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CHICKEN EGGSHELL BIOMINERALIZATION
Structure, composition and role of the organic matrix

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Molecular medecine, University of Ottawa
Ottawa, Canada

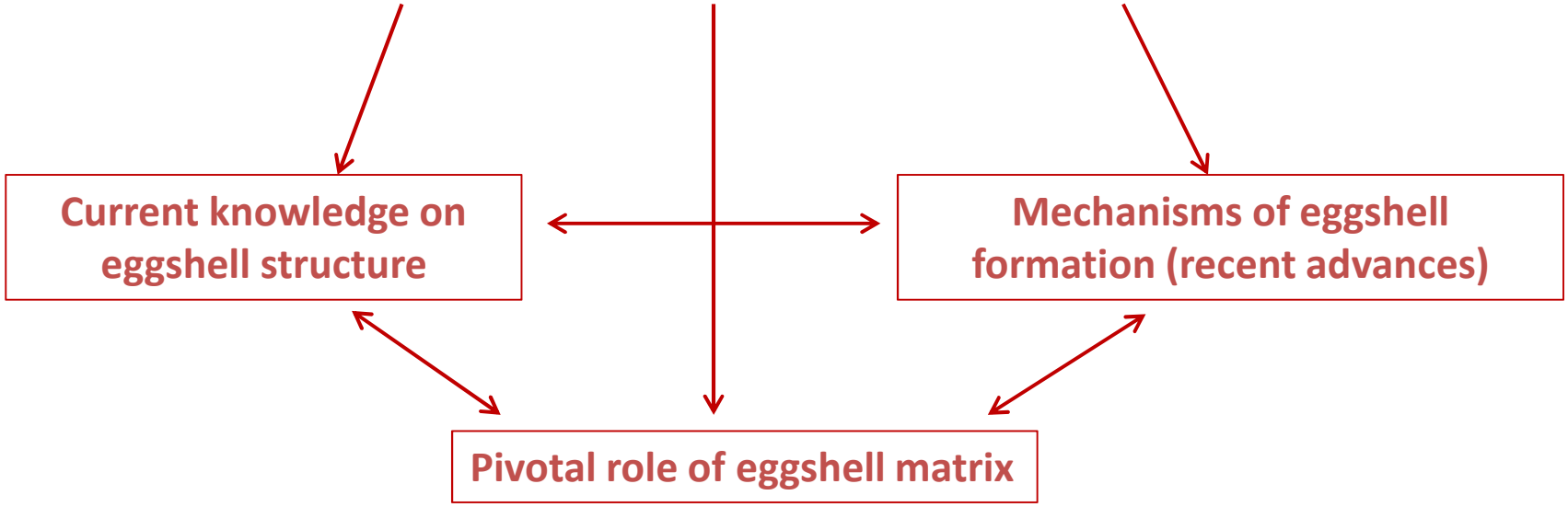
A. Rodriguez-Navarro, J.M. Garcia-Ruiz
University of Granada, **CSIC**, Spain

V. Labas
UMR INRA 85, Proteomics facilities, 37380 Nouzilly France

The chicken eggshell

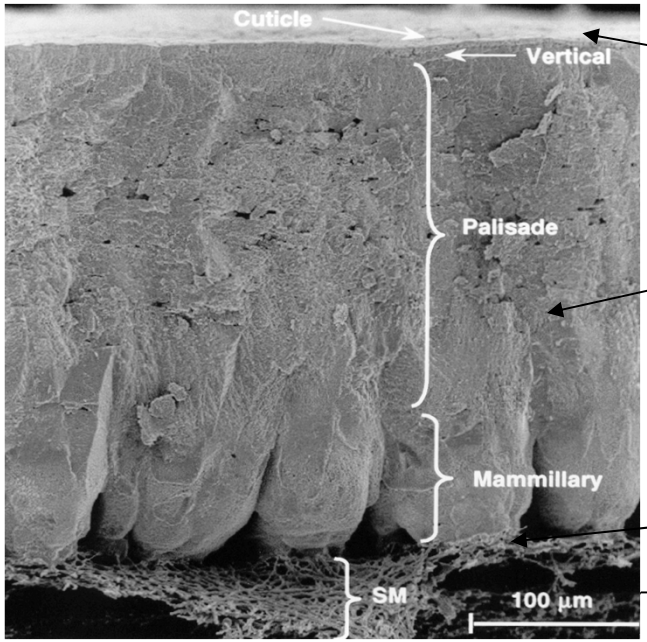
- ✓ Natural envelope to ensure physical defence of egg
  - *Protects the developing embryo*
  - *Ensures that table eggs remains free of pathogens*

- ✓ Natural ceramic formed at ambient temperature and pressure
  Model of biomineralization on membrane support



The chicken eggshell

- ✓ Eggshell biomineralization in uterus (one of the fastest on earth)
- ✓ 5-6 g of mineral are deposited within a 20 h period
- ✓ 95 % calcium carbonate (calcite polymorph)
- ✓ 3.5 % proteins and proteoglycans (organic matrix)



