Lexicon of histological structures found in the ovaries and during the oogenesis of the four-spot megrim, *Lepidorhombus boscii* (Risso, 1810)

HEUDE-BERTHELIN Clothilde
KELLNER Kristell
SAUGER Carine

August 2024

Cover photo: Picture of a female four-spot megrim (*Lepidorhombus boscii*). Personal collection. C. Sauger©

Lexicon of histological structures found in the ovaries and during the oogenesis of the four-spot megrim, *Lepidorhombus boscii* (Risso, 1810)

The digital version of this document is available on the Archimer Web site.

The digital version of the reading protocol that accompanies this document is available on the Archimer Web site.

Version	Date	Comments
1.0	March 2020	Conception of the document
1.1	April 2020	First review
1.2	June 2020	Alcian blue review
2.0	April 2023	Minor corrections + Summary sheets
2.1	August 2024	Change zp (*zona pellucida*) to zr (*zona radiata*)

Summary

Abbreviations

aoA : undischarged atretic oocyte
aoB : discharged atretic oocyte
bv : blood vessel
CA : cortical alveoli
cao : cortical alveoli oocyte
chr : condensed chromosome
chrom : chromatin
ct : connective tissue
E : unnatural emptiness (from the section's cut)
fc : follicular cell
gw : gonadal wall
ho : hydrated oocyte
ic : inner cytoplasm
Is : Intercellular space
L : lysis
ld : lipid droplet
mc : macrophage cell
N : nucleus
NC : Nucleo-Cytoplasmic
nl : nucleolus
oc : outer cytoplasm
og : oogonium
oih : oocyte in hydration
po1 : primary oocyte stage 1
po2 : primary oocyte stage 2
POF : Post-Ovulatory Follicle
SI : sexually immature
SM : sexually mature
som : somatic cell
T : theca
U : undetermined structure
vd : vitellus droplet
vi : vitellus
vtg1 : oocyte in stage 1 of vitellogenesis
vtg2 : oocyte in stage 2 of vitellogenesis
vtg3 : oocyte in stage 3 of vitellogenesis
zr : *zona radiata*

Introduction

During this study, the terminology used was that of Brown-Peterson *et al.* (2011), following the criteria set by the working groups of the International Council for the Exploration of the Sea (ICES) that described the different maturity phases for European stock species (ICES 2008, 2010, 2012, 2018, 2019).

For the description of the female germinal cells that will become gametes, the terms **oogonia** (before meiosis) or **oocyte** (meiosis has begun) will be used. An oocyte is characterized by an **ooplasm** encased in an **oolemma** (plasmic membrane) (Tyler and Sumpter, 1996). A ***zona radiata*** will also be present once the cell becomes dependent on follicle-stimulating hormone (FSH).

The term **follicle** will be used to designate an oocyte encased in somatic cells: **follicular cells** and **theca cells** (Tyler and Sumpter, 1996).

For the description of geminal cells development, the term **stage** will be used to designate the different gametogenesis stages (Brown-Peterson *et al.*, 2011) :

- Oogonia
- Primary growth oocytes
- Secondary growth oocytes
- Oocyte maturation
- Ovulation stage

The sexual maturity cycle (*Figure 1*) is split into two **states**. The Sexually Immature (**SI**) state and the Sexually Mature (**SM**) state. The terminology **phase** will be used for the gonadal development. The reproduction cycle of fish is divided into seven **phases** (Brown-Peterson *et al.*, 2011; ICES, 2012 ; 2018).

- Immature
- Developing
- Spawning
- Regressing
- Regenerating
- Omitted spawning
- Abnormal

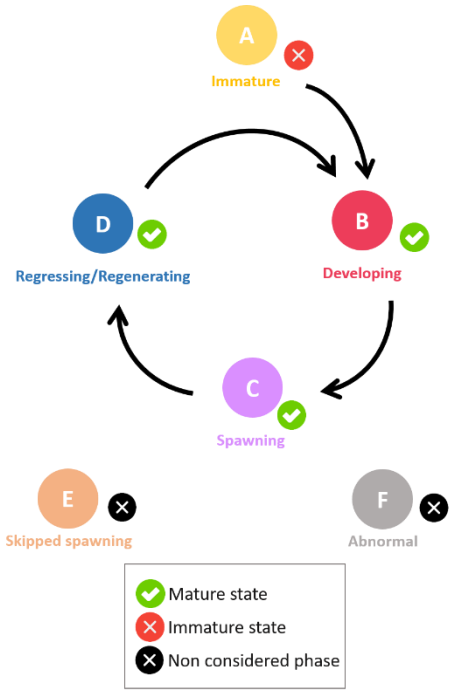


Figure 1 : Different phases found in the teleost maturation cycle, from the ICES (2018). The phases being: Immature (A), Developing (B), Spawning (C), Regressing/Regenerating (D), Skipped spawning (E) and Abnormal (F)

Sub-divisions/Sub-phases (Ba, Bb, Ca, Cb, Da, Db) should be used for specific particularities, meaning that their use is optional and must be evaluated for each species (ICES, 2012).

Table 1 : Macroscopic and microscopic descriptions of the phases in the reproductive cycle of female fish, from Brown-Peterson *et al.* (2011). Timing within each phase is species dependent. Some criteria listed for phases may vary depending on species, reproductive strategy, or water temperature. Subphases that apply to all fishes are listed; additional subphases can be defined by individual researchers. With CA = cortical alveoli, GVBD = germinal vesicle breakdown, GVM = germinal vesicle migration, OM = oocyte maturation, PG = primary growth, POF = post-ovulatory follicle complex, Vtg1 = primary vitellogenic, Vtg2 = secondary vitellogenic and Vtg3 = tertiary vitellogenic

Phase	Previous terminology	Macroscopic and histological features
Immature (never spawned)	Immature, virgin	Small ovaries, often clear, blood vessels indistinct. Only oogonia and PG oocytes present. No atresia or muscle bundles. Thin ovarian wall and little space between oocytes.
Developing (ovaries beginning to develop, but not ready to spawn)	Maturing, early developing, early maturation, mid-maturation, ripening, previtellogenic	Enlarging ovaries, blood vessels becoming more distinct. PG, CA, Vtg1, and Vtg2 oocytes present. No evidence of POFs or Vtg3 oocytes. Some atresia can be present. *Early developing subphase*: PG and CA oocytes only.
Spawning capable (fish are developmentally and physiologically able to spawn in this cycle)	Mature, late developing, late maturation, late ripening, total maturation, gravid, vitellogenic, ripe, partially spent, fully developed, prespawning, running ripe, final OM, spawning, gravid, ovulated	Large ovaries, blood vessels prominent. Individual oocytes visible macroscopically. Vtg3 oocytes present or POFs present in batch spawners. Atresia of vitellogenic and/or hydrated oocytes may be present. Early stages of OM can be present. *Actively spawning subphase*: oocytes undergoing late GVM, GVBD, hydration, or ovulation.
Regressing (cessation of spawning)	Spent, regression, postspawning, recovering	Flaccid ovaries, blood vessels prominent. Atresia (any stage) and POFs present. Some CA and/or vitellogenic (Vtg1, Vtg2) oocytes present.
Regenerating (sexually mature, reproductively inactive)	Resting, regressed, recovering, inactive	Small ovaries, blood vessels reduced but present. Only oogonia and PG oocytes present. Muscle bundles, enlarged blood vessels, thick ovarian wall and/or gamma/delta atresia or old, degenerating POFs may be present.

For the histological slides, fresh ovaries were placed in a Davidson solution before their inclusion in paraffin. 3 µm thick sections were cut using a microtome (HM330) and were either trichrome stained with a Prenant-Gabe (Gabe, 1968) solution or Alcian blue stained.

The histological pictures found in this lexicon were taken with a Nikon Eclipse 80i microscope using the NIS-Elements D (v. 5.02.03) software. Mean cell diameters and mean *zona radiata* widths were taken with an Olympus AX70 microscope using the Olympus CellSens© software, on a minimum of 20 oocytes found throughout multiple slides.

As seen in Table 1, different **phases** are correlated with the presence or absence of specific cell types. Following the terminologies and definitions of Brown-Peterson *et al.* (2011) and the ICES (2012; 2018). For the four-spot megrim (*Lepidorhombus boscii*), the different cell types described in this work will be shown in bold (**og**, **po1**, **po2**, **ldo**, **cao**, **vtg1**, **vtg2**, **oih**, **ho**, **POF**) while their matching *Figures* will be indicated in italic.

