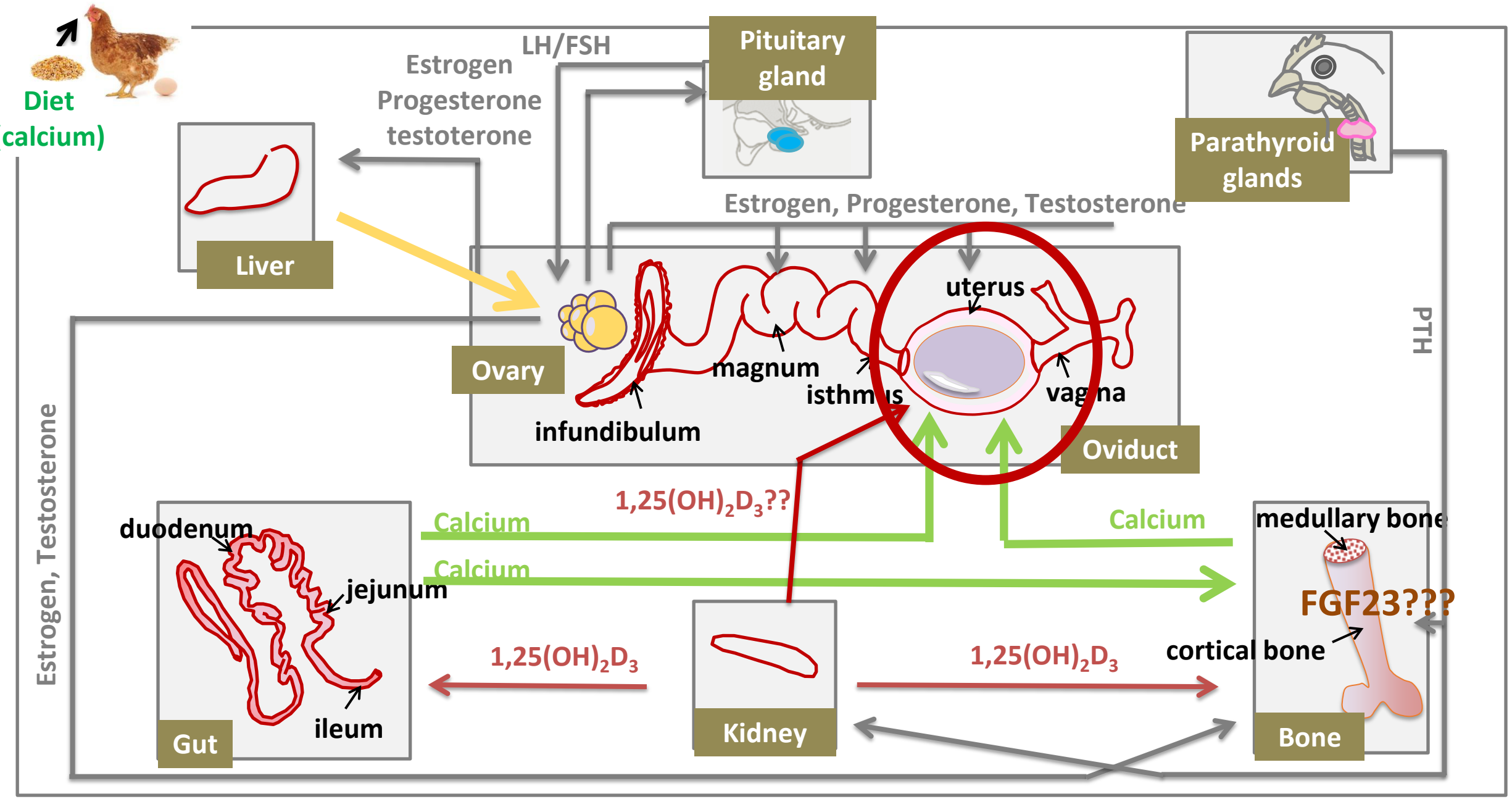
Calcium

Calcium

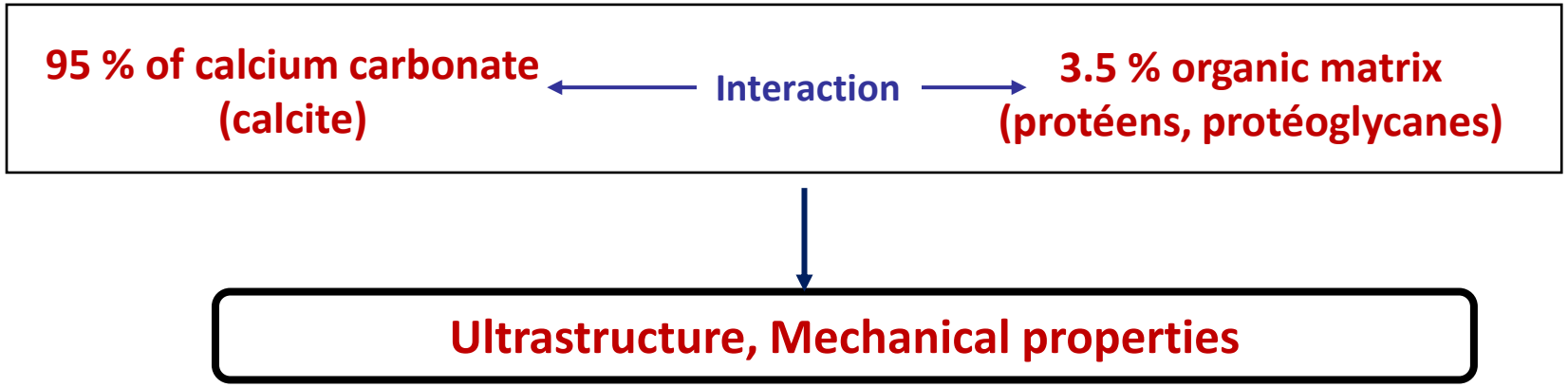
Desynchronization between calcium intakes and requirements

Need for shell calcification	**0**	**--+**	**+++**	**+++**	**+++**	**+++**
Availability of calcium	**+++**	**+++**	**0**	**0**	**0**	**0**

Régulation of calcium metabolism in laying hens



Improve the eggshell quality



✓Quantity About 60 %

✓Control of the mineralisation process About 40%

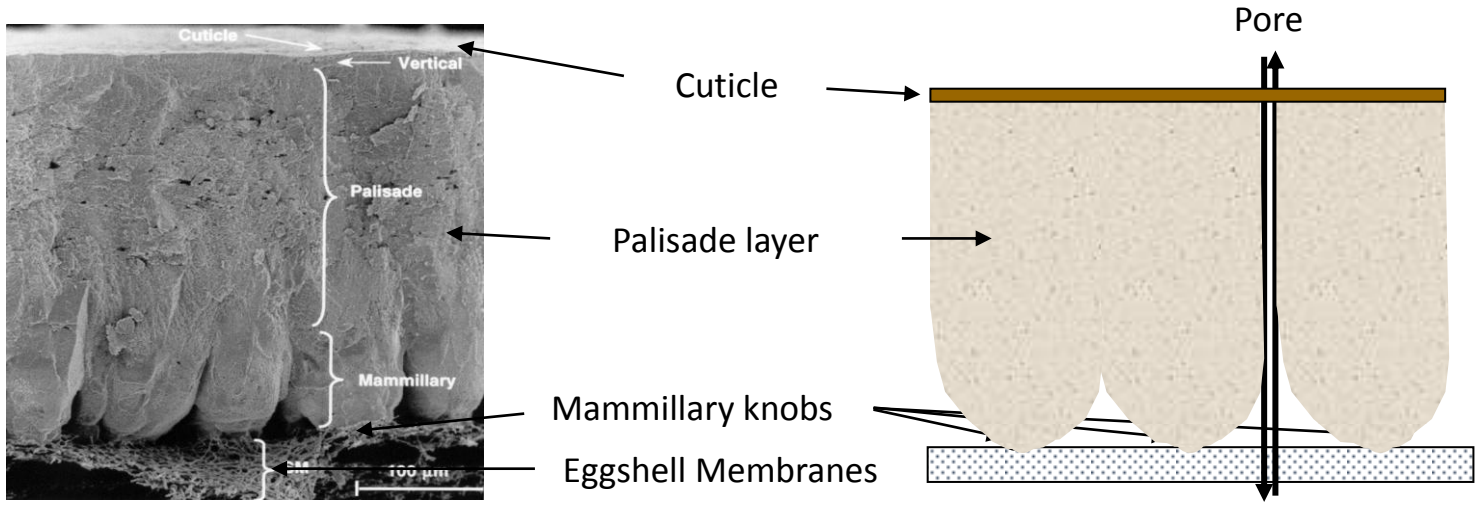
Mass or fabric

☞ Mass : nutrition, genétic, environemment, lightning programs

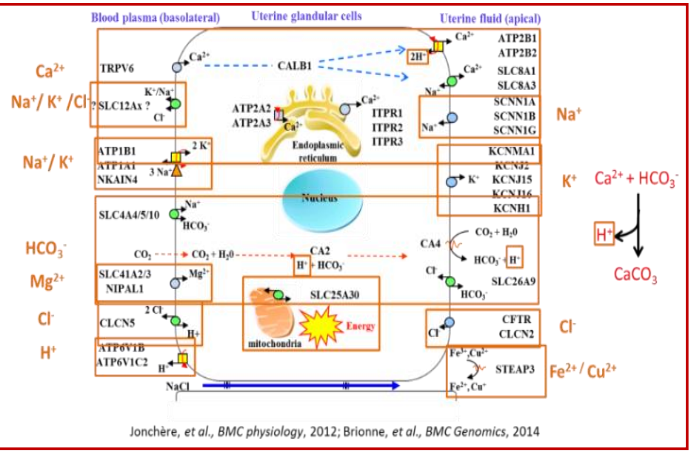
☞ fabric : genetic (eggshell matrix proteins)