SEM

Cuticle

Palissadic layer

Mammilary knobs

Eggshell membranes

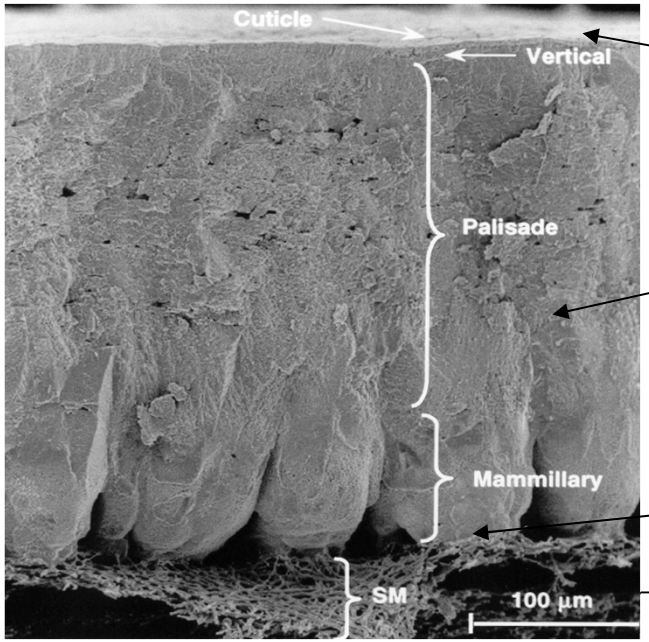


Cross-polarized light

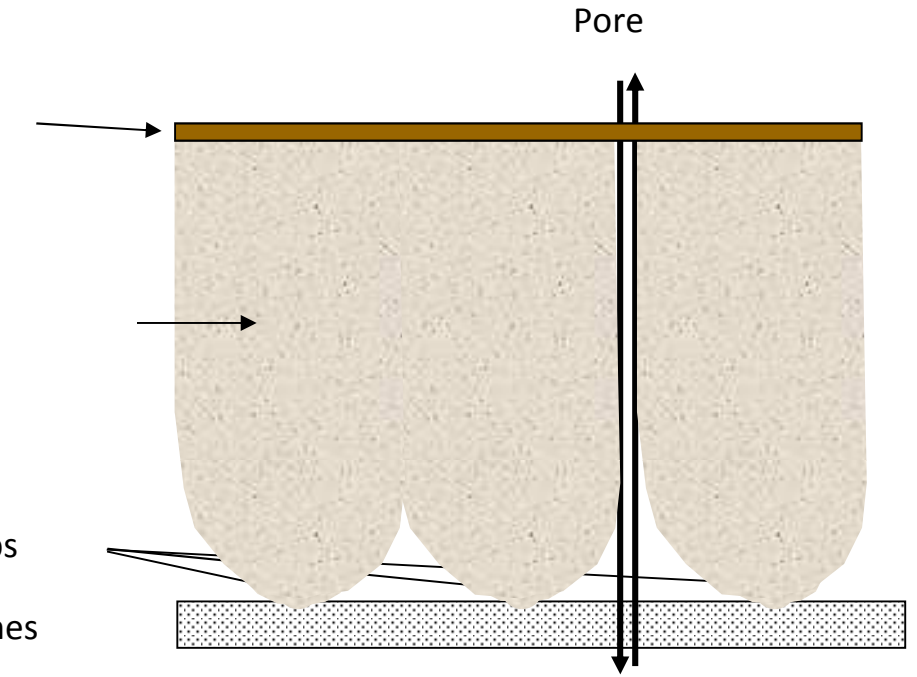
Images: J.M. Garcia-Ruiz, Granada

The chicken eggshell

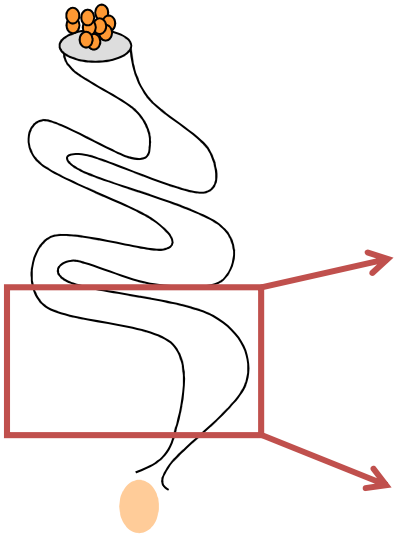
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SEM



Eggshell biomineralization in uterus

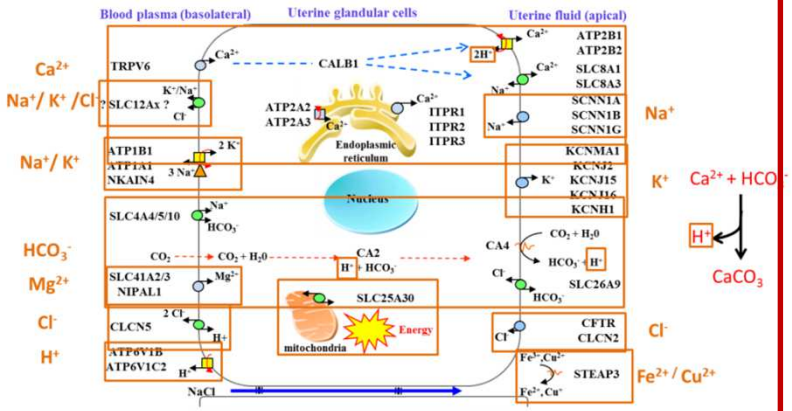


Supply of minerals for shell mineralization

General model describing uterine ion transporters during eggshell calcification

Jonchere et al., 2012, Bmc physiology

Brionne et al., 2014, Bmc Genomics



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

Biomineralisation process

Interaction matrix-minerals

* ***In situ physical measurements***
* ***In Vitro*** **analysis**

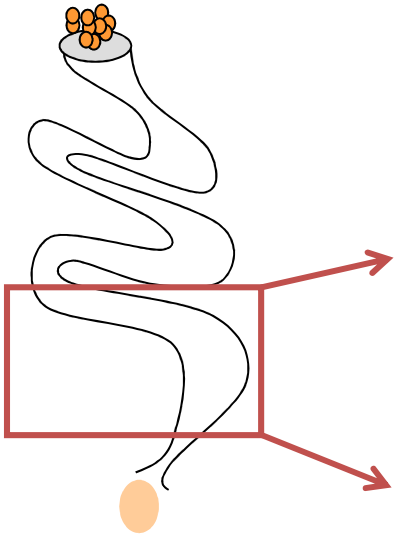
Characterisation of eggshell matrix proteins

* **Chicken, Guinea fowl**
* **Proteomics, NGS (trancripts)**

IMPACT (2013-2017)

INRA, URA, Tours (coordinator)
INRA, PRC-PAIB, Tours
INRA, SIGENAE, Toulouse,
INRA, GetPlage, Toulouse
University Granada, Espagne
University Ottawa, Canada