- Oogonium stage :
  - oogonium (**og**) (*Figure 6*)

- Primary growth oocytes :
  - Primary stage 1 oocyte (**po1**) (*Figures 7 to 9*)
  - Primary stage 2 oocyte (**po2**) (*Figures 10 & 11*)

- Secondary growth oocytes :
  - cortical alveoli oocytes with lipid droplets (**cao**) (*Figures 12 & 13*)
  - vitellogenic oocytes
    - subphase vtg1 : **vtg1** (*Figures 14 to 16*)
    - subphase vtg2 : **vtg2** (*NA*)

- Oocyte maturation :
  - nucleus migration :
  - germinal vesicle breakdown : oocyte enters metaphase I of meiosis
  - vitellus coalescence : oocyte in hydration (**oih**) (*NA*)
  - hydration : hydrated oocytes (**ho**) (*NA*)

- Ovulation stage : discharge of the hydrated oocytes into the ovary's lumen, leaving behind a post-ovulatory follicle (**POF**) (*NA*)

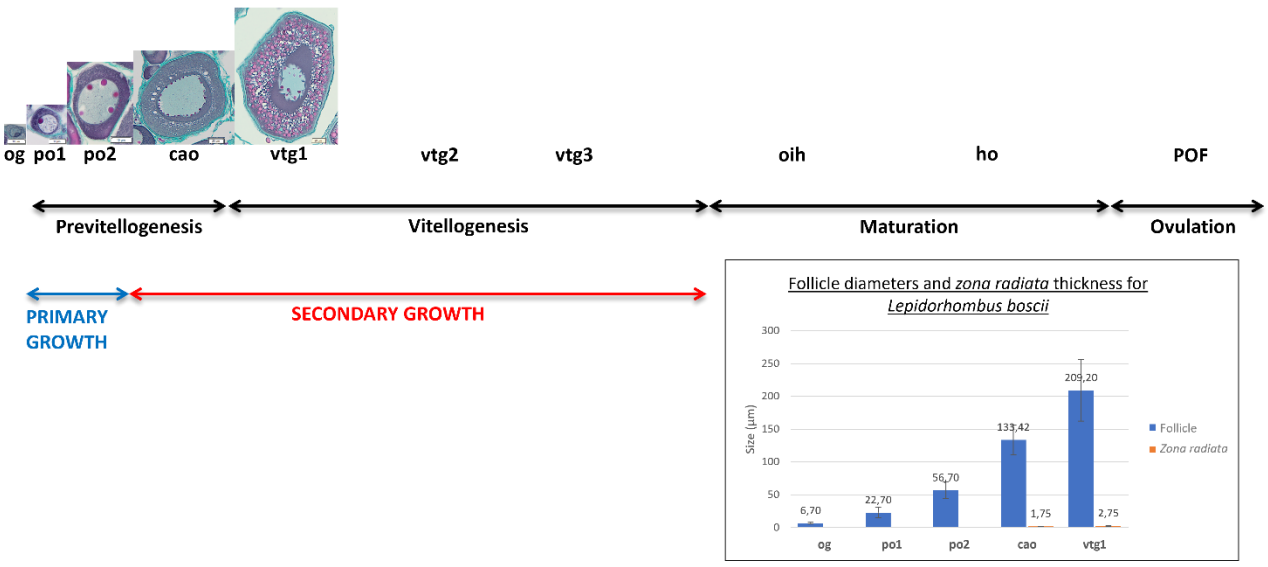


Figure 2 : Determination key of the different female germinal cells that can be found in the ovaries of *Lepidorhombus boscii* at different oogenesis stages. With average oogonium and oocyte diameters (µm ± standard deviation), and *zona radiata* widths (µm ± standard deviation)

Overall ovarian organization

The four-spot megrim is a **total spawner** species, meaning that the females will release all of their oocytes as a unique event during the breeding season.

Figure 3 : Dissected four-spot megrim, *Lepidorhombus boscii*, with the dorsal ovary uncovered

Criteria like the gonad wall (**gw**) thickness, connective tissue (**ct**) quantity, gonad vascularization (**bv**) or the surface area of the **lumen**, will depend on the maturity phase the fish is in. The histological cross sections will visually be quite different, with oocytes at various stages of gametogenesis.

Each gonad is organized concentrically (*Figure 4* and *Figure 5*), with the ovarian lamellas delimited by the germinal epithelium. The germinal cells (oogonia and oocytes) are found within these ovarian lamellas, inside follicles, at different stages of ovogenesis. The connective tissue (**ct**) will hold the germinal cells together, and the gonad wall (**gw**) can vary in thickness. Finally, the **lumen** is the naturally empty space found between the germinal epithelium lamellas inside the ovary.

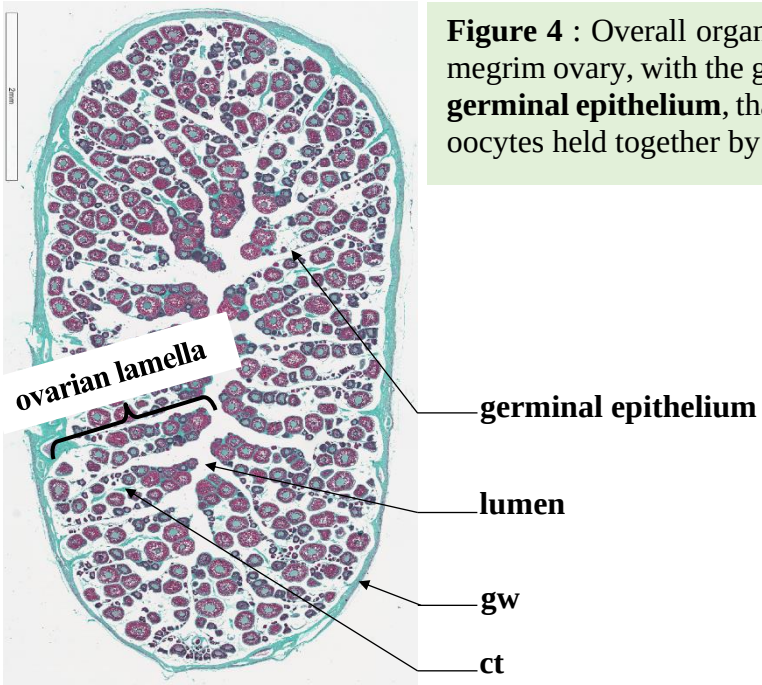


Figure 4 : Overall organization of a cross section of a four-spot megrim ovary, with the gonad wall (**gw**), the **lumen**, and a folded **germinal epithelium**, that makes the **ovarian lamella**, filled with oocytes held together by connective tissue (**ct**)