The eggshell formation

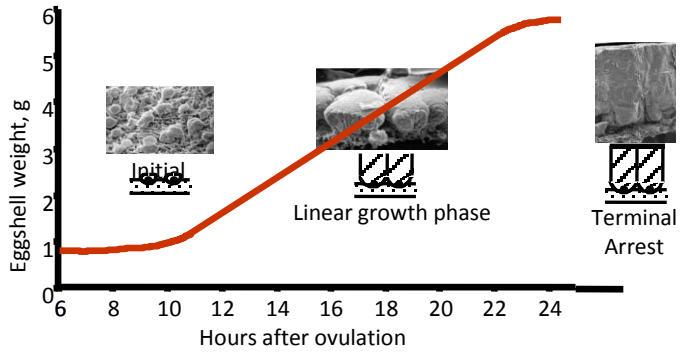
- ✓ Eggshell biomineralization in uterus (fast process)
- ✓ 5-6 g of mineral (calcium carbonate) are deposited within a 20 h period



Mineral supply



Biomineralization



Mineral supply

3 Potential pathways

Transcellular

- *Carbonic Anhydrase 2*
- *Carbonic Anhydarse 4*
- *SLC4A4-A5-A10*
- *SLC26A9*
- *TRPV2-3*
- *Calbindin-1*
- *ATPA2/3*
- *ITPR1/2/3*
- *ATP2B1-B2*
- *SLC8A1-A3*

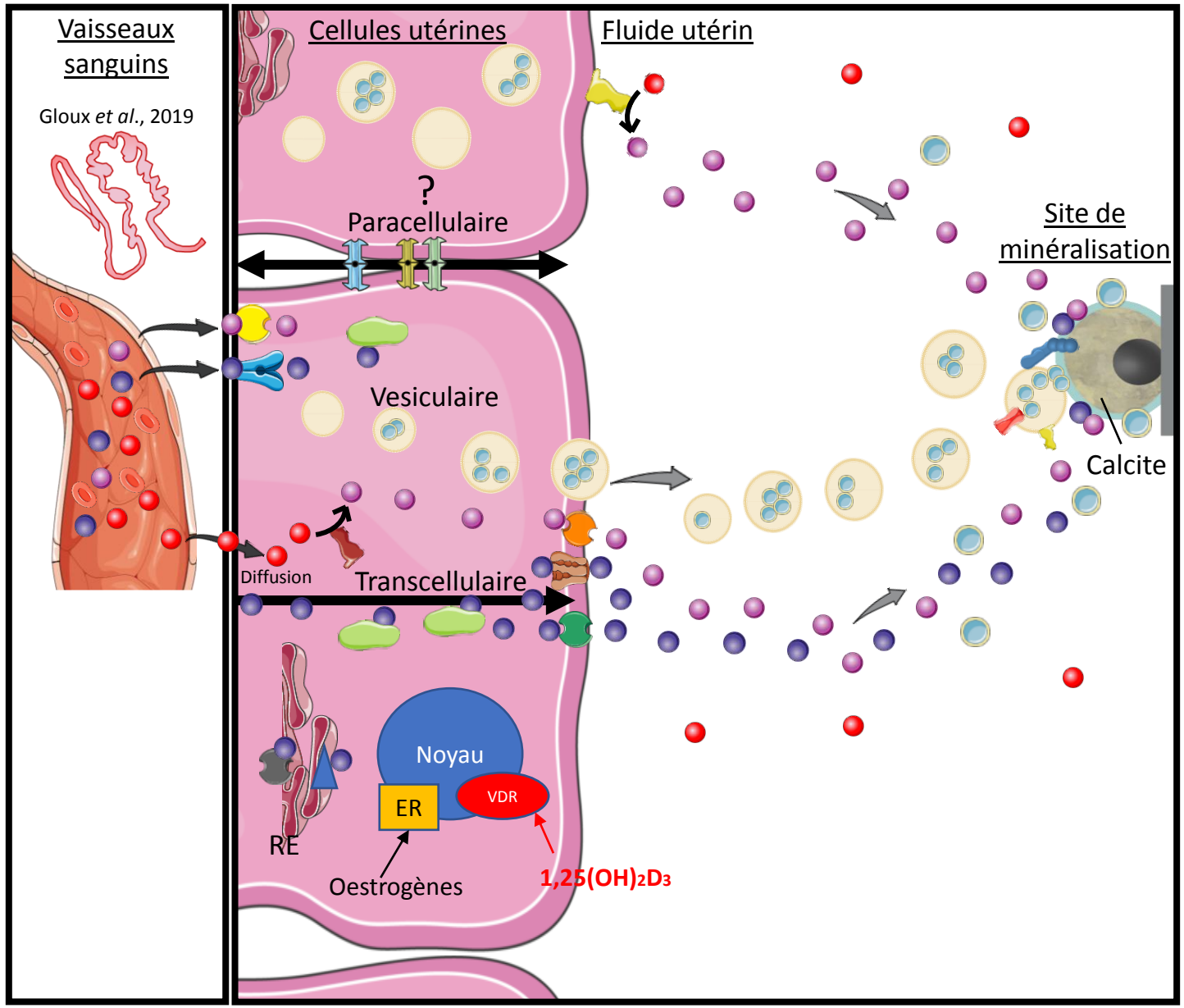
Vésicular

- Extra and intra cellular vesicles
- *Annexines*
- *EDIL3/MFGE8*

Paracellular ?

- *Claudins*
- *JAM*
- *Occludin/TJP*

- HCO_3^-
- Ca^{2+}
- CO_2
- ACC

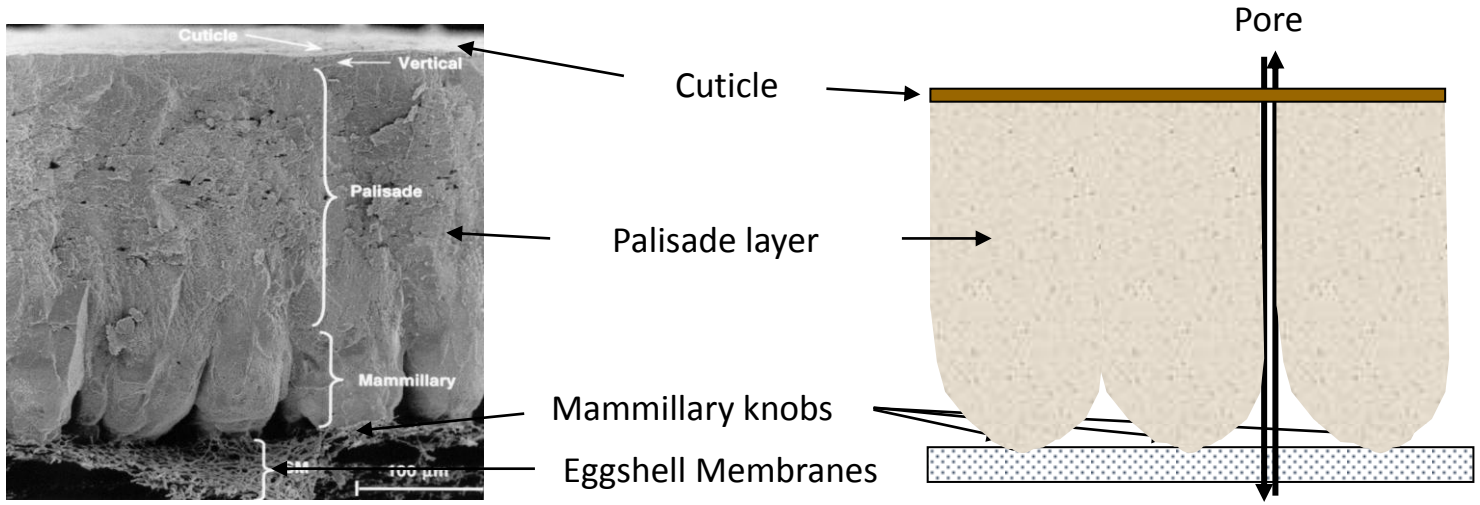


Adapted from L..Stapane-D-17/12/19

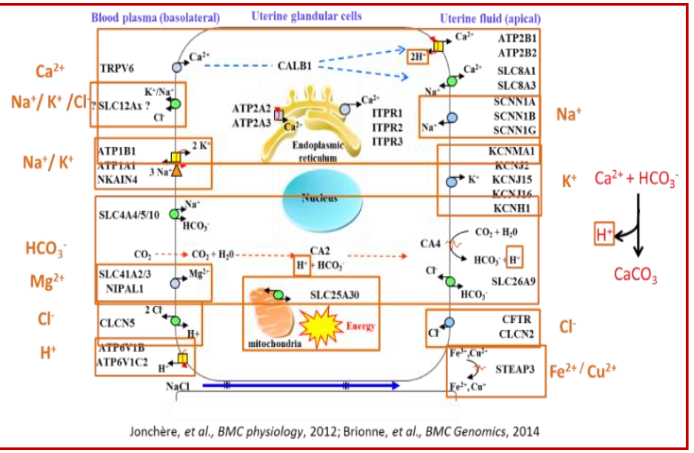
Hodges et Lörcher 1967; Jonchère *et al.*, 2012; Brionne *et al.*, 2014; Nys et Le Roy, 2018