Eggshell biomineralization in uterus

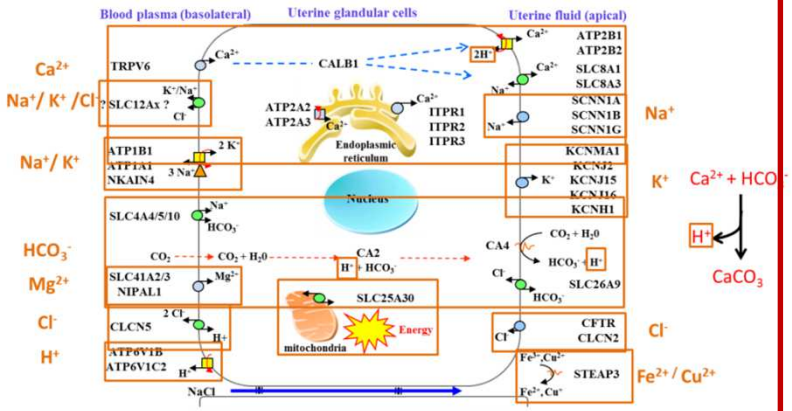


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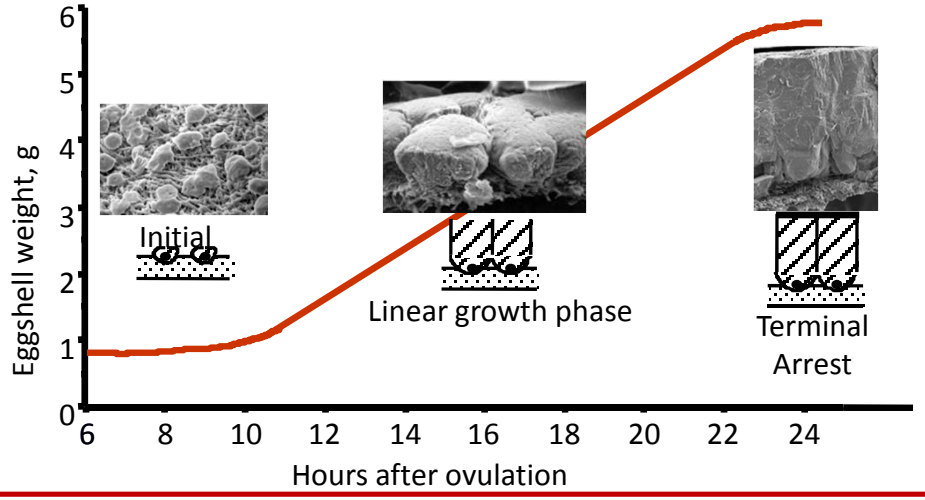
Brionne et al., 2014, Bmc Genomics



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

Biomineralisation process

3 main phases in the uterine fluid (acellular milieu)



Role of organic matrix

✓ **Organic matrix plays a key role**

determine the texture of the shell and its resulting mechanical properties

95% of $CaCO_3$ (calcite)

quantity

Interaction

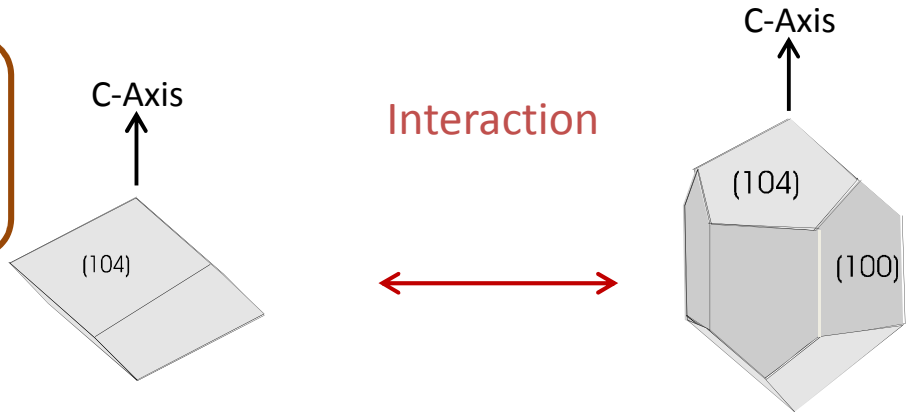
control of biomineralisation process

3.5% of organic matrix (proteins, proteoglycans)

Ultrastructure
Mechanical properties

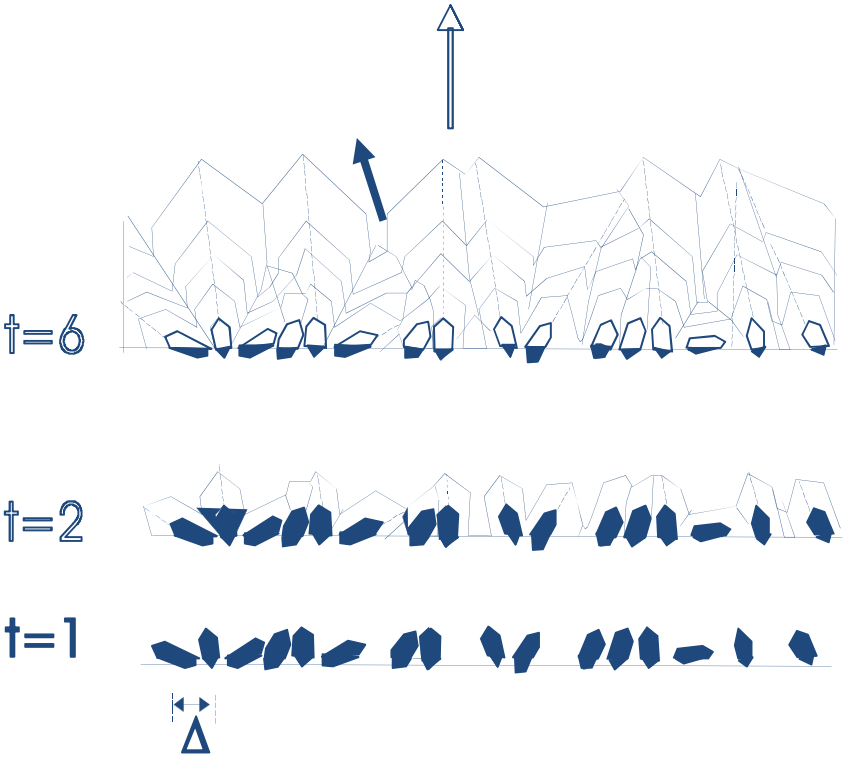
Calcium carbonate (calcite) ⟷ Organic matrix

Orientation and stabilisation of calcium carbonate polymorphs Drive the calcite morphology