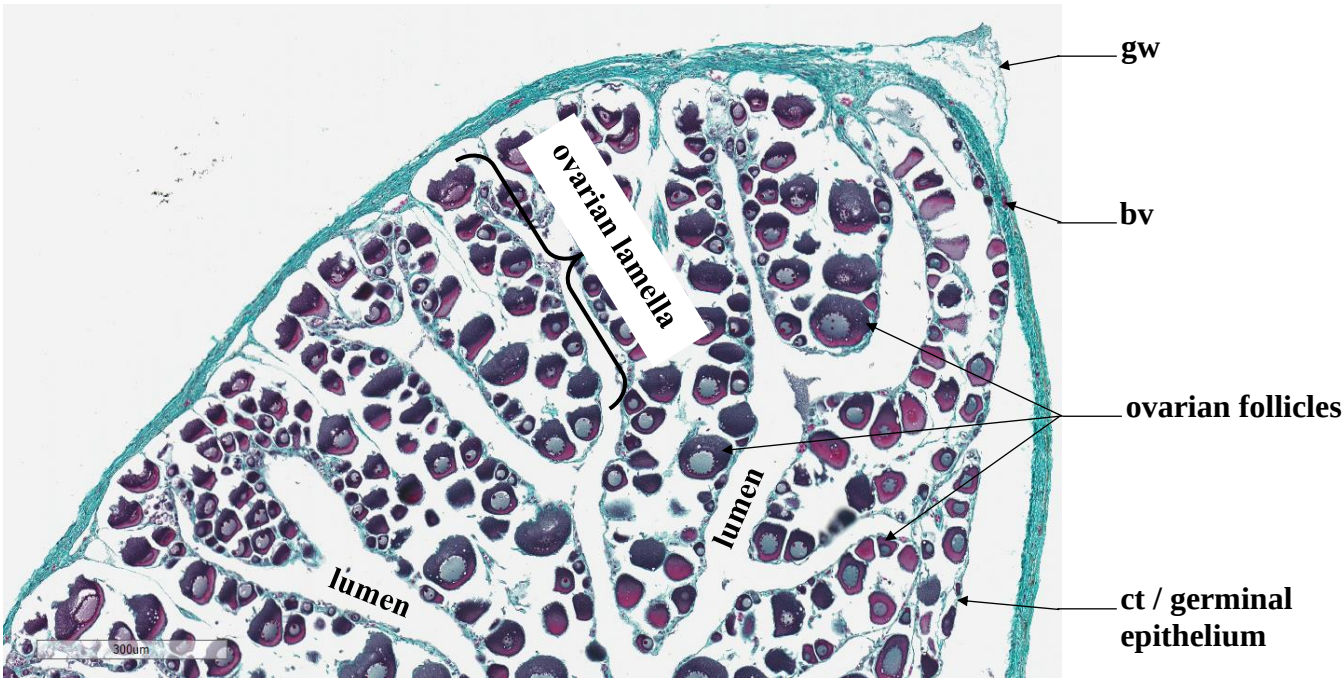
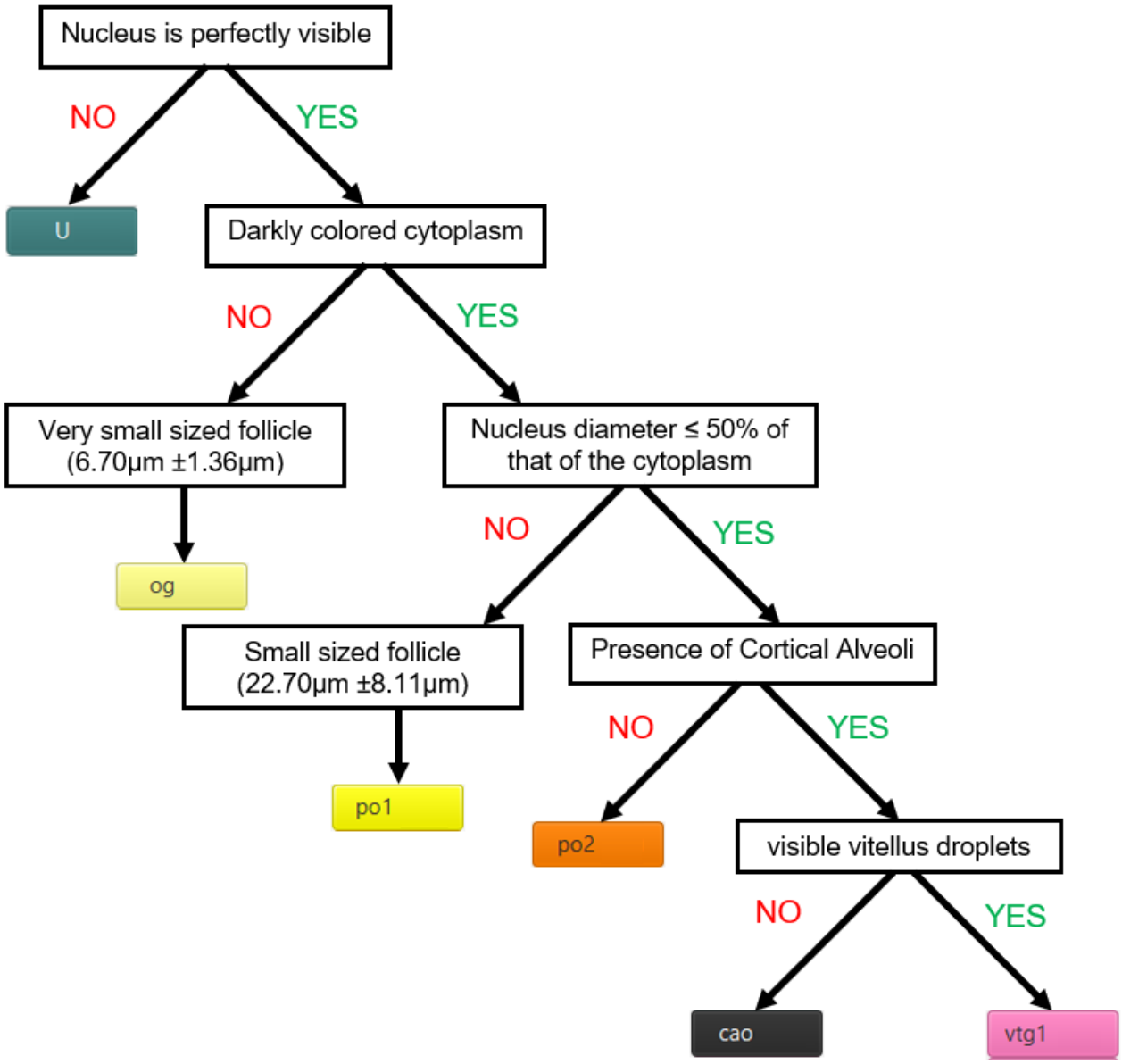


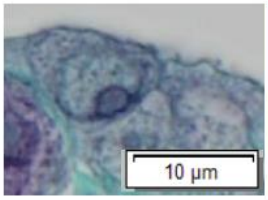
Figure 5 : Partial view of a four-spot megrim ovary cross section, with the gonad wall (**gw**), blood vessels (**bv**), the **lumen**, and oocytes in **follicles** held together by connective tissue (**ct**), organized inside **ovarian lamellas**

Decision Tree



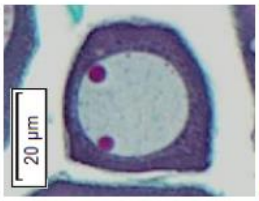
Follicle Summary Sheet

og



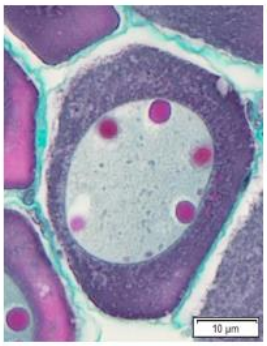
6.70µm (±1.36µm)
Light gray nucleus taking most of the space, barely visible light-colored cytoplasm with chromatin. Single nucleolus may be present

po1



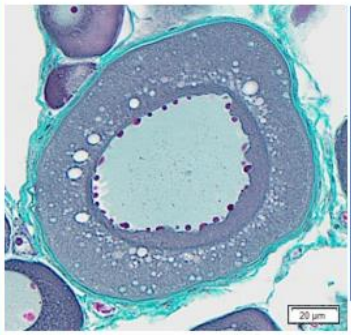
22.70µm (±8.11µm)
Nucleus diameter >50% of the cytoplasm's. Nucleus is visible, smooth and round, one or two nucleoli can be present. Dark colored cytoplasm

po2



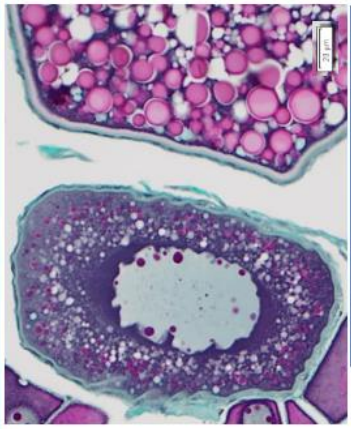
56.70µm (±12.37µm)
Nucleus is visible, smooth and round. Many nucleoli can be present. Lipid droplets may be present. Dark colored cytoplasm

cao



133.42µm (±22.45µm)
Cortical alveoli near the outer periphery, green *zona radiata* (1.75µm±0.27µm), scalloped nucleus with multiple nucleoli, growing white lipid droplets in the cytoplasm

vtg1



209.20µm (±47.16µm)
First appearance of small pink vitellus droplets amongst the cortical alveoli, under the thin greenish *zona radiata* (2.75µm±0.69µm). Scalloped nucleus with multiple nucleoli. Enlarging lipid droplets and vitellus droplets around the nucleus.

Oogonium (og)

An oogonium (**og**) has a pale nucleus (**N**), due to low chromosome condensation, and a high nucleo-cytoplasmic (NC) ratio (very little cytoplasm). Chromatin clusters can be seen near the edges of the nucleus. Inside this nucleus, a single large nucleolus can generally be found. The cytoplasm (**c**) is light-colored (*Figure 6*). Oogonia stem from germinal cells through gonial mitosis. They can be found alone or in a germinal niche within the germinal epithelium, accompanied by somatic cells (**som**).

Identification : It is uncommon to fall on this very small sized cell. The **nucleus is light-gray and fully apparent** while the **cytoplasm is barely visible and very light-colored**. A **single** light-colored and smooth **nucleolus** can be present. The diameter of the nucleus of an oogonium is greater than the surface area of the cytoplasm (>50 %).

Average size

og : 6.70µm (±1.36µm)

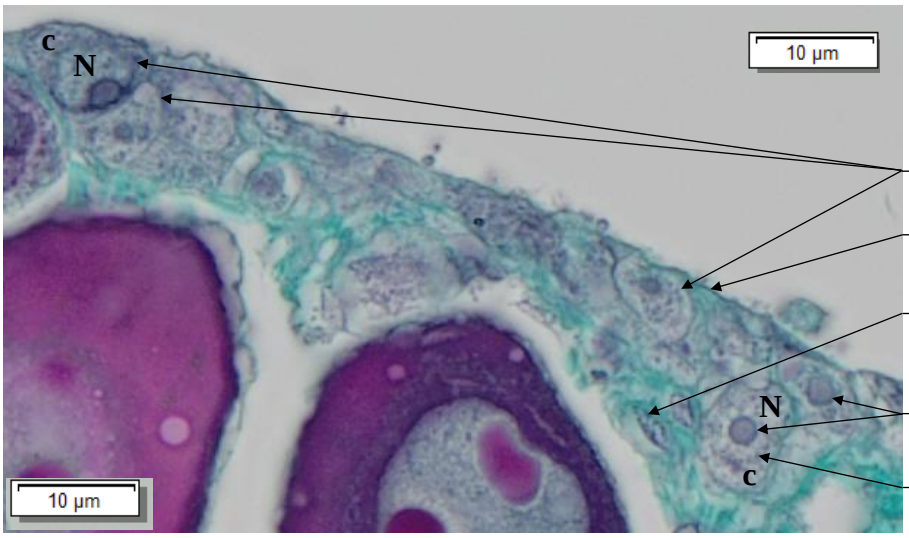


Figure 6 : Oogonia (**og**) and their apparent, decondensed, nucleus (**N**) with a single large nucleolus (**nl**) and clusters of chromatin (**chrom**). Light-colored cytoplasm (**c**). Presence of somatic cells (**som**)

Primary oocyte stage 1 (po1)

A primary oocyte stage 1 (**po1**) has a darker cytoplasm (**c**) compared to an oogonium (**og**), with a smaller **NC** ratio. The diameter of the cytoplasm is inferior or equal to 50 % of the nucleus' diameter. The nucleus (**N**) is spherical, smooth, with the possibility of a nucleolus to be present. As the cell grows in size, 2 or 3 nucleoli can be present, colored magenta from the trichrome stain.