The eggshell formation

- ✓ Eggshell biomineralization in uterus (fast process)
- ✓ 5-6 g of mineral (calcium carbonate) are deposited within a 20 h period

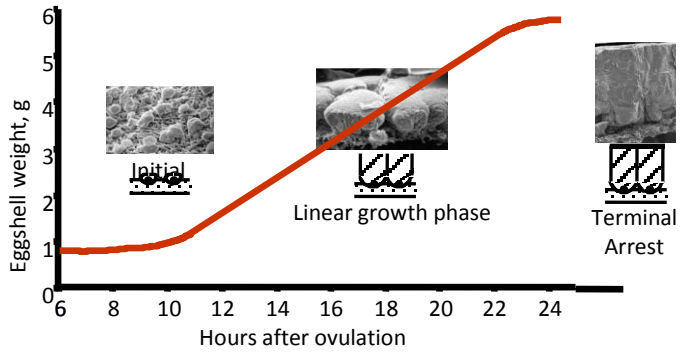


Apports de minéraux

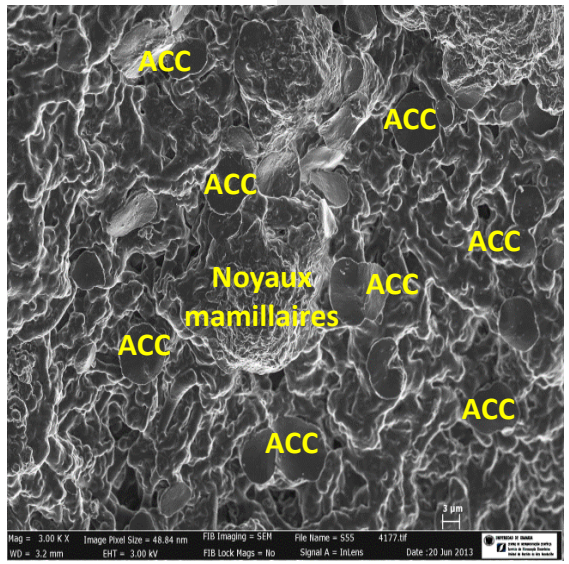


Jonchère, *et al., BMC physiology*, 2012; Brionne, *et al., BMC Genomics*, 2014

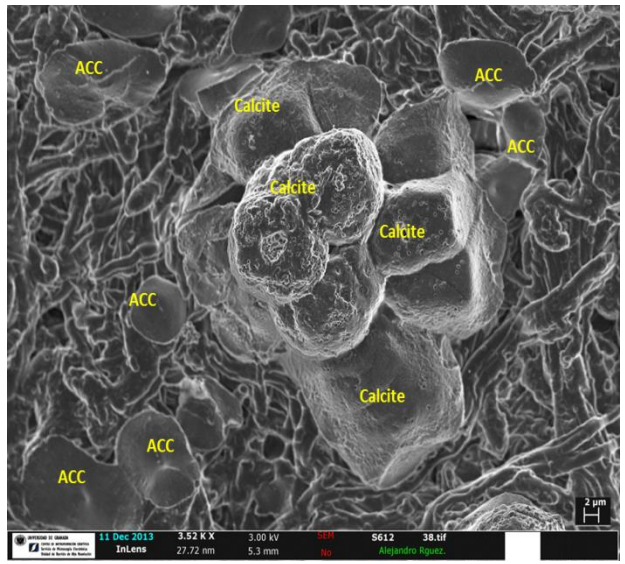
Biominéralisation



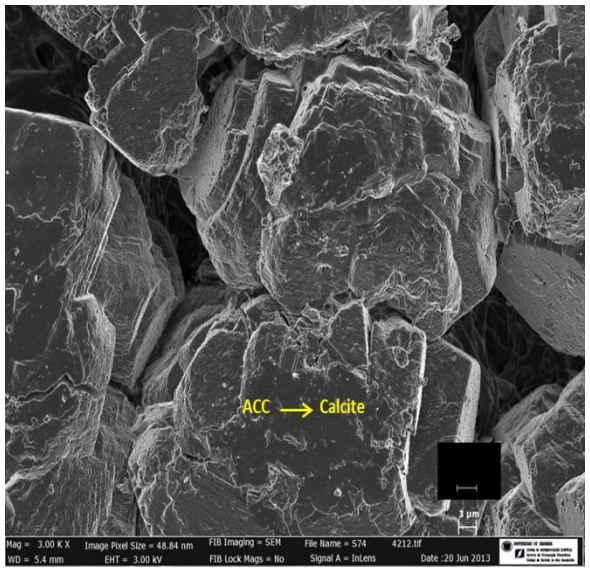
Eggshell biomineralization



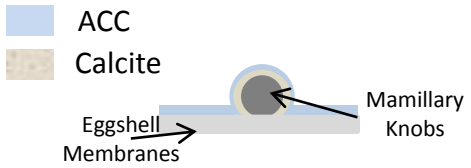
First events of nucléation

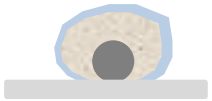


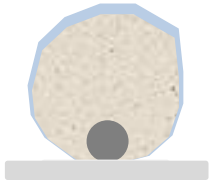
Calcite formation



Larger calcite crystal units deposition







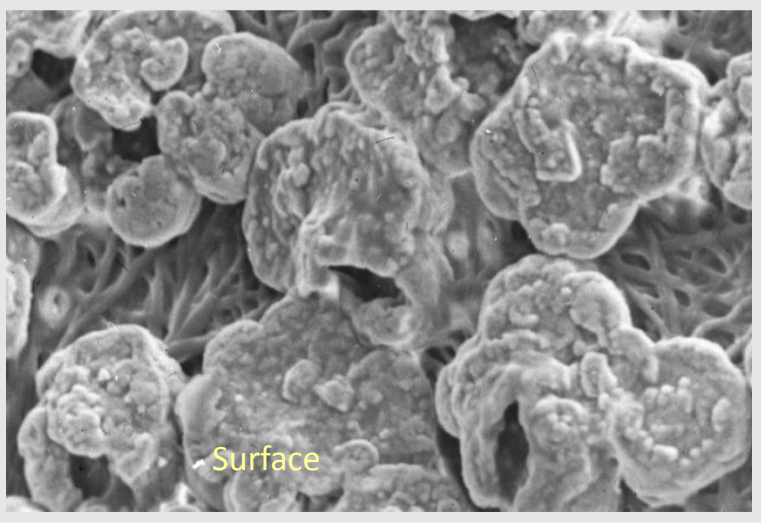
Time 1 (5-6 h Post ovulation):
ACC particles nucleate on the whole eggshell membranes.
Form massive deposits

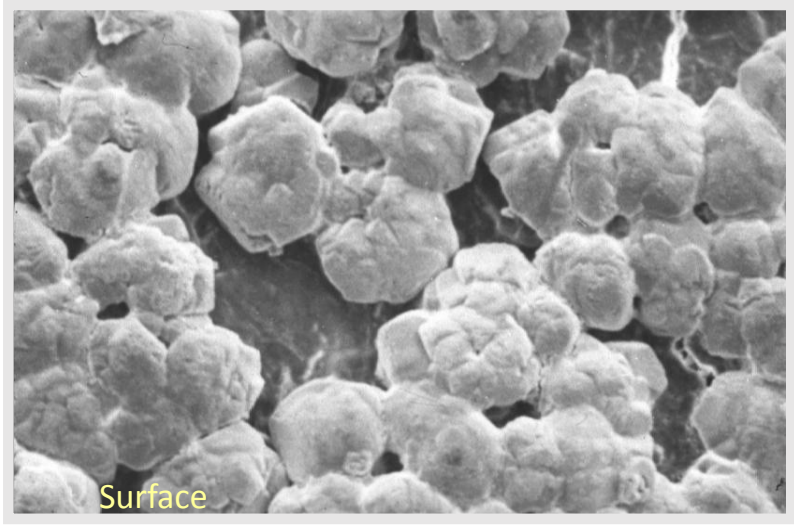
Time 2 (6-7 h post ovulation):
Interface-coupled dissolution precipation process
Direct transformation of ACC into calcite aggregates on mammillary knobs

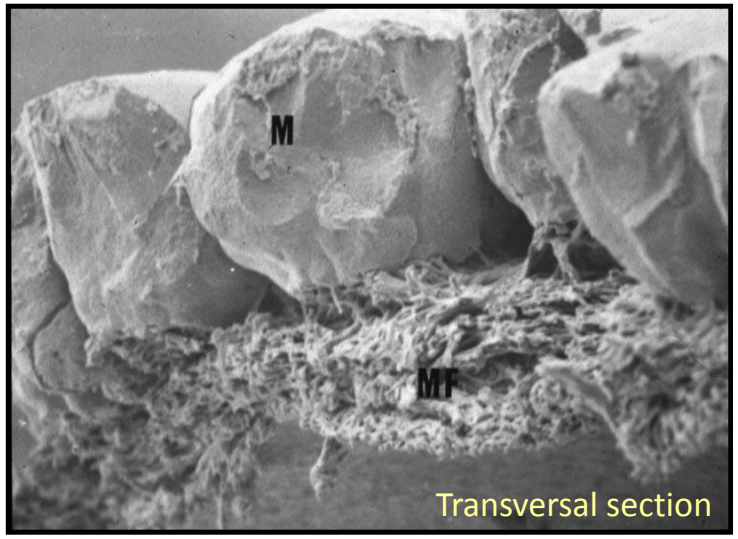
Time 3 (>7h post ovulation):
Additional cristallisation events on calcite template