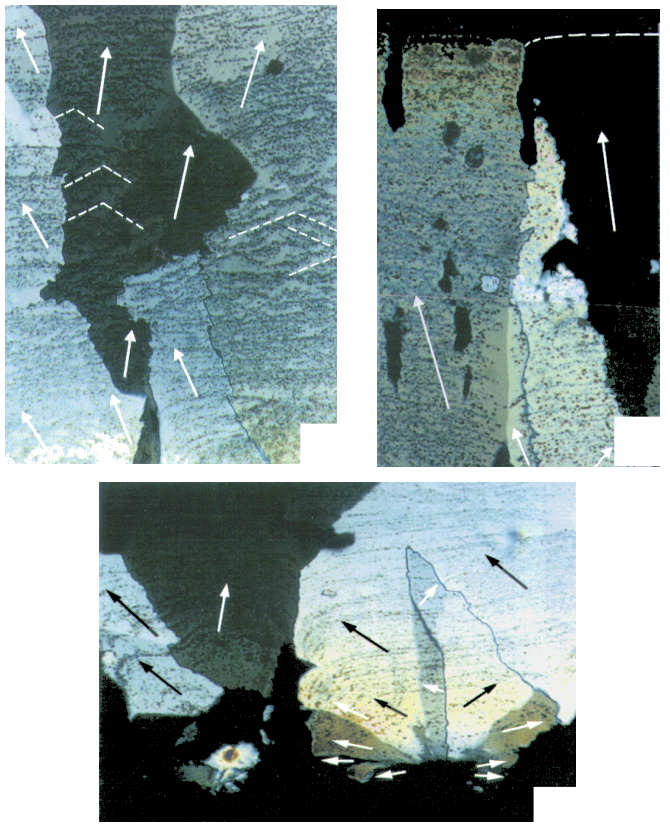


Eggshell microstructure

- ✓ The avian eggshell is a complex and highly structured calcitic bioceramic
- ✓ Competitive crystal growth
- ✓ Mineral-binding proteins guide crystal growth (organic matrix control)



Rodriguez-Navarro et al., 2000; Nys et al., (2004)



Images: J.M. Garcia-Ruiz, Granada

Identification of eggshell matrix proteins

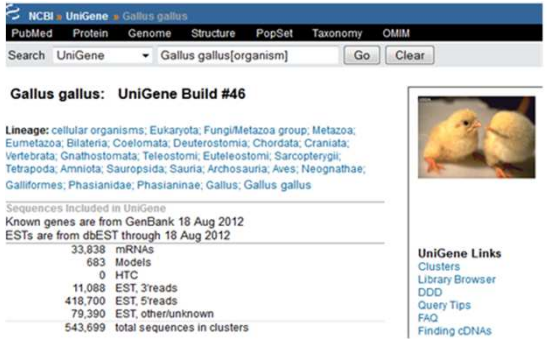
- **11 eggshell proteins were identified in 2006**
- **Major advances in last decade**

✓2004, Publication of the chicken genome sequence



Availability of « omics » high-throughput techniques and data mining tools to identify novel egg proteins

✓ Genome-wide non redundant catalog of 33 838 different genes



✓ cDNA and ESTs libraries (600 434 functional genes in chickens)



Eggshell biomineralization and matrix proteins

IMPACT

95% of $CaCO_3$ (calcite)

↑ Interaction ↓

Control of the calcification process

3,5% of organic matrix (proteins, proteoglycans)

Global and non hierarchical approaches

✓ More than 670 proteins

✓ More than 700 specific transcripts

Only a few numbers are abundant and active on calcification process

Quantify eggshell matrix proteins and their variation according to mineralisation

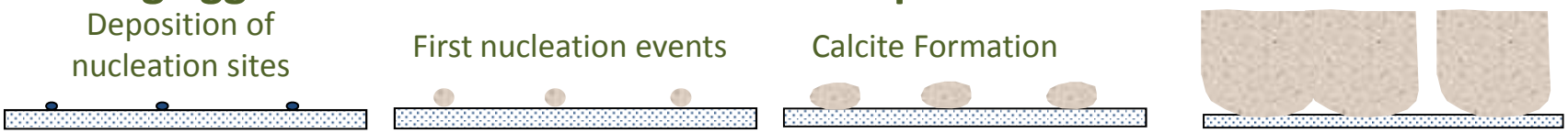
Determine the molecular actors with a pivotal role during the mineralisation process

Quantification of matrix proteins at pivotal events

Importance of the first events of mineralisation

- Initial stages are crucial for ultrastructure of the biomineral
- Determine eggshell mechanical properties

Forming eggshell collected at different time points of the onset of calcification



***In situ* physical measurements** (University of Granada)

- SEM
- XRD 2D
- FTIR

High-throughput quantitative and statistical analyses of matrix proteins

- mass spectrometry

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

List of protein candidates involved in particular key points of the mineralisation

In situ physical measurements

IMPACT

✓ Role of amorphous calcium carbonate (ACC)

- Calcite is a common material to build highly sophisticated protective mineral structures

  ➢ $Ca + CO_3 \longrightarrow$ calcite

  Direct formation of calcite

  ➢ $Ca + CO_3 \longrightarrow$ ACC $\longrightarrow$ calcite

  ACC as a transient phase

  More soluble, more reactive, used as a temporary calcium source

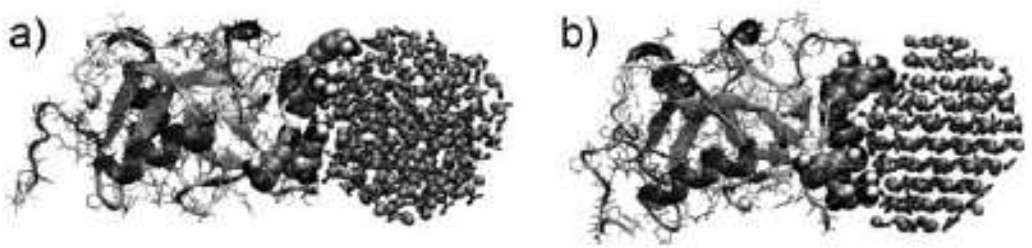


Figure 1. Ovocleidin-17 bound to an amorphous (a) and a crystallized (b) calcium carbonate nanoparticle containing 192 formula units. The