Identification : This cell type is slightly bigger than an oogonium, but is still relatively small in size compared to the other cellular structures found inside the ovary. Falling on this follicle in stereology is still uncommon. The **nucleus must be fully visible, smooth,** and the **cytoplasm is darkly-colored**. The nucleus diameter of a **po1** is inferior or equal to 50 % of the cytoplasm's diameter, and can contain up to 4 nucleolus. If there is a hesitation between a **po1** and a **po2**, the cell size can be taken into consideration.

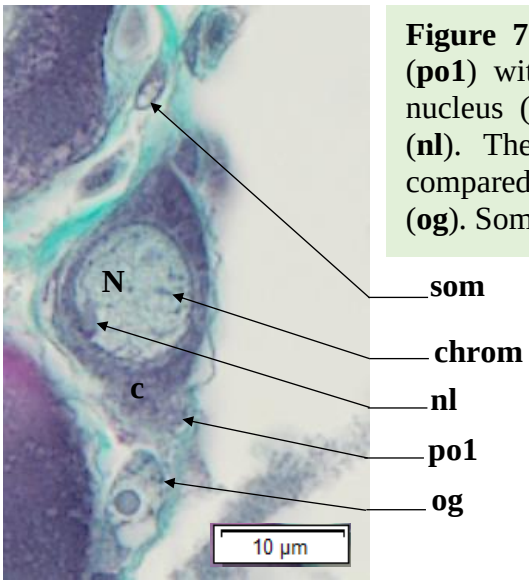


Figure 7 : Primary oocyte stage 1 (**po1**) with a spherical and smooth nucleus (**N**) and a single nucleolus (**nl**). The cytoplasm (**c**) is darker compared to that of an oogonium (**og**). Somatic cells (**som**) can be seen

Average size

po1 : 22.70µm (±8.11µm)

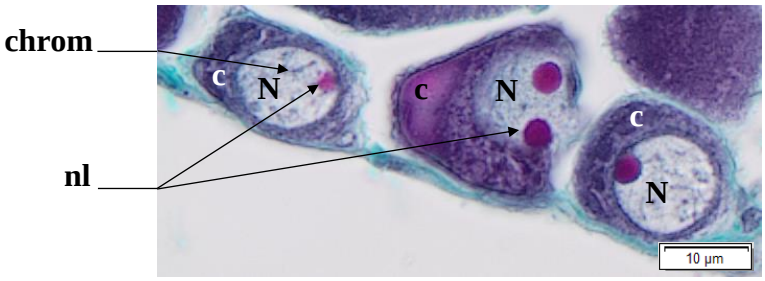


Figure 8 : Three premature stage 1 oocytes (**po1**) with spherical and smooth nucleus (**N**) containing 1 or 2 nucleoli (**nl**) and condensed chromosomes (**chrom**). The cytoplasm (**c**) is darker compared to that of an oogonium (**og**)

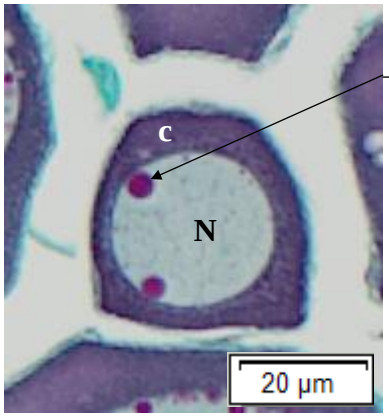


Figure 9 : Primary oocyte stage 1 (**po1**) with a spherical and smooth nucleus (**N**) with two nucleoli (**nl**) and a colored cytoplasm (**c**)

Primary oocyte stage 2 (po2)

Inside the cytoplasm (**c**) of a primary oocyte stage 2 (**po2**), lipid droplets (**ld**) (*cf Figure 11*) may be seen. It is also possible to see lampbrush chromosomes inside the nucleus, typically found in immature diplotene oocytes.

Identification : This ovarian follicle varies in size, between the **po1** and **cao** stage. This cell has a **single cytoplasm**, with a **spherical and smooth nucleus** that shows **multiple nucleoli against the nuclear envelope**. Lipid droplets may be seen inside the cytoplasm.

Average size

po2 : 56.70µm (±12.37µm)

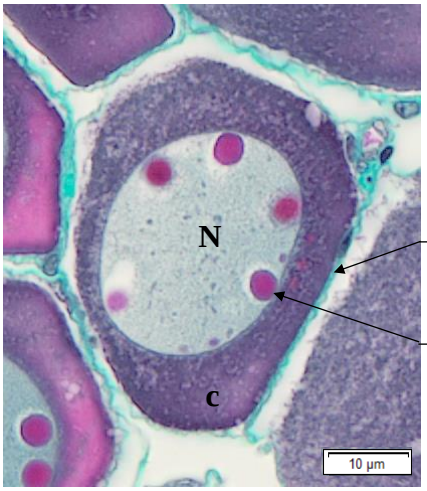


Figure 10 : A **po2**, with a spherical and smooth nucleus (**N**) filled with multiple nucleoli (**nl**) along its nuclear envelope. The theca (**T**) encases the cytoplasm (**c**)

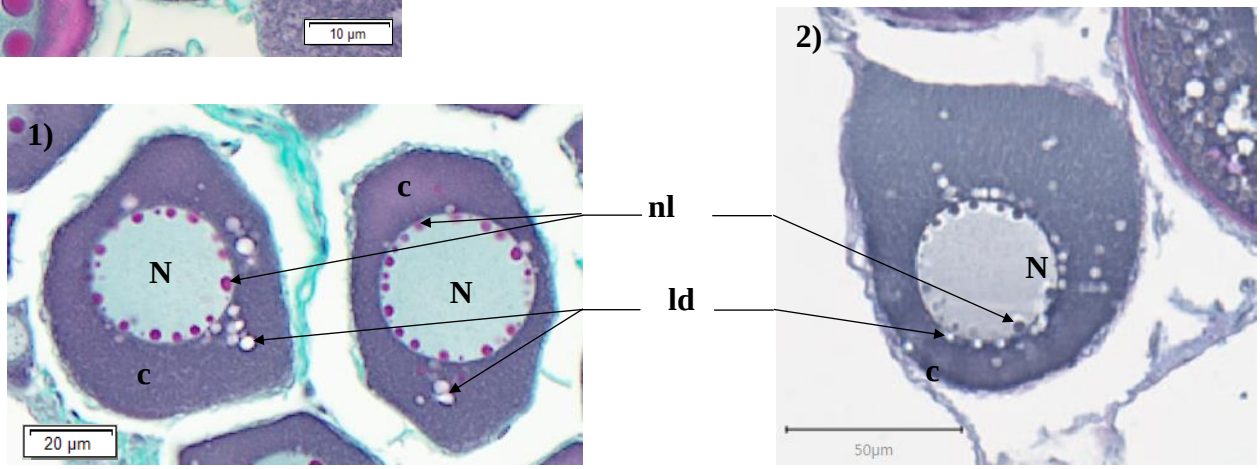


Figure 11 : Three **po2**, with a spherical and smooth nucleus (**N**) filled with multiple nucleoli (**nl**) along the inside of its nuclear envelope. Lipid droplets (**ld**) can be seen inside the cytoplasm (**c**). **1)** Trichrome stained ; **2)** Alcian blue stained **po2** showing that the vesicles in the cytoplasm are unstained : do not contain acidic polysaccharides and probably consist in lipid droplets

Cortical alveoli oocytes (cao)

An oocyte with cortical alveoli (**cao**) is defined by : (1) a cytoplasm (**c**) divided in two areas : internal cytoplasm (**ic**) and external cytoplasm (**ec**) ; (2) cortical alveoli (**CA**) present in the external cytoplasm, that first appear under the follicular cell layer; (3) a nucleus (**N**) in the center of the cytoplasmic mass, with a slightly scalloped nuclear envelope and multiple nucleoli (**nl**) against the nuclear envelope. Sometimes, it is possible to see lampbrush chromosomes inside the nucleus, typically found in immature diplotene oocytes. Finally, the *zona radiata* (**zr**) will start developing between the cytoplasm and the follicular cells (**fc**) + theca (**T**). Lipid droplets (**ld**) are still present in the **ec**, and will grow in size as they are gathered against the **ic**.

The **CA** will appear in the outer periphery of the outer cytoplasm. The Alcian blue coloration, as well as the trichrome stain, allows us to differentiate them from lipid droplets. With the trichrome stain, the **CA** will take a mint color, while they will take a purple color with the Alcian blue stain (*cf Figure 12*), revealing the presence of acidic polysaccharides.