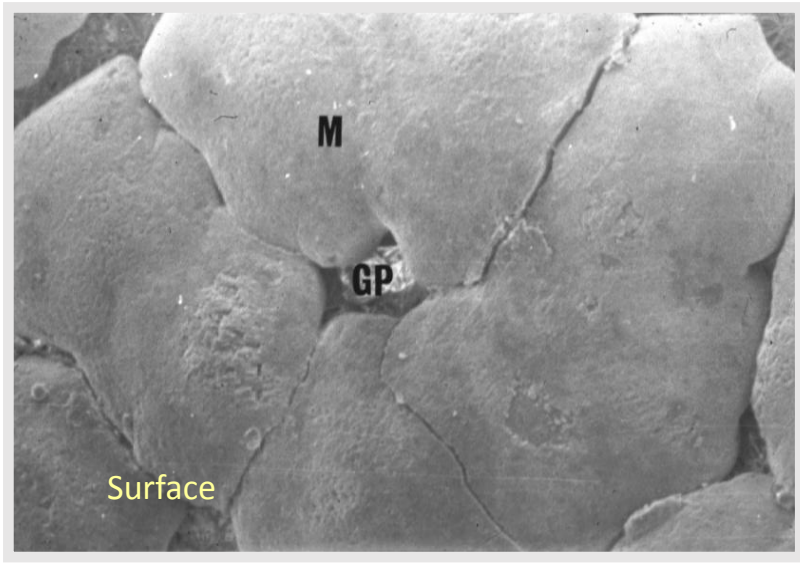
Rodriguez-Navarro et al., Journal of structural Biology, 2015

Eggshell biomineralization



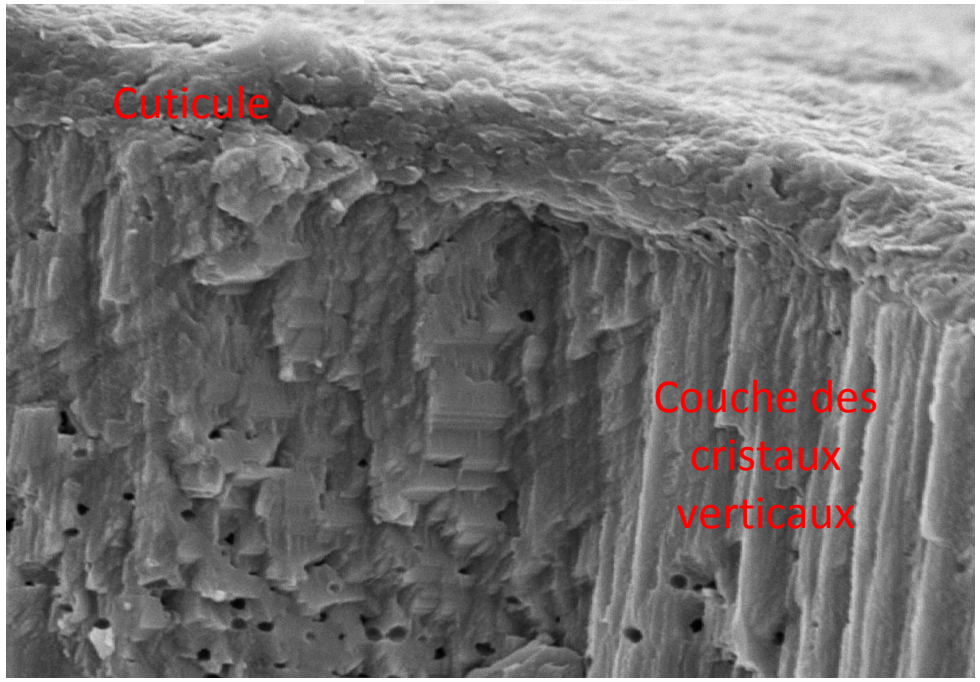


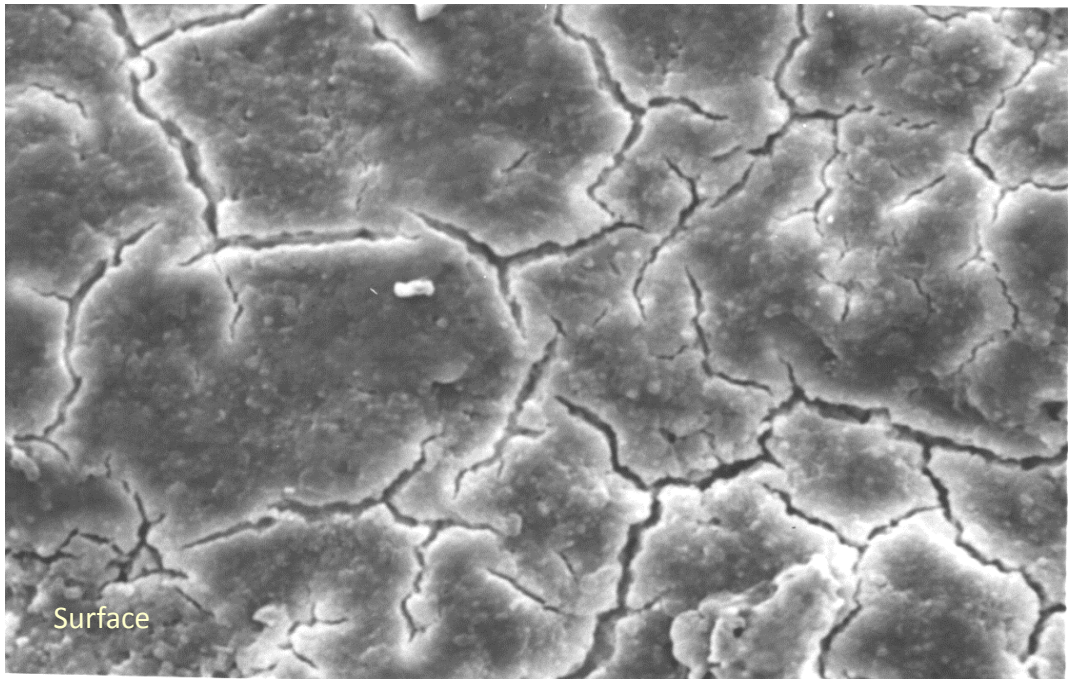




Time 4 (7-10h post ovulation):
Caclcite deposition and fusion of adjacent cônes

Eggshell biomineralization





Time 5 (11 to Oviposition):

- Formation of palisade layer. Generation of a compact layer with crystals all oriented perpendicular to the surface
- Deposition of a thin layer of vertical structure
- Cuticle deposition
- Oviposition, drying and cracking of cuticle

Eggshell biomineralization

95 % of calcium carbonate (calcite) ⟷ **Interaction** ⟶ **3.5 % organic matrix (protéines, protéoglycanes)**

✓ **Quantity**

✓ **Control of the mineralisation process**

Ultrastructure, Mechanical properties

First events of shell mineralisation are crucial

Role of amorphous calcium carbonate (ACC)

- $Ca + CO_3$ ⟶ calcite

Calcite direct formation

- $Ca + CO_3$ ⟶ ACC ⟶ calcite

ACC is a transient phase

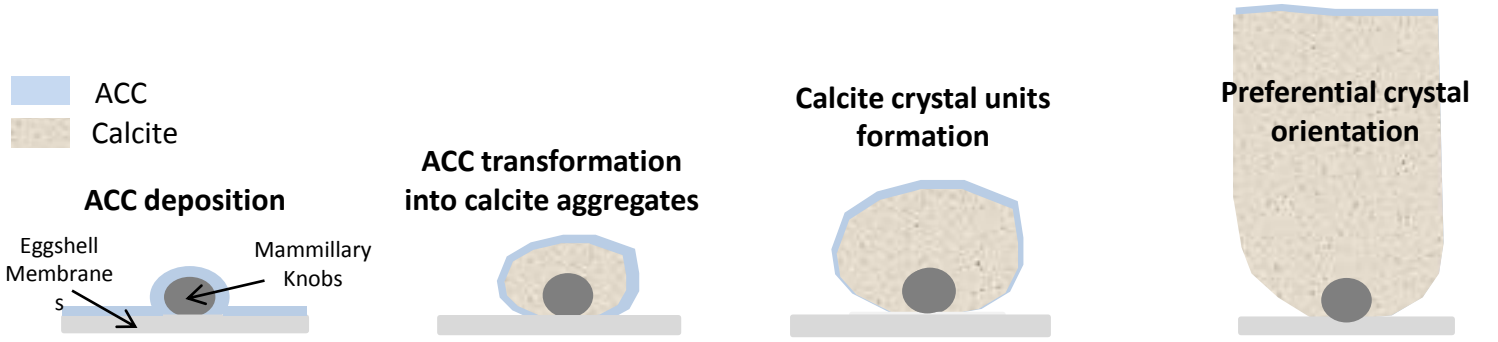
More soluble, more reactif,

Used as a source of temporary calcium storage

Rodriguez-Navarro et al., Journal of structural Biology, 2015

Eggshell biomineralization

Pivotal stages of shell formation, crucial role of amorphous calcium carbonate (ACC)



First events of shell mineralization (initiation 2 h)

Rapid growth phase (12 h)