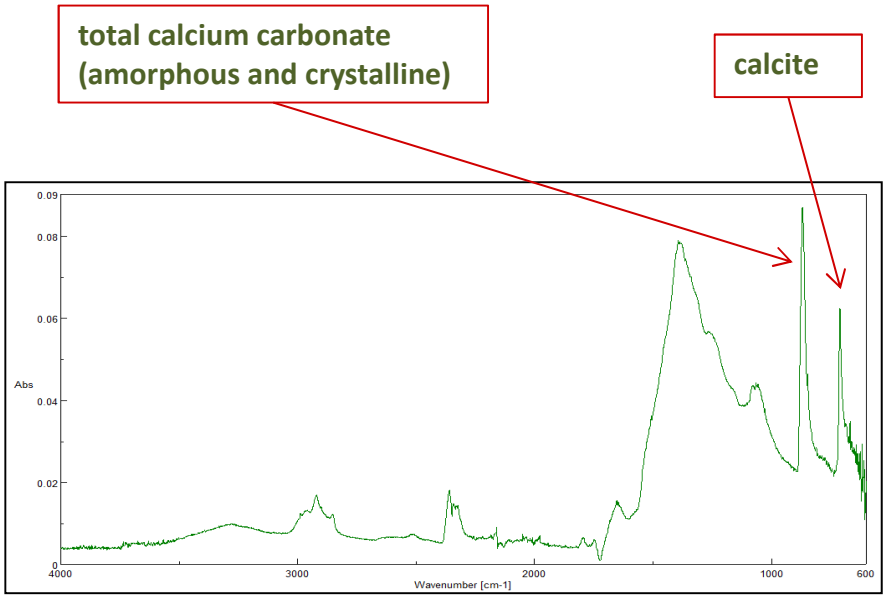
Freeman et al, 2010

In situ physical measurements

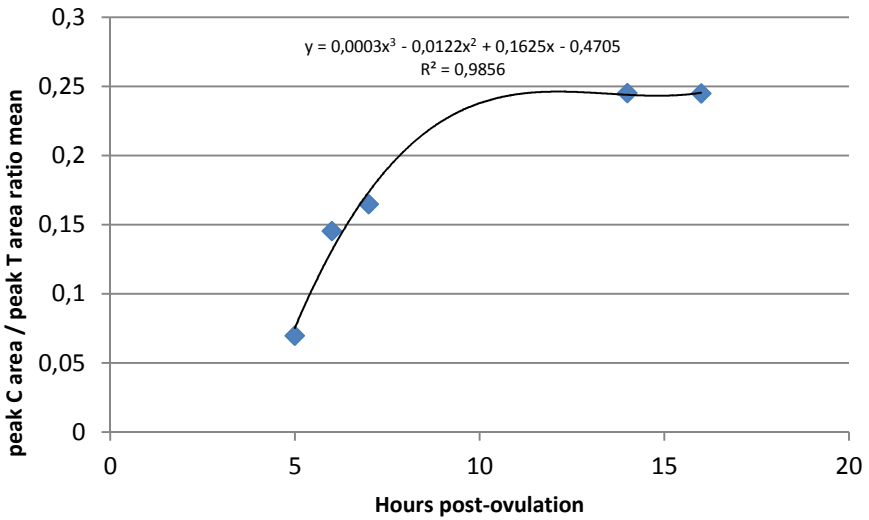
IMPACT

✓ Role of amorphous calcium carbonate (ACC)

FTIR (Fourier Transformed InfraRed spectroscopy)



- Evolution of crystalline $CaCO_3$ / total $CaCO_3$ ratio



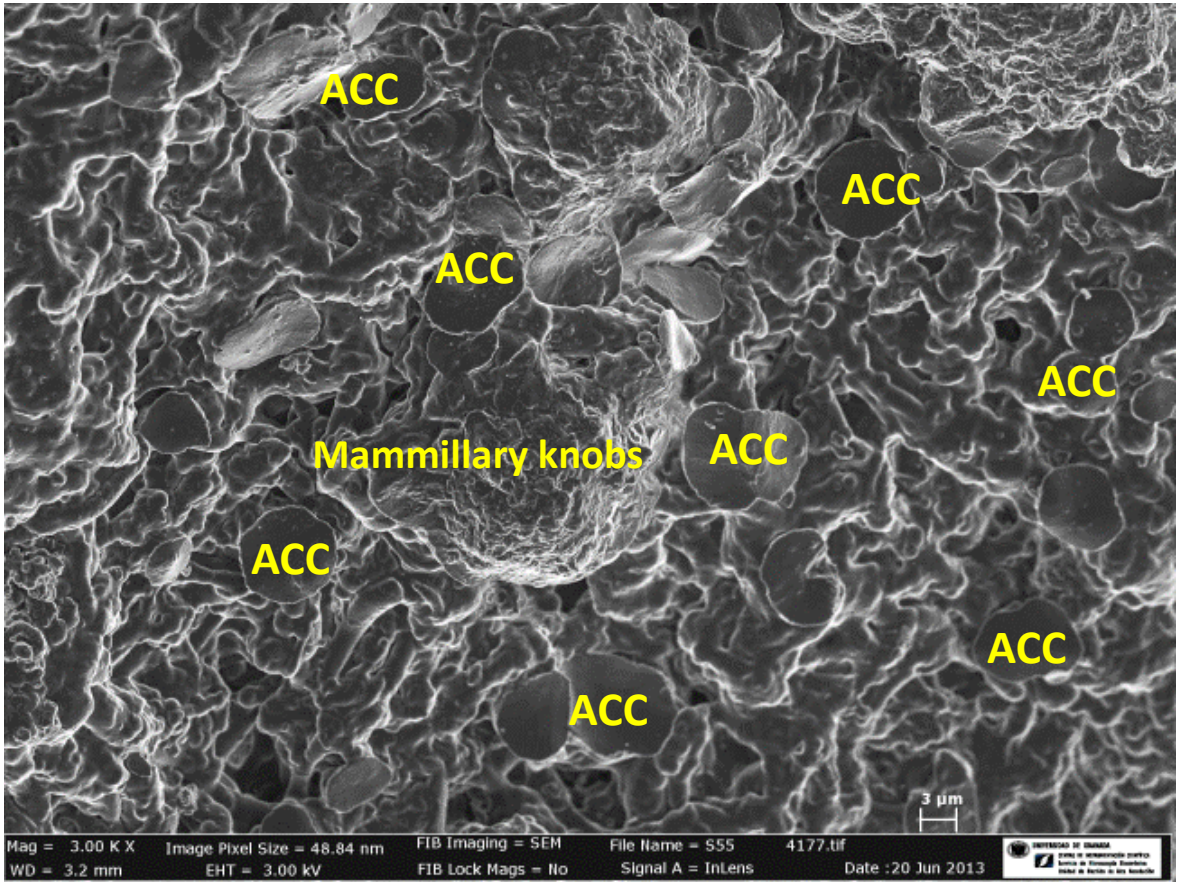
➢ **Presence of an amorphous transient phase at the front of mineralization, which is more important during initiation phase (95%) and still important in later phases (75%)**