Identification : An oocyte with cortical alveoli is primarily defined by a **two-zoned cytoplasm** with the **presence of cortical alveoli in the external cytoplasm**. The **nucleus in the center of the follicle contains multiple nucleoli** along its scalloped nuclear envelope, and the **nuclear envelope will become scalloped as the cell approaches the vitellogenic stage.** It is possible to see condensed chromosomes (small irregular dark-gray lines inside the nucleus), as lipid droplets grow in size. Finally, around the oocyte, **the *zona radiata* will start to form**.

Average size

cao : 133.42µm (±22.45µm)

zr : 1.75µm (±0.27µm)

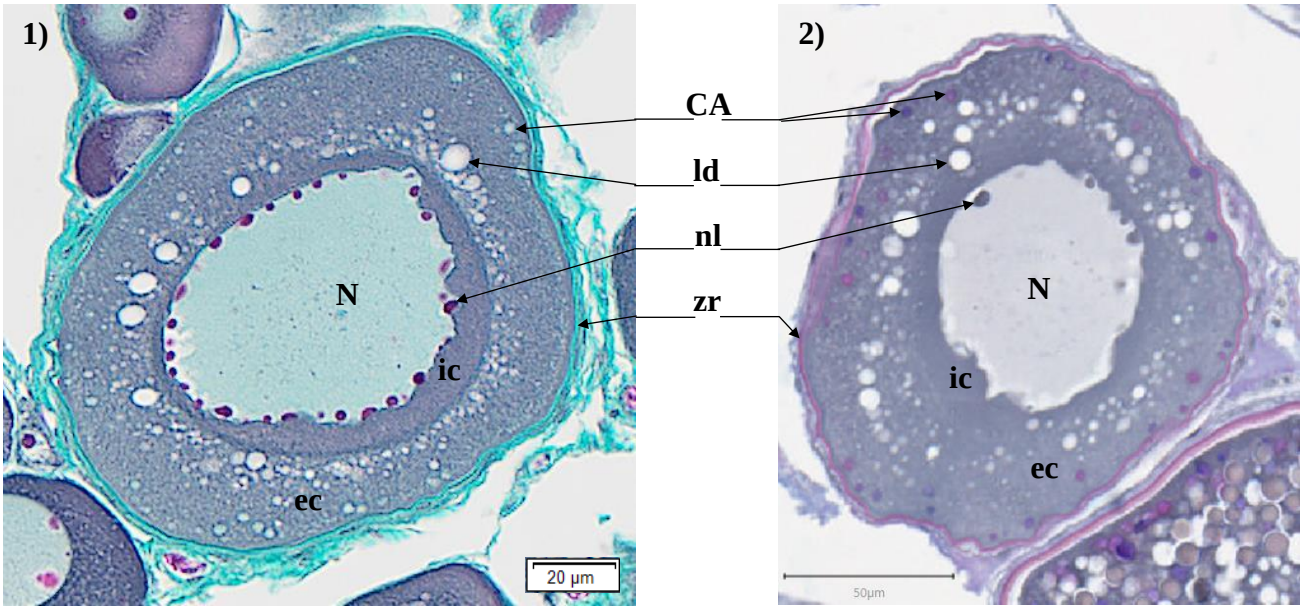


Figure 12 : Cortical alveoli oocyte (**cao**) with a scalloped nucleus (**N**) holding multiple nucleoli (**nl**). Cortical alveoli (**CA**) build-up inside the external cytoplasm (**ec**), against the *zona radiata* (**zr**). Lipid droplets (**ld**) can be seen on the inner periphery of the **ec**. **1)** Trichrome stained ; **2)** Alcian blue stained

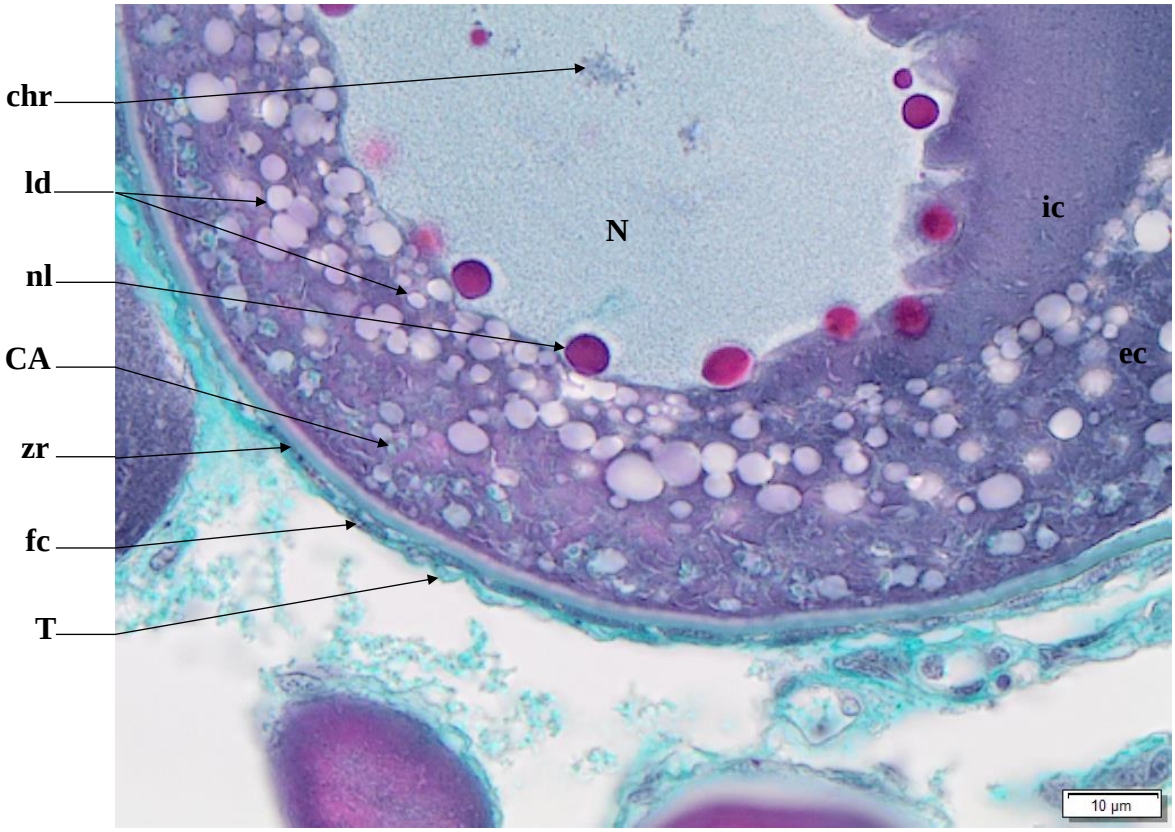


Figure 13 : Cell wall of an oocyte with cortical alveoli (**cao**). The nucleus (**N**) is scalloped, contains multiple nucleoli (**nl**) and condensed chromosomes (**chr**) can be seen. Lipid droplets (**ld**) build-up in the external cytoplasm (**ec**), against the internal cytoplasm (**ic**), while cortical alveoli (**CA**) form in the outer periphery of the **ec**. Beginning of the *zona radiata* (**zr**) growth, presence of follicular cells (**fc**) under the theca (**T**)

Oocyte in early vitellogenesis (vtg1)

An oocyte at the beginning of the vitellogenic stage (**vtg1**) is defined by the presence of vitellus droplets (**vd**), pink eosinophilic droplets, that first appear on the edge of the external cytoplasm (**ec**), under the *zona radiata* (**zr**). The nucleus (**N**) is still centered in the follicle, with multiple nucleoli (**nl**) along the scalloped nuclear envelope. The *zona radiata* (**zr**) is thin and green (not acidophilus).

As the cell matures, the vitellus droplets (**vd**), cortical alveoli (**CA**) and lipid droplets (**ld**) will grow in size and the internal cytoplasm (**ic**) will be pushed back towards the nucleus (**N**) (*cf Figure 16*).

Identification : This oocyte in early vitellogenesis is recognizable by the appearance of a **peripheral ring of pink vitellus droplets in the external cytoplasm**. At the **first appearance of a vitellus droplet**, the oocyte is considered to be in the **vtg1** stage. The **nucleus is still scalloped and centered inside the follicle** and the **vitellus droplets will run along the edge of the cell wall** (under the *zona radiata*). Even if the nucleus is not visible, if the vitellus droplets are visible, this follicle should be placed in the **vtg1** category.