Role of organic matrix proteins at pivotal events

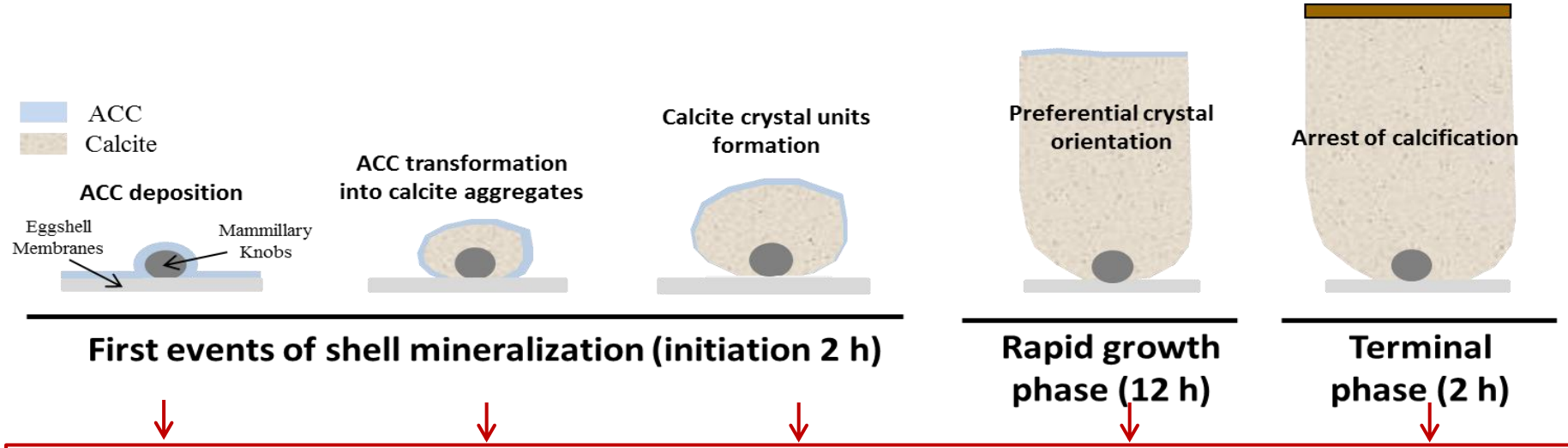
- ✓ *Stabilization of amorphous calcium carbonate (ACC)*
- ✓ *Polymorphs, morphology and size of crystals*

PROJET FINANCÉ PAR L'ANR – ANR – PROJECT FUNDED BY THE ANR

Impact (2013-2017)

Eggshell texture and mechanical properties

Eggshell biomineralization



444 Main candidate proteins to be involved in the shell biomineralization process

Predicted functional activities of the identified matrix proteins ?

(Marie et al., 2014, 2015a,b)

Classification in 3 different groups according to their potential functions

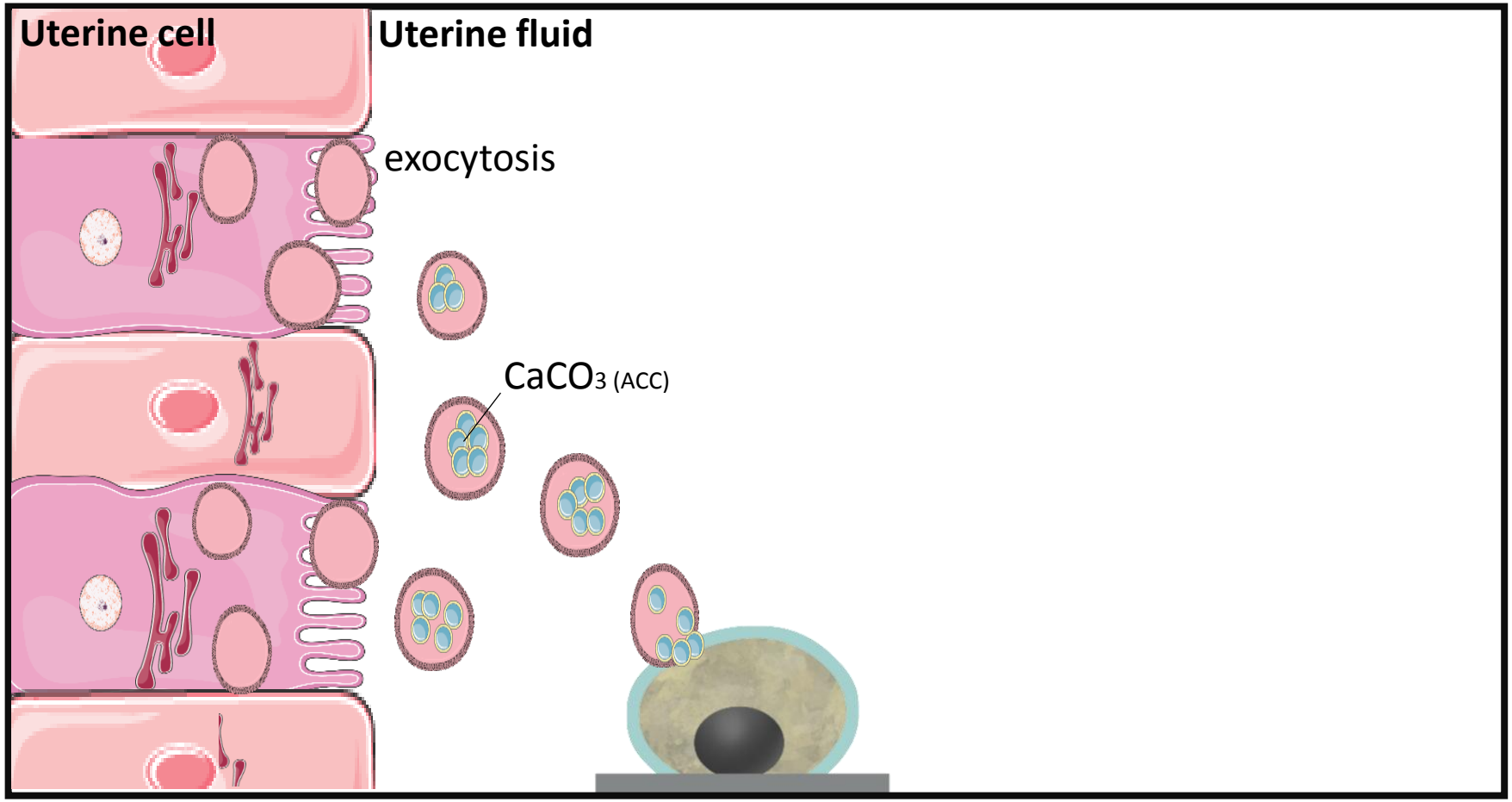
- **Associated to mineralization process**
- **Involved in the regulation of activity of proteins**
- Antimicrobial and other proteins

PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR

Impact (2013-2017)

Eggshell biomineralization

Involvment of vesicular system to transport and stabilize Amorphous calcium carbonate (ACC)



Stapane 2019

Eggshell biomineralization

Involvment of vesicular system to transport and stabilize Amorphous calcium carbonate (ACC)

EDIL3

ANXA1

ANXA2

ANXA8

CA4

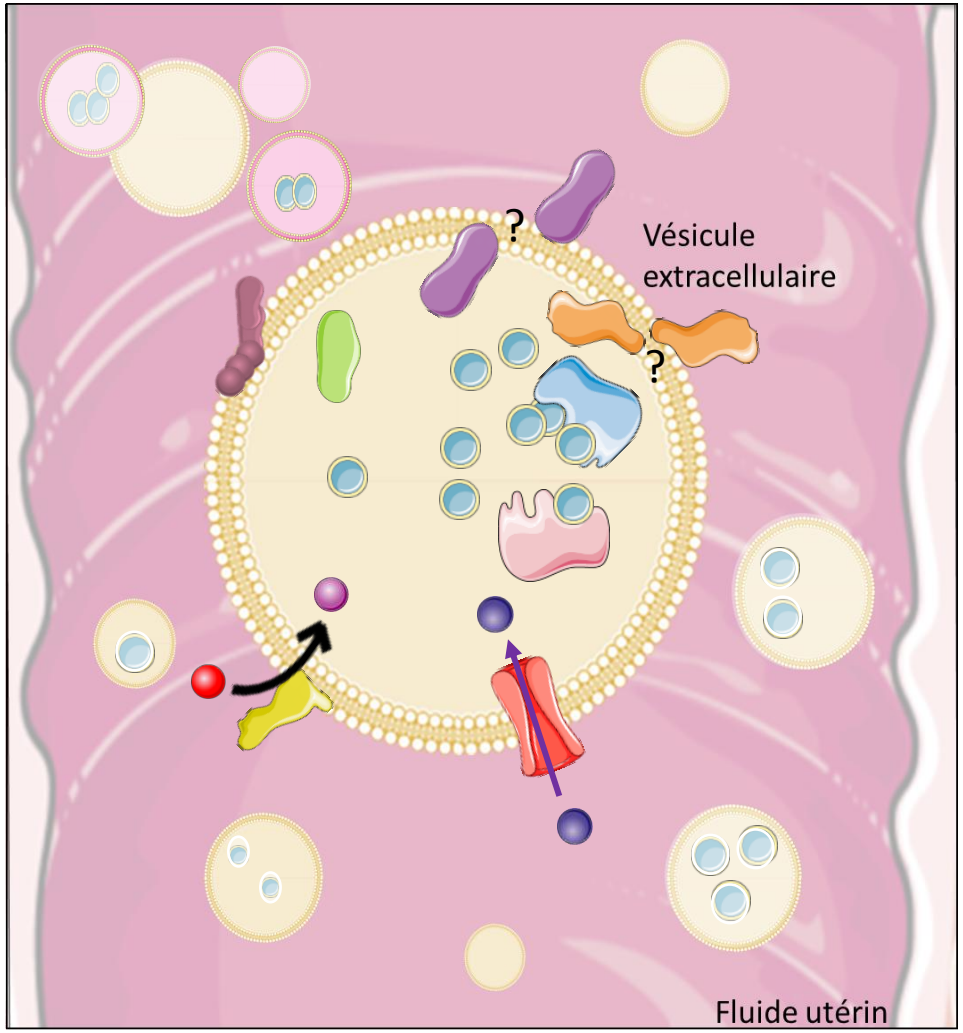
PDCD6IP

Syntenin-1

Ovalbumin

Ezrin

Lysozyme



ANX supplied Ca^{2+} ?