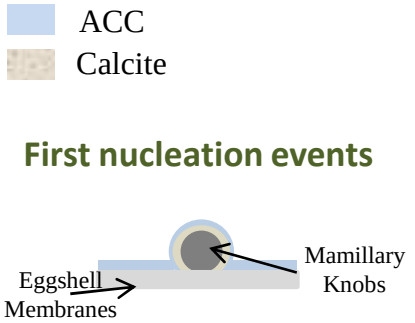
Rodriguez-Navarro et al., Journal of structural Biology, Submitted

In situ physical measurements

IMPACT

✓ Role of amorphous calcium carbonate (ACC)





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

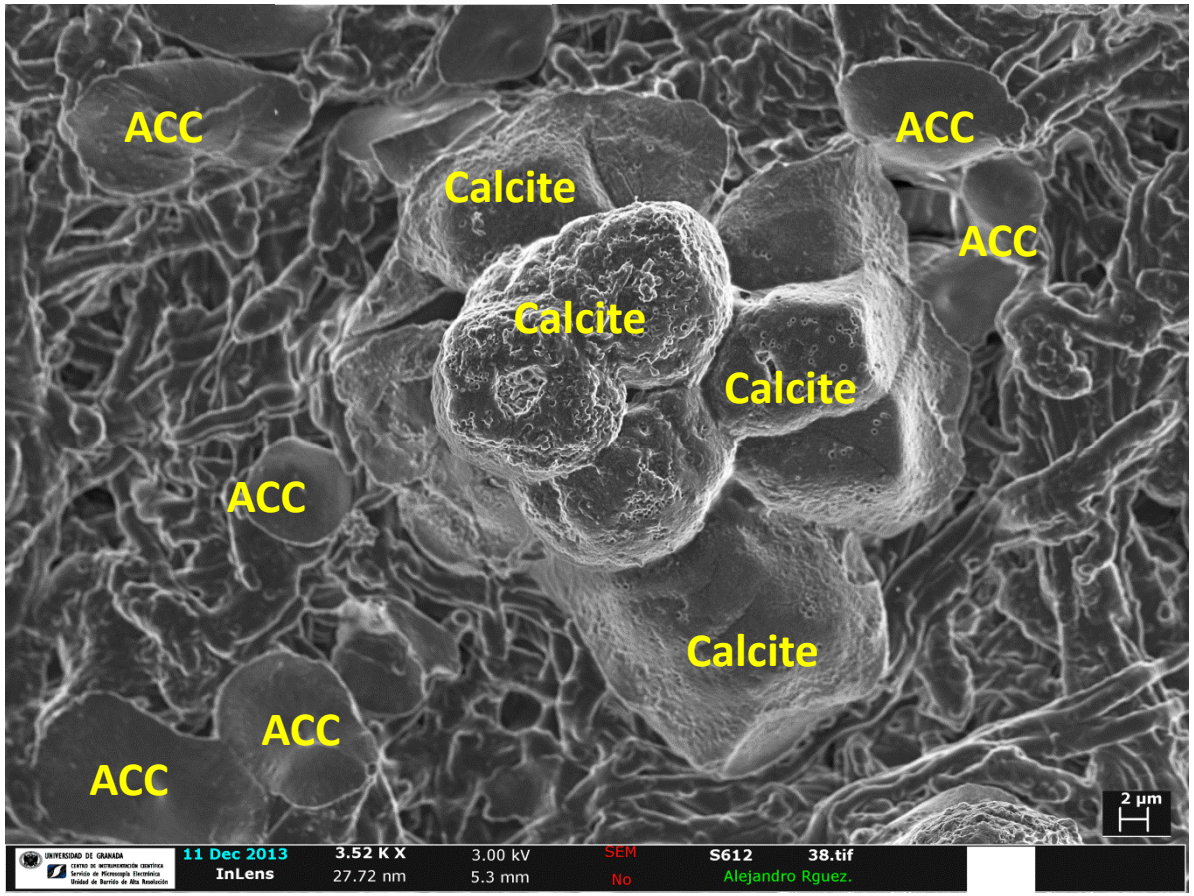
Images: A. Rodriguez-Navarro, University of Granada

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

In situ physical measurements

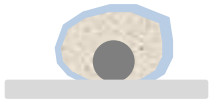
IMPACT

✓ Role of amorphous calcium carbonate (ACC)