Average size

vtg1 : 209.20µm (±47.16µm)

zr : 2.75µm (±0.69µm)

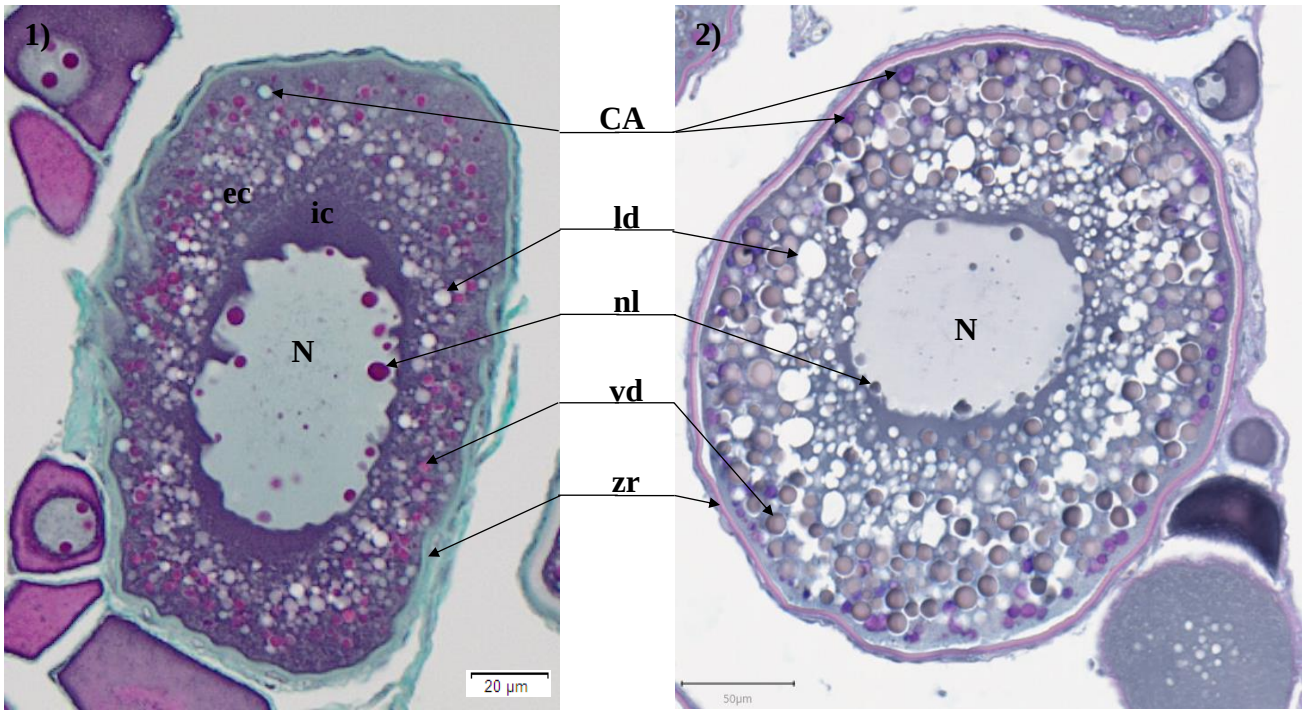


Figure 14 : Stage **vtg1** oocytes, with a scalloped nucleus (**N**) envelope containing multiple nucleoli (**nl**). Vitellus droplets (**vd**) will form on the edge of the external cytoplasm (**ec**). Cortical alveoli (**CA**) are amongst the **vd**, while the lipid droplets (**ld**) take up most of the space in the **ec**. The *zona radiata* (**zr**) is developing. **1)** Trichrome stained ; **2)** Alcian blue stained

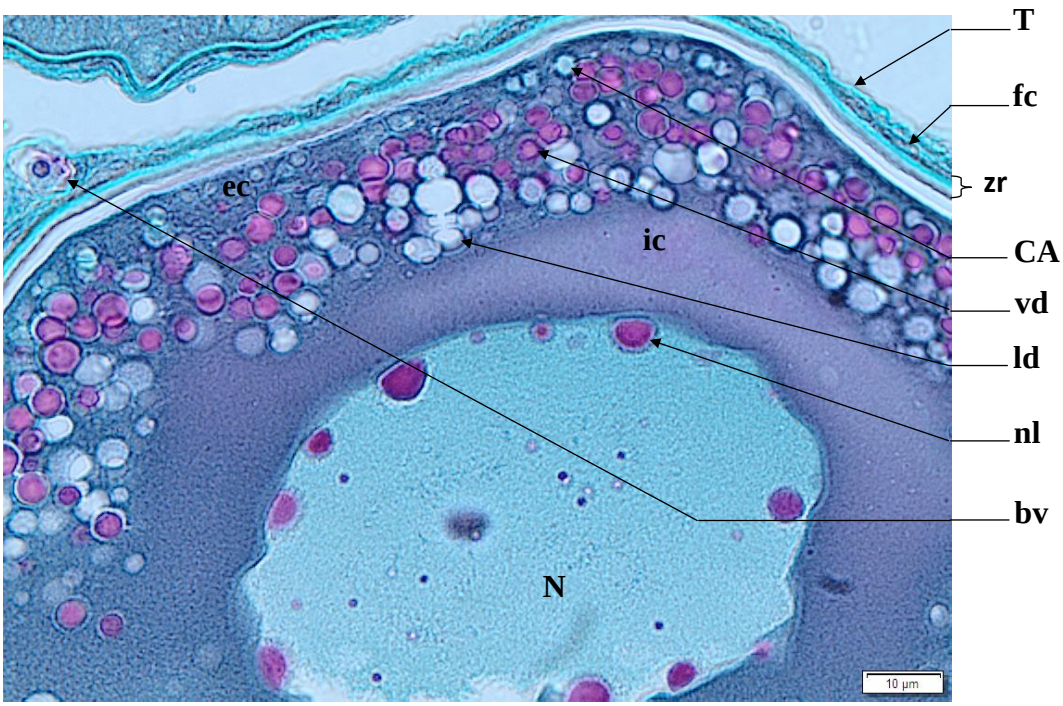


Figure 15 : Cell wall of a **vtg1** oocyte with a slightly scalloped nucleus (**N**) envelope containing multiple nucleoli (**nl**). Vitellus droplets (**vd**) will form on the edge of the external cytoplasm (**ec**), amongst the cortical alveoli (**CA**) and lipid droplets (**ld**). Follicular cells (**fc**) are present in the theca (**T**), with the *zona radiata* (**zr**) under these cell layers. The internal cytoplasm (**ic**) is still present, and blood vessels (**bv**) near the **T** can be seen

Figure 16 : A **vtg1** oocyte with enlarged vitellus droplets (**vd**), lipid droplets (**ld**), and cortical alveoli (**CA**) that have pushed the internal cytoplasm (**ic**) back towards the nucleus (**N**). The **N** is centered, scalloped and contains numerous nucleoli (**nl**)

Oocyte in vitellogenesis stage 2 (vtg2)

During the **vtg2** stage, …

Identification :

Average size

vtg2 : µm (±µm)

zr : µm (±µm)

N/A

Oocyte in vitellogenesis stage 3 (vtg3)

An ovarian follicle enters stage 3 of vitellogenesis (**vtg3**) when …

Identification :

Average size

vtg3 : µm (±µm)

zr : µm (±µm)

Oocyte in hydration (oih)

The …

Identification :

Average size

oih : µm (±µm)

zr : µm (±µm)

Hydrated oocyte (ho)

A hydrated oocyte (**ho**) is defined by …

Identification :

Average size

ho : µm (±µm)

zr : µm (±µm)

Post-Ovulatory Follicle (POF)

A Post-Ovulatory Follicle (**POF**) …

Identification :

Atresia (aoA & aoB) and Lysis (L)

Torres-Martìnez *et al.* (2017) define atresia as the degeneration, reabsorption, or even ablation, of ovarian follicles. In general, the first sign of atresia is the degeneration of the germinal vesicle of the oocyte, followed by the fragmentation of the *zona radiata* (**zr**) that will take an irregular appearance. This will lead to an hypertrophy of the follicular cells. There can be cell differentiations of the follicular cells (**fc**) into phagocytes and/or macrophages.

In this study, we will define two types of atresia. Oocytes in early atresia (**aoA**) and oocytes in late atresia (**aoB**). **aoA** are all germinal cells in lysis, from the oogonium (**og**) stage until the **undischarged** hydrated oocyte (**oh**) stage. They will always be encased in somatic cells (follicular cells (**fc**) and/or theca (**T**)). **aoB** are all hydrated oocytes in lysis that have been **discharged**, and are thus not encased in somatic cells anymore. Since an **aoB** is a cellular structure in lysis in the middle of the **lumen**, their shapes will often be warped, scattered and surrounded by lysis (**L**).

Identification : An **aoA** is a **follicle in lysis**. This means that the **oocyte is still encased inside a theca**, **within the ovarian lamella**.