CA4 supplied HCO^{3-} ?

LYZ and OVA stabilized ACC ?
EDIL3 adress vésicles ?

CO_2

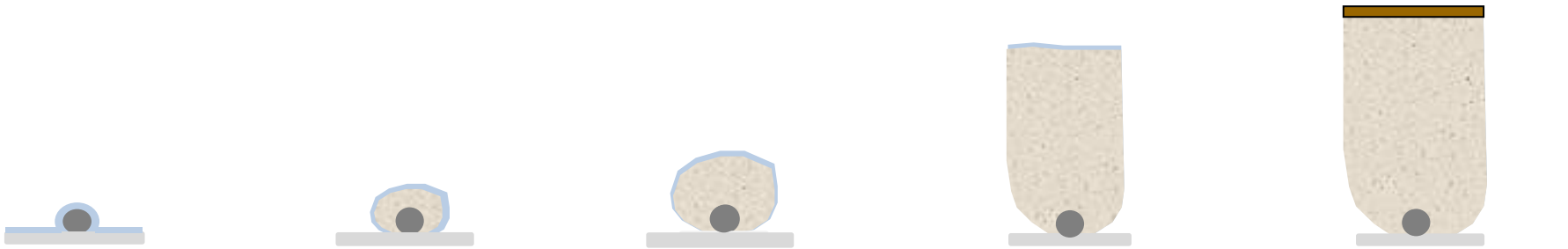
HCO_3^-

Ca^{2+}

ACC

Adapté de L.Stapane-Doctorat-17/12/19

Eggshell biomineralization



ACC deposition

ACC transformation into calcite aggregates

Calcite crystal units formation

Preferential crystal orientation

Arrest of calcification

First events of shell mineralization (initiation)

Rapid growth phase

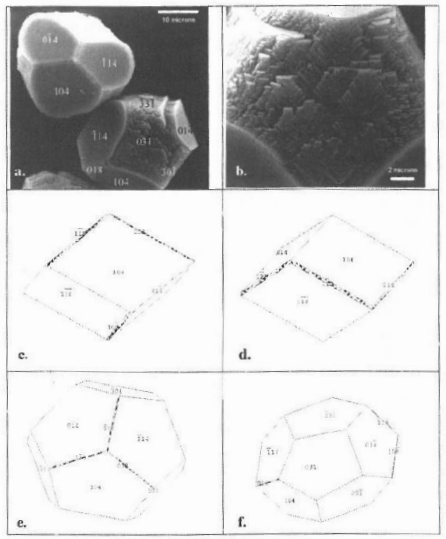
Terminal phase

PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR
ANR
Impact (2013-2017)

Eggshell biomineralization

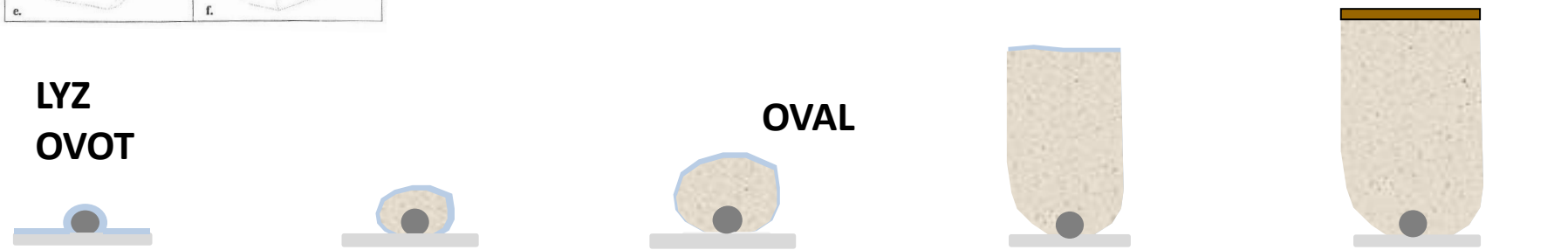
❑ Proteins having a direct involvement in eggshell mineralization

✓ Proteins with established role in the **biomineralisation**



Ovotransferrin is a Matrix Protein of the Hen Eggshell Membranes and Basal Calcified Layer

J. GAUTRON[a], M.T. HINCKE[b], M. PANHELEUX[a], J.M. GARCIA-RUIZ[c], T. BOLDICKE[d] and Y. NYS[a,*]



PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR
ANR
Impact (2013-2017)

Formation de l'œuf et biominéralisation

❑ Proteins having a direct involvement in eggshell mineralization

✓ Proteins with established role in the **biomineralisation**

Freeman et al, 2010

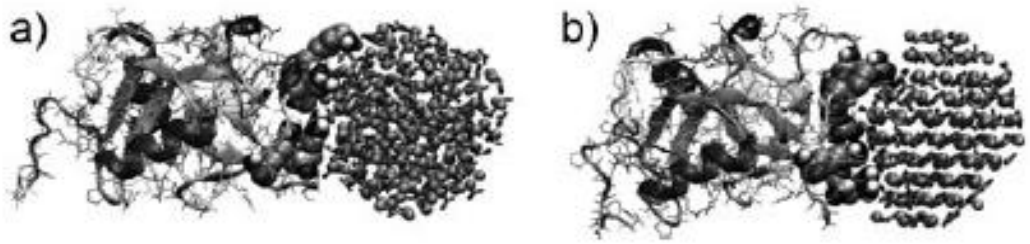
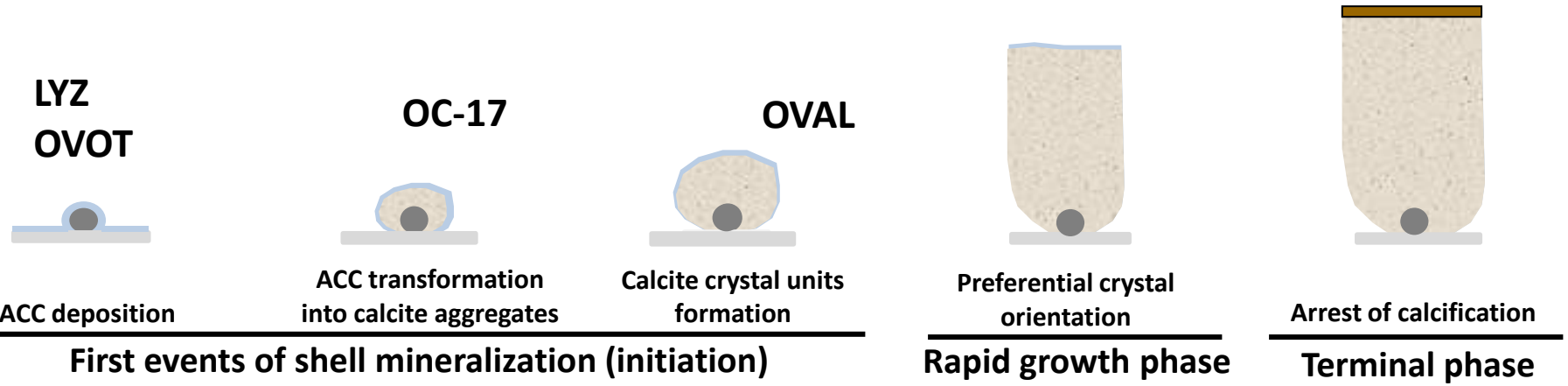


Figure 1. Ovocleidin-17 bound to an amorphous (a) and a crystallized (b) calcium carbonate nanoparticle containing 192 formula units. The



PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR
ANR
Impact (2013-2017)