ACC
Calcite

Calcite formation



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

Images: A. Rodriguez-Navarro, University of Granada

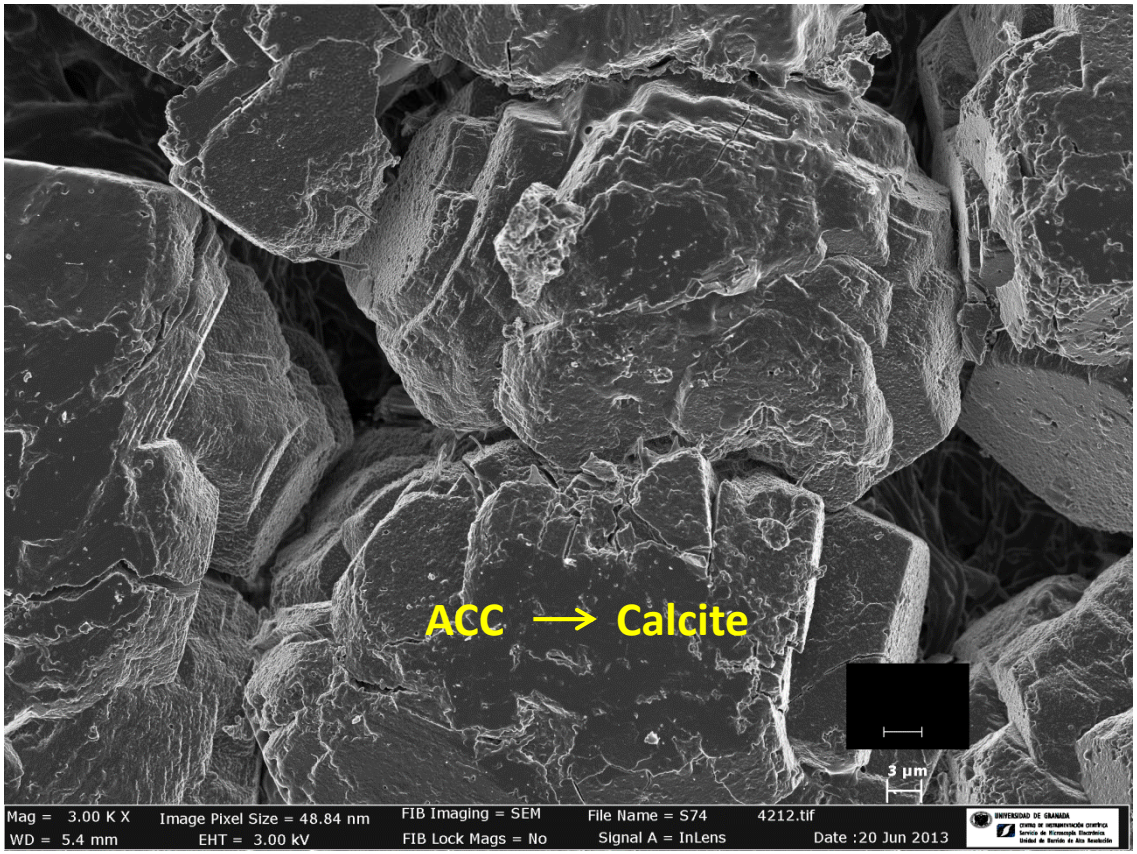
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In situ physical measurements

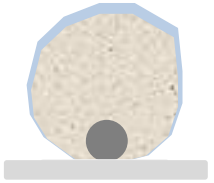
IMPACT

✓ Role of amorphous calcium carbonate (ACC)

ACC
Calcite



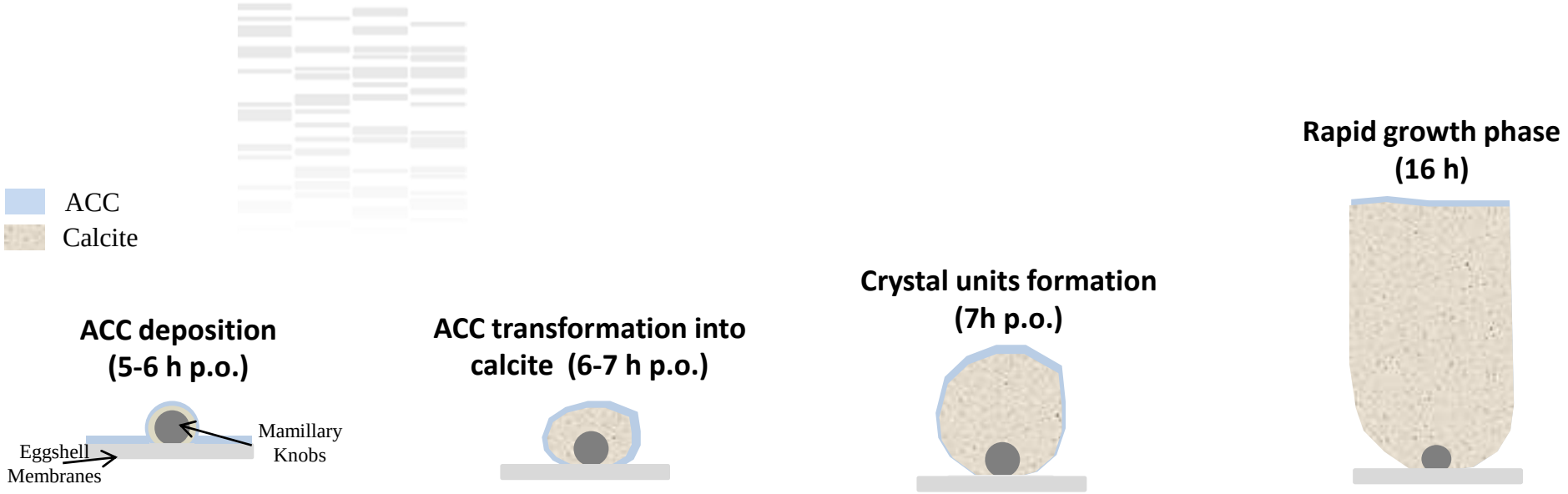
Large calcite crystal units deposition



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

Images: A. Rodriguez-Navarro, University of Granada

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



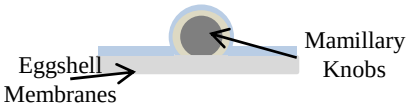
Identification and quantification of proteins at the four stages (mass spectrometry)

Statistical analysis at the four stages

Identification and quantification of matrix proteins

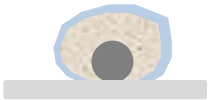
ACC
Calcite

ACC deposition (5-6 h p.o.)



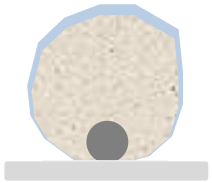
27 overabundant proteins

ACC transformation into calcite (6-7 h p.o.)



13 overabundant proteins

Crystal units formation (7h p.o.)



47overabundant proteins

Rapid growth phase (16 h)



70 overabundant proteins

Functions and biological activities of the identified matrix proteins ?