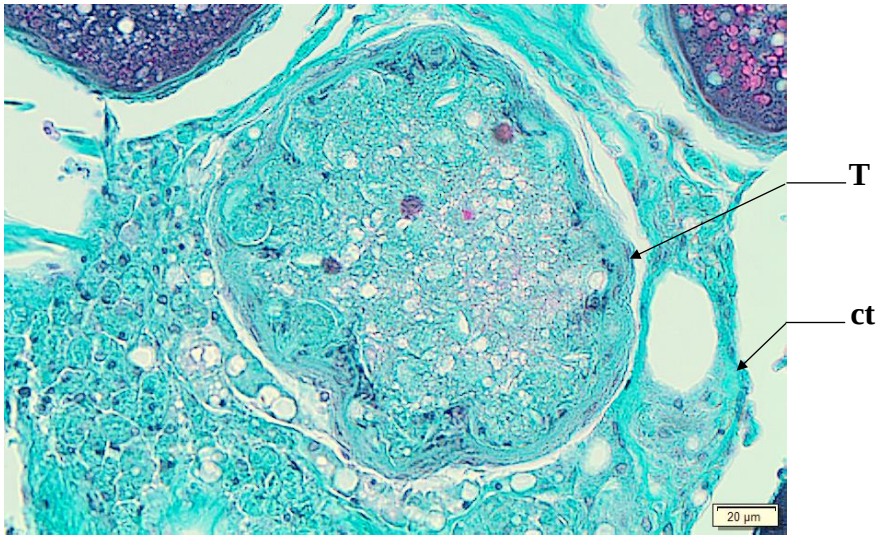


Figure 17 : Oocyte in early atresia (**aoA**). The oocyte has not been discharged into the **lumen**, and the follicle is still within the connective tissue (**ct**) of the ovarian lamella, encased by its theca (**T**)

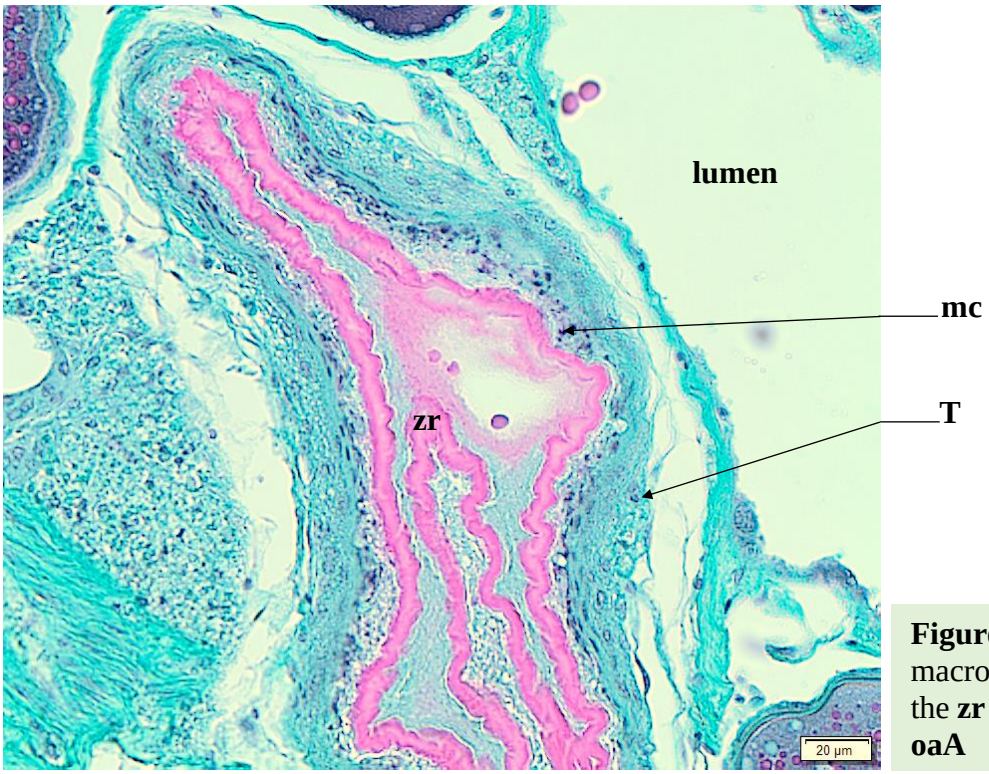


Figure 18 : Zoom on macrophages (**mc**) between the **zr** and the theca (**T**) of an **oaA**

Identification : An **oaB** is an **oocyte in lysis**.

N/A

Identification : Lysis can be difficult to identify, especially with the presence of atresia. They can be found **anywhere inside the gonad**, **at any maturity phase**. This **cluster of cells in lysis** can contain macrophages, are of **different shapes and sizes**, and do **not possess a theca and/or *zona radiata***

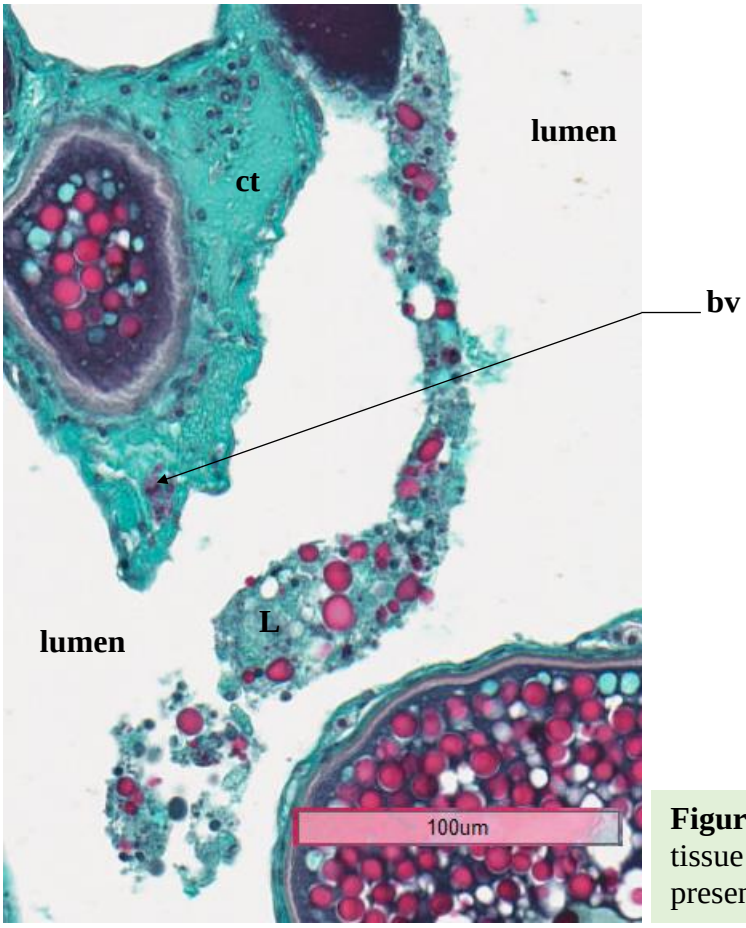


Figure 19 : Lysis (**L**) in the **lumen**. Connective tissue (**ct**) and blood vessels (**bv**) are also present

Connective tissue (ct) & Gonadal wall (gw)

The connective tissue (**ct**) links all of the ovarian structures together to form the ovarian lamellas. Follicles can be found inside the ovarian lamellas, encased in **ct**.

Connective tissue (**ct**) is made of green-colored cells, just like the cells that make up the gonad wall (**gw**). Gonad wall cells are muscular tissue while **ct** is not. Be wary not to mix the both up! Even if a sampling point falls on a very small piece of **ct** in the middle of a wide expanse of intercellular space (**Is**), this sampling point will be classified under **ct**.

The gonad wall (**gw**) will have the same green tint as the **ct**. Muscle bundles and blood vessels (**bv**) can be found in the **gw**. If a sampling point falls on muscular tissue within the **gw**, then the sampling point will be put into the **gw** category. On the other hand, if a sampling point within the **gw** falls on a blood vessel (**bv**), then this structure will be classified under **bv**.

The boundary of the **gw** starts from the outer most cell layer of the gonad and ends at the inner most cell layer of the gonad wall (**gw**), just before entering into the ovarian lamellas. If the **gw** has been stretched, ripped or spread, but the sampling point lands between the inner and outer most cell layers before the ovarian lamellas, then the sampling point will be classified under the **gw** category. Do not hesitate to unzoom in order to obtain a more general view of where the sampling point is in the gonad. This will allow the reader a better view of where the **gw** ends and the **ct** starts.

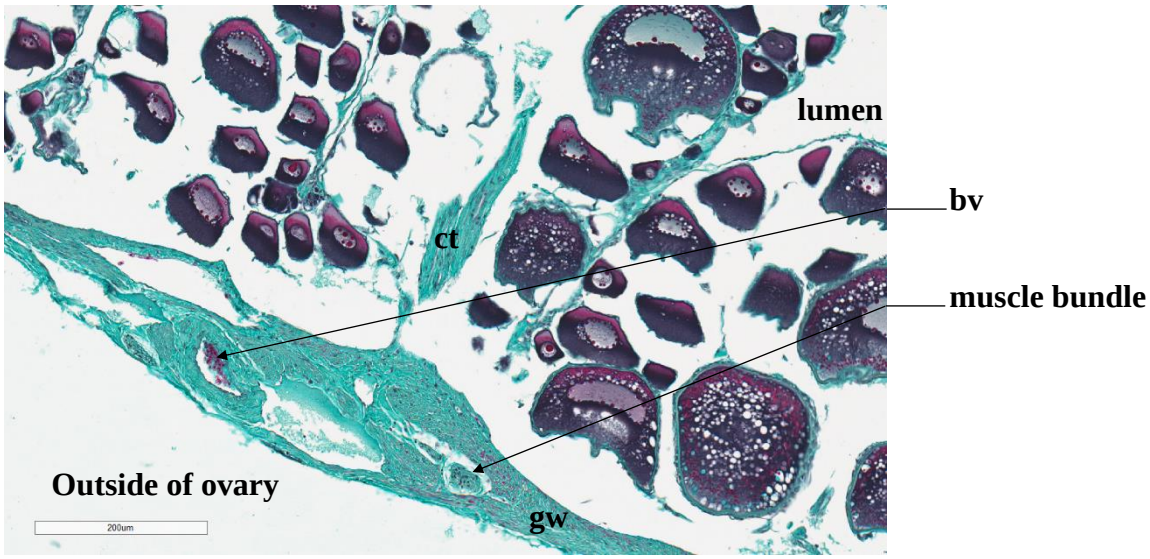


Figure 20 : Cross section of a four-spot megrim ovary with its gonad wall (**gw**). The ovarian lamellas are held together by connective tissue (**ct**), delimiting the **lumen** and the outside of the ovary. Blood vessels (**bv**) and **muscle bundles** can be seen

Blood vessel (bv)

Blood vessels (**bv**) are more or less small, with a size that can vary from that of a single, cell to over 100µm.