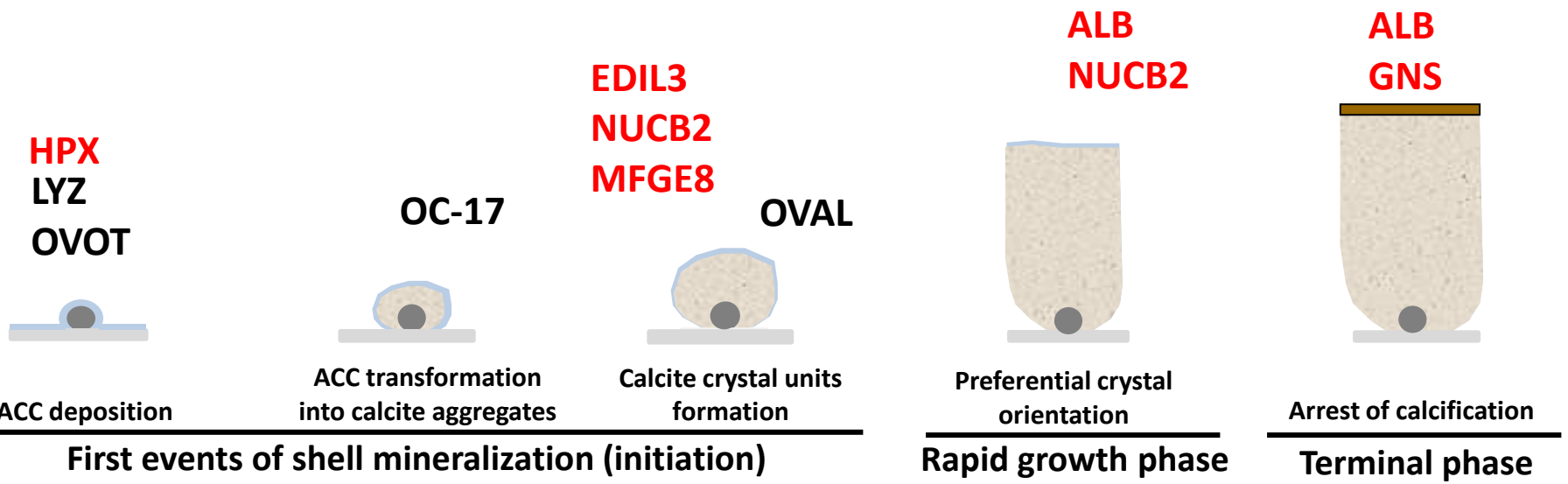
Eggshell biomineralization

❑ Proteins having a direct involvement in eggshell mineralization

✓ Proteins with established role in the **biomineralisation**

✓**Calcium binding proteins (CaBPs)** interacting with calcium, favoring crystal nucleation and driving the morphology of crystals

- *Proteins with EF-hand and EGF-like calcium binding domains*



PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR
ANR
Impact (2013-2017)

Eggshell biomineralization

❑ Proteins having a direct involvement in eggshell mineralization

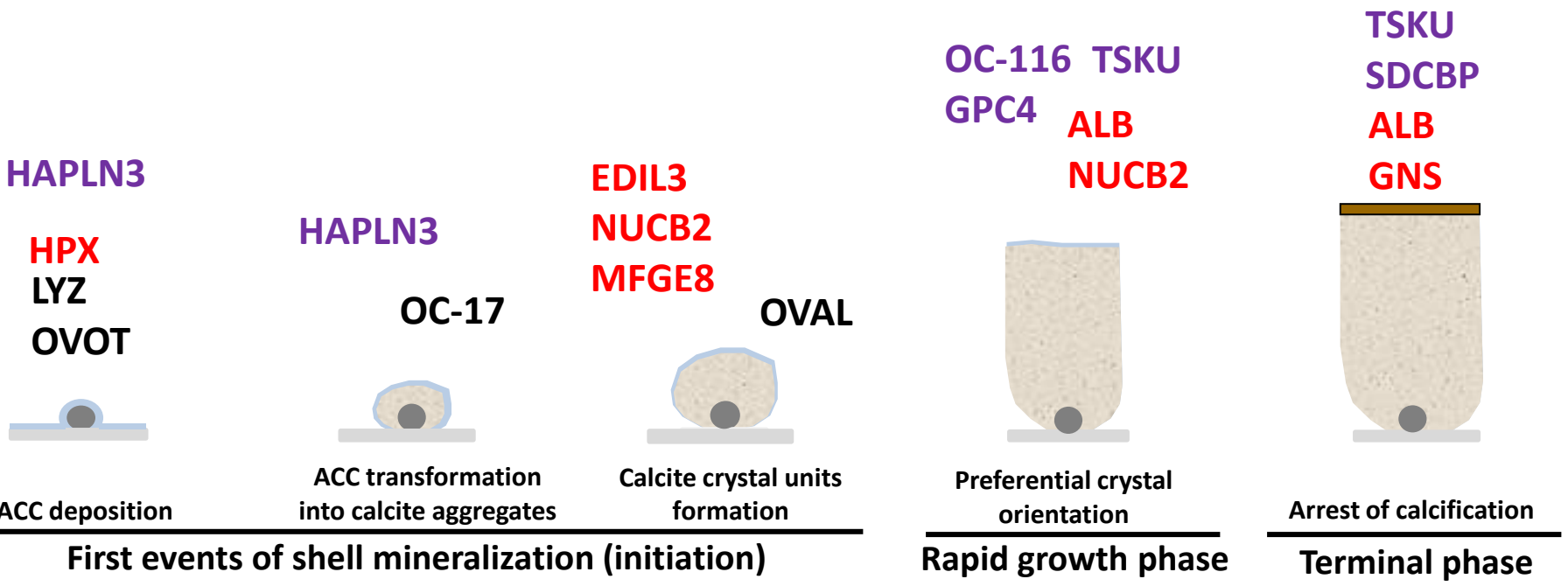
✓ Proteins with established role in the **biomineralisation**

✓**Calcium binding proteins (CaBPs)** interacting with calcium, favoring crystal nucleation and driving the morphology of crystals

- *Proteins with EF-hand and EGF-like calcium binding domains*

✓ **Proteoglycans** and proteoglycan binding proteins

- proteoglycans have a negative charge to attract Ca2+ ions



PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR
ANR
Impact (2013-2017)

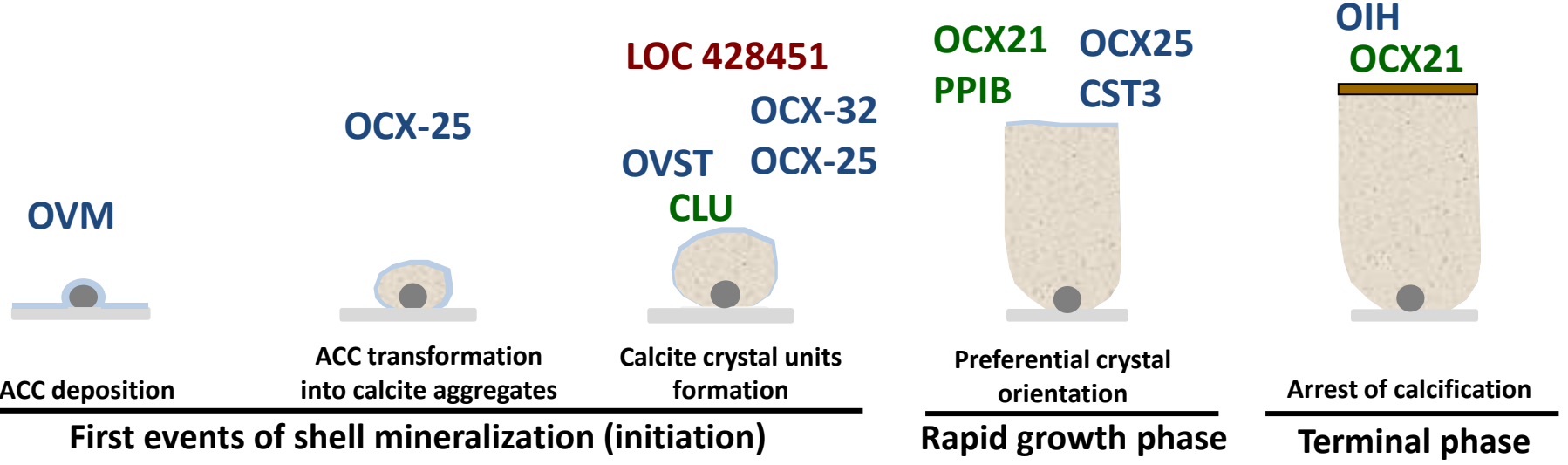
Eggshell biomineralization

❑ Proteins involved in the regulation of proteins driving mineralization

- ✓ Proteins involved in the **proper folding of the eggshell matrix** to ensure calcium and mineral interactions and to ensure template to the mineralized structure
- ✓ Proteins **inhibiting or activating proteins present in the mineralization milieu (non cellular).**

  → *Direct interaction with other proteins.*

  - ***Molecular chaperone interact with proteins driving mineralization***
  - ***Proteases and protease inhibitors (specific and controlled role during calcification process, either by degrading proteins or regulating processing of proteins into their mature forms)***
- ✓ Mineralization depends of the **degree of protein phosphorylation**
  - ***Kinases and Phosphatases***



PROJET FINANCÉ PAR L'ANR - PROJECT FUNDED BY THE ANR
ANR
Impact (2013-2017)

And now ? How to improve shell quality ?