Literature, data mining and bioinformatics tools

↓

Classification in 3 different groups according to their potential functions

↙ ↓ ↘

- Associated to Mineralization process
- Involved in the regulation of activity of proteins
- Antimicrobial proteins

Functions of eggshell matrix proteins

❑ Proteins associated to shell mineralization process

✓ Proteins involved in the **biomineralization** of shell or other biominerals (bones, molluscan…)

✓**Calcium binding proteins (CaBPs)** interact with calcium to favour crystal nucleation or drive the morphology of crystals

- Identification of numerous novel CaBPs

  Numerous proteins with EF-hand and EGF-like calcium binding domains are present in the shell

✓ **Proteoglycans** et proteoglycan binding proteins

- proteoglycans have a negative charge to attract Ca2+ ions

✓ **Eggshell membranes** proteins

Functions of eggshell matrix proteins

❑ Proteins involved in the regulation of activity of proteins driving mineralisation

✓ Proteins involved in the **proper folding of the eggshell matrix**

- *An appropriate conformation of proteins is required to ensure calcium and mineral interactions and to ensure template to the mineralized structure*
  - *Molecular chaperone*
  - *Protein assisting folding*
  - *Proteins with interactive properties related to proteoglycans*

✓ **Regulation of the activity of proteins** related to the shell deposit

- *Shell mineralisation occurs in a non cellular milieu*
- *Direct action of proteins to inhibit or activate the molecular actors present in the milieu.*
  - *Molecular chaperone interact with proteins driving mineralisation*
  - *Proteases and protease inhibitors (specific and controlled role during calcification process, either by degrading proteins or regulating processing of proteins into their mature forms)*

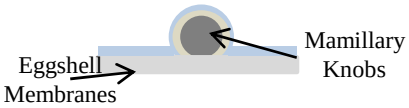
✓ Mineralisation depends of the **degree of protein phosphorylation** (Osteopontin, Hincke et al.)

- *Kinases*
- *Phosphatases*

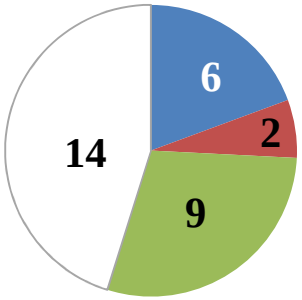
Identification and quantification of matrix proteins

ACC
Calcite

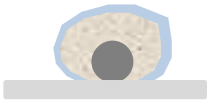
ACC deposition (5-6 h p.o.)



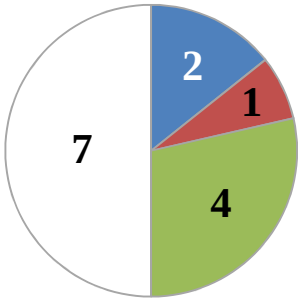
27 overabundant proteins



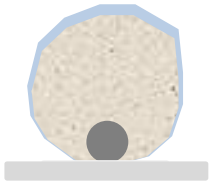
ACC transformation into calcite (6-7 h p.o.)



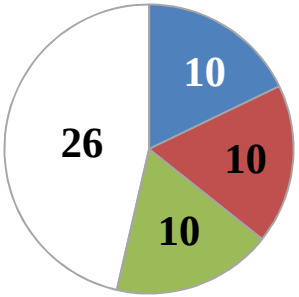
13 overabundant proteins



Crystal units formation (7h p.o.)



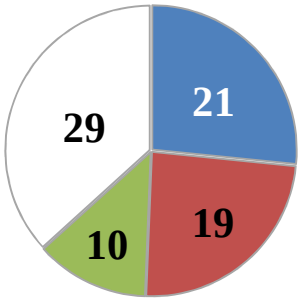
47overabundant proteins



Rapid growth phase (16 h)



70 overabundant proteins



Proteins associated to mineralisation

Proteins involved in the regulation of activity of proteins driving mineralisation

Antimicrobial proteins

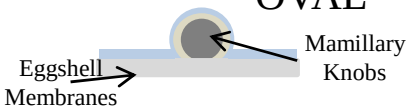
Others or unknown roles

Eggshell matrix proteins at key points of shell calcification process

ACC
Calcite

LYZ
OVOT
OC-17
OVM
OVAL

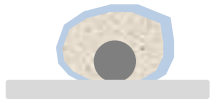
LOXL2
HAPLN3
CST3
HPX



ACC deposition (5-6 h p.o.)

EDIL3
ALB
OVM
CST3
HAPLN3
CLU
OCX-25

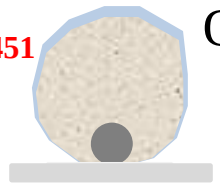
LYZ
OVOT
OC-17
OVAL



ACC transformation into calcite (6-7 h p.o.)

EDIL3
ALB
CLU
OC-116
CST3
LOC 428451
NUCB2
MFGE8
OCX-32
OCX-25

OVAL
LYZ
OVOT
OC-17



Crystal units formation (7h p.o.)

ALB
OVAL
CST3
CLU
NUCB2
OCX21
PPIB
GPC4
OCX25



LYZ
OC-116
OC-17
EDIL3
OVOT

Rapid growth phase (16 h)

XVII èmes Journées Françaises de Biologie des Tissus Minéralisés

Clermont-Ferrand (4-6 Février 2015)

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https://colloque.inra.fr/jfbtm2015

SUPER-BESSE Ski ressort

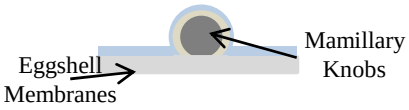


The French Mineralized Tissue Biology Days (JFBTM) are an opportunity to meet and exchange between researchers from more than 30 French research teams. A clear emphasis is put on the participation of young investigators, and it is often their first opportunity to present their work to the scientific community, including distinguished international speakers invited by the organizers.

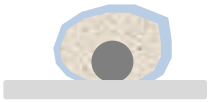
Identification and quantification of matrix proteins

ACC
Calcite

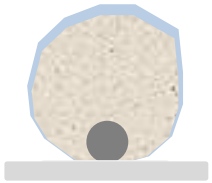
ACC deposition (5-6 h p.o.)



ACC transformation into calcite (6-7 h p.o.)



Crystal units formation (7h p.o.)



Rapid growth phase (16 h)



- 175 proteins with different abundance according to stages
- Hierarchical cluster analysis (10 clusters)