Found within the theca (**T**), inside connective tissue (**ct**) or the gonad wall (**gw**), blood vessels (**bv**) are identifiable by the presence of blood cells (small red cells with a darker nucleus). If a sampling point falls in the « white zone » inside a cavity containing blood cells, this sampling point will be classified under the **bv** category.

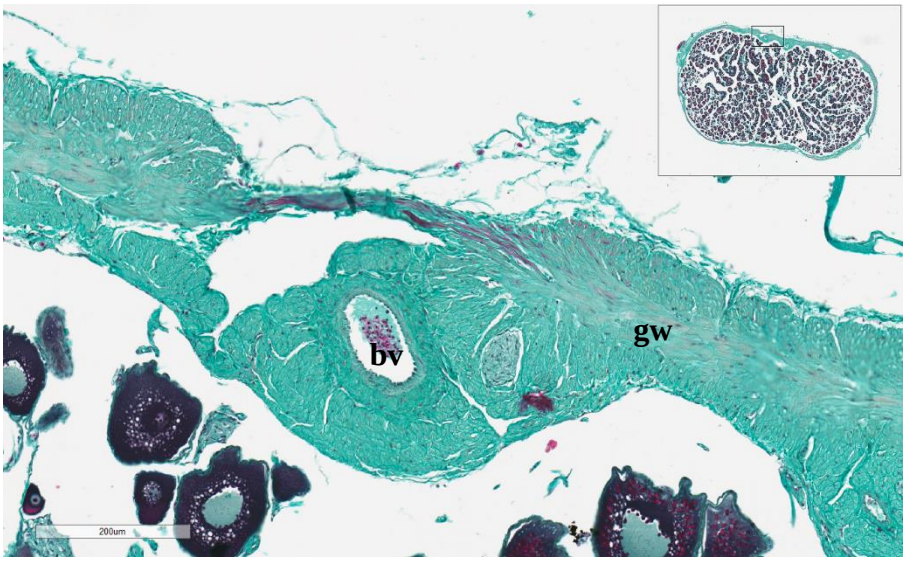


Figure 21 : Presence of blood vessels (**bv**) in the gonadal wall (**gw**)

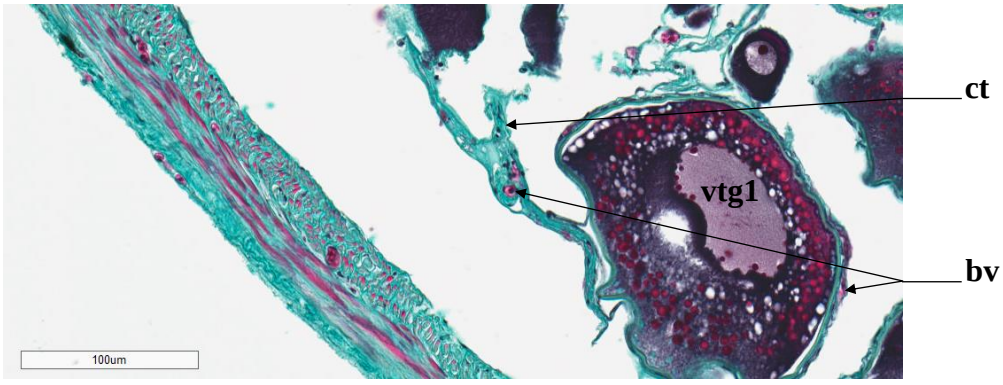


Figure 22 : Presence of a blood vessel (**bv**) in the Theca (**T**) of a **vtg1** and inside connective tissue (**ct**)

Bibliography

Anderson, **E.**, 1969. Oocyte-follicle Cell Differentiation in Two Species of Amphineurans (Mollusca), *Mopalia mucosa* and *Chaetopleura apiculata*. J. Morphol. 129, 89-126.

Anderson, **E.**, 1968. Cortical alveoli formation and vitellogenesis during oocyte differentiation in the Pipefish, *Syngnathus fuscus*, and the Killifish, *Fundulus heteroclitus*. J. Morphol. 125, 23-60.

Aragón, **L.**, **Aranda**, **G.**, **Santos**, **A.**, **Medina**, **A.**, 2010. Quantification of ovarian follicles in bluefin tuna *Thunnus thynnus* by two stereological methods. J. Fish Biol. 77, 719-730.

Brown-Peterson, **N.J.**, **Wyanski**, **D.M.**, **Saborido-Rey**, **F.**, **Macewicz**, **B.J.**, **Lowerre-Barbieri**, **S.K.**, 2011. A Standardized Terminology for Describing Reproductive Development in Fishes. Mar. Coast. Fish. 3, 52-70.

Domínguez-Castanedo, **O.**, **Uribe**, **M.C.**, **Rosales-Torres**, **A.M.**, 2016. Morphological development of the structures related to annualism in the ovarian follicle of the killifish *Millerichthys robustus* (Costa,1995) (Teleostei: Cyprinodontiformes). J. Morphol. 277, 1219-1230.

Gabe, **M.**, 1968. Techniques Histologiques, 2ème ed. Masson et C., Paris.

ICES, 2008. Report of the Workshop on Maturity Ogive Estimation for Stock Assessment (WKMOG), 3-6 June 2008, Lisbon, Portugal. ICES CM2008/ACOM:33. 72 pp.

ICES, 2010. Report of the ICES/HELCOM Workshop on Flatfish in the Baltic Sea (WKFLABA), 8-11 November 2010, Öregrund, Sweden. ICES CM 2010/ACOM:68. 85pp.

ICES, 2014. Report of the Workshop for maturity staging chairs (WKMATCH), 11-15 June 2012, Split, Croatia. ICES CM 2012/ACOM:58. 57 pp.

ICES, 2018. Report of the Workshop for Advancing Sexual Maturity Staging in Fish (WKASMSF), 30 April - 4 May 2018, ICES Headquarters, Copenhagen, Denmark. ICES CM/EOSG: 38. 75 pp.

ICES, 2019. Working Group on Biological Parameters (WGBIOP), 7-10 October 2019, Lisbon, Portugal. ICES Scientific Reports. 1:85. 93 pp.

Lowerre-Barbieri, **S.K.**, **Brown-Peterson**, **N.J.**, **Murua**, **H.**, **Tomkiewicz**, **J.**, **Wyanski**, **D.M.**, **Saborido-Rey**, **F.**, 2011. Emerging Issues and Methodological Advances in Fisheries Reproductive Biology. Mar. Coast. Fish. 3, 32-51

Robson, **S.M.**, 2004. Age, growth, reproductive biology and population dynamics of the common megrim *Lepidorhombus whiffiagonis* (Walbaum, 1792) from off the west coast of Ireland.Unpublished thesis (Doctor of Philosophy in Fisheries Biology), Galway-Mayo Institute of Technology.

Sauger, **C.**, **Quinquis, J.**, **Dubroca**, **L.**, **Parrad, S.**, **Kellner**, **K.**, **Heude-Berthelin, C.**, 2019. Sample collection protocol for the extraction of female gonads in the megrim (*Lepidorhombus spp.*) for maturity staging through histology.

Sauger, **C.**, **Dubroca**, **L.**, **Villain-Naud, N., Kellner**, **K.**, **Heude-Berthelin, C.**, **Elie**, **N.**, 2020. Protocol for the determination of histological structures found in the ovaries and during the oogenesis of the four-spot megrim, *Lepidorhombus boscii* (Risso, 1810). IFREMER

Torres-Martínez, **A.**, **Hernández-Franyutti**, **A.**, **Uribe**, **M.C.**, **Contreras-Sánchez**, **W.M.**, 2017. Ovarian structure and oogenesis of the extremophile viviparous teleost *Poecilia mexicana* (Poeciliidae) from an active sulfur spring cave in Southern Mexico. J. Morphol., Wiley Periodicals 278, 1667-1681.

Tyler, **C.R.**, **Sumpter**, **J.P.**, 1996. Oocyte growth and development in teleosts. Rev. Fish Biol. Fish., Chapman & Hall 6, 287-318.