- Mapping genes coding matrix proteins to detect polymorphisms and haplotype related to good quality shell

Physiology

Understand the mechanisms of shell manufacturing and determine the origin of its weaknesses

Genetics

Classical and genomic selection

Recent Developments and Future Prospects :

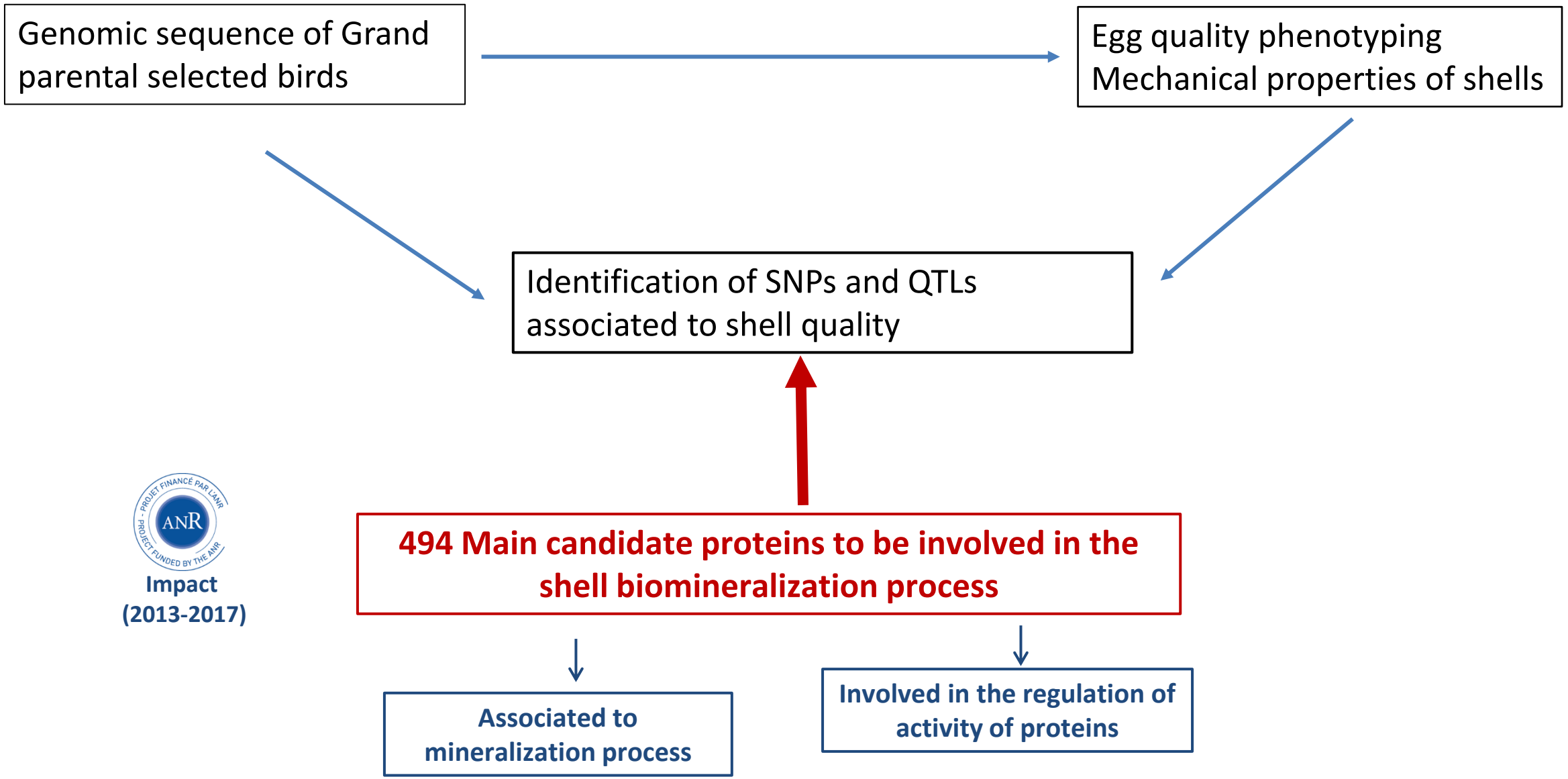
- ✓ Genomic selection (precision, taking into account the male effect)
- ✓ Taking into account scientific advances in the knowledge of mechanisms
  - ✓ Candidate gene approach

Candidate Genes of eggshell calicification in laying hens (CACAO)

Défenses de l'œuf
DOVE
Valorisation Evolution

Eggshell Calcification Polymorphism Candidates (POLCACAO)

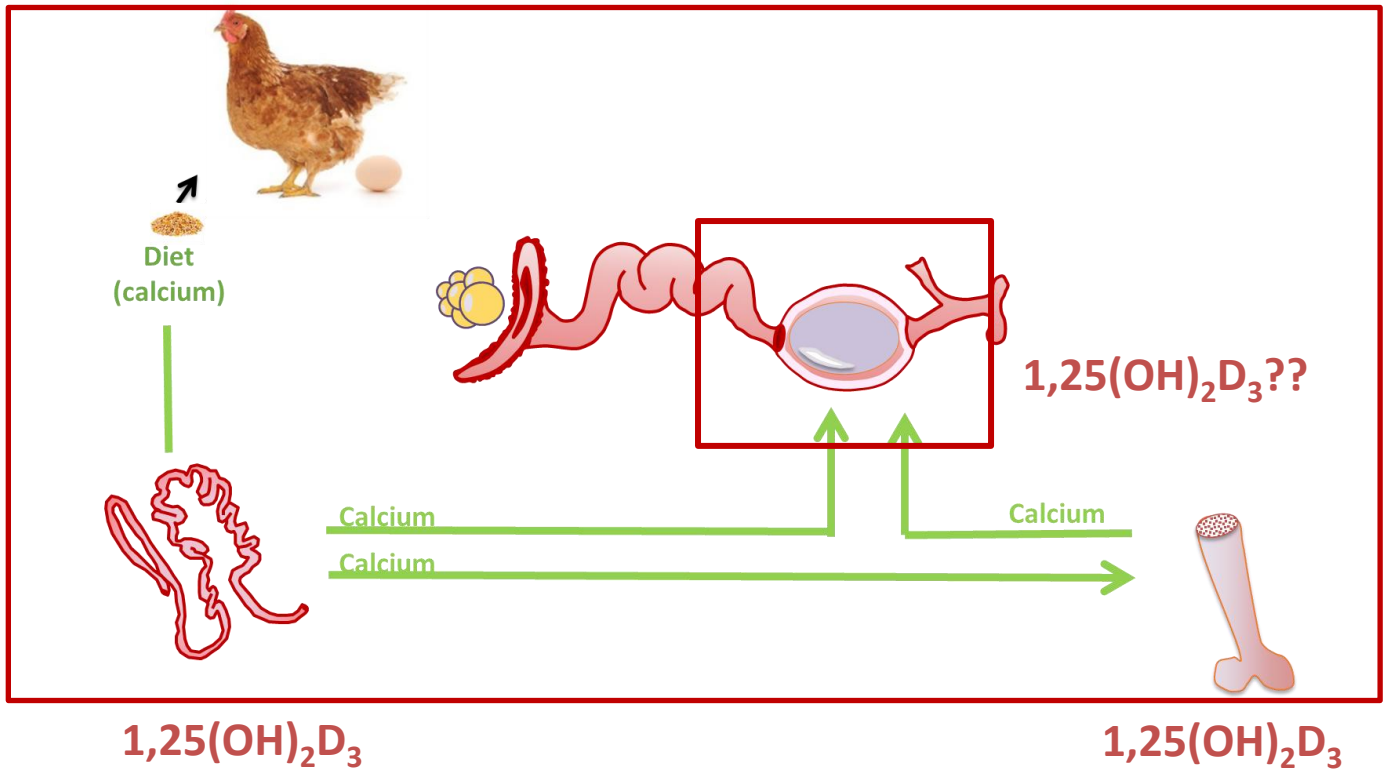
Complementarity of information between proteomics (candidate genes) and genomics results (QTL / Sequencing).



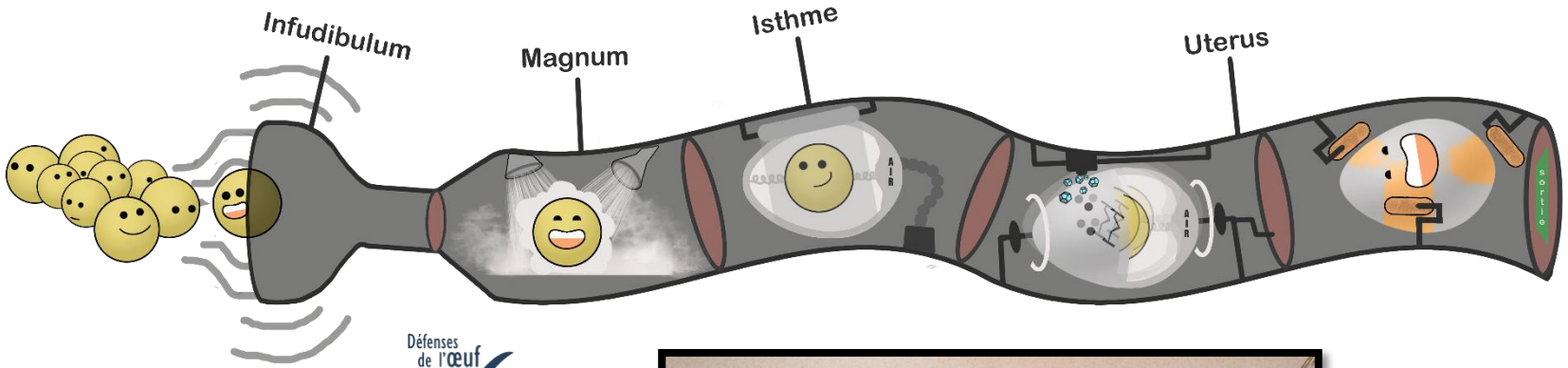
Impact (2013-2017)

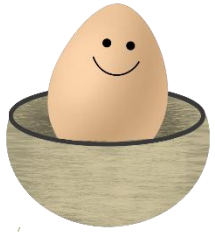
And then? How to improve shell quality ?

- Maping genes coding proteins to detect polymorphisms and haplotype related to good quality shell
- Identification of early biological markers associated to shell calcification.
- Influence of Vit D status (form of Vit D)
    - Age effect



Thank you for your attention





- L'équipe DOVE
- Directeur de thèse: J. Gautron
- A. Rodriguez-Navarro
- N. Le Roy, J. Ezagal
- Y. Nys et M. Hincke
- P. Leroy, F. Heraud, C. Diot
- BOA
- PAIB2
- PEAT
- Pegase
- GeT-Genotoul
- Universidad de Granada