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WGMEGS Manual for the AEPM and DEPM estimation of fecundity in mackerel and horse mackerel

Version 11.0

The Working Group on Mackerel and Horse Mackerel Egg surveys

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CIEM Conseil International pour l'Exploration de la Mer

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Contents

1 Changes in fecundity and atresia estimation methods for mackerel and horse mackerel since 2007

2007	2010	2013	2016
Mackerel			
On board, ovaries are weighted and pipette subsamples of known volume and weight taken and fixed in formaldehyde solution.		Samples are taken for screening for spawning markers and atresia. The results from the histology are used to decide which samples will be analysed for fecundity and which for atresia. Only samples that contain spawning markers and/or early alpha atresia will be embedded from the cassettes for further atresia analyses.	
Gravimetric fecundity estimation. Sub samples preserved in 3.6% buffered formaldehyde. F = O * C * S (F = fecundity, O = Ovary weight, C = count follicles > 185 μm in subsample, S = subsample weight) (Hunter *et al*., 1989)		Each cruise will collect 10 samples of one fish (stages 3 to 6) for the fecundity ring test.	
Stereometric method		Ovaries need to be pierced with a fine needle before fixation in formaldehyde.	Cut off both ends (1–2 cm depending on the size of the ovary) before fixation in formaldehyde.
H&E -PAS – Toluidine blue			

2007	2010	2013	2016
Mackerel and Horse mackerel			
In 2007 count all oocytes > 185 μm and measure 1/3 of the oocytes.	Measure the oocyte diameters automatically using ImageJ software provided for the fecundity analysis. Count all the oocytes > 185 μm in the sample that are not automatically detected.	Measure the oocyte diameters automatically using ImageJ software provided for the batch fecundity analysis. Count and measure all the oocytes > 500 μm in the sample that are not automatically detected.	
Spawning markers: hydrated, > 5 POFs	ImageJ and macros will be made available during the workshop to all participants and they should use this for analysis of the samples.		
	Distribute the sample randomly in the tray. If it is not possible to separate the oocytes, exclude the sample for fecundity analysis.		If possible, try using an ultrasound pen to separate the oocytes in whole mounts.
	For 10 mackerel and 10 horse mackerel (two from each survey), six subsamples will be taken and used for calibration between the institutes.		Five mackerel slides will be provided for POF staging calibration between institutes.
	Spawning markers: hydrated (> 800 μm) oocytes or POFs, or all oocytes diameter < 400 μm in the whole sample		Examine the screening sample for the most advanced oocyte stage, POFs, hyaline eggs, early alpha atresia, massive atresia, if it is spent and if it should be discarded. Oocyte development stages are changed to Stage 1–5. Hyaline eggs are taken out of the oocyte stage as well as the spent stage.

2007	2010	2013	2016
Mackerel and Horse mackerel			
			New screening and POFs staging template
Horse mackerel			
Gravimetric fecundity estimation. Sub samples preserved in 3.6% buffered formaldehyde. F = O * C * S (F = fecundity, O = Ovary weight, C = count follicles > 185 μm in subsample, S = subsample weight) (Hunter *et al*., 1989)	IPMA will perform a DEPM survey for horse mackerel.	From 2013 and onwards, no samples for potential fecundity are collected. Only DEPM adult parameter samples will be collected.	
On board, ovaries are weighed and pipette subsamples of known volume and weight taken and fixed in formaldehyde solution	Batch fecundity: Gravimetric method. Take whole fixed ovary to the lab, take 3 subsamples, weigh and count all the hydrated oocytes in subsample.		
	Spawning fraction: migratory nucleus, hydrated, POF's		Spawning fraction based on POFs; POFs staging/ageing based on both histomorphological and metric (POFs cross-sectional area) criteria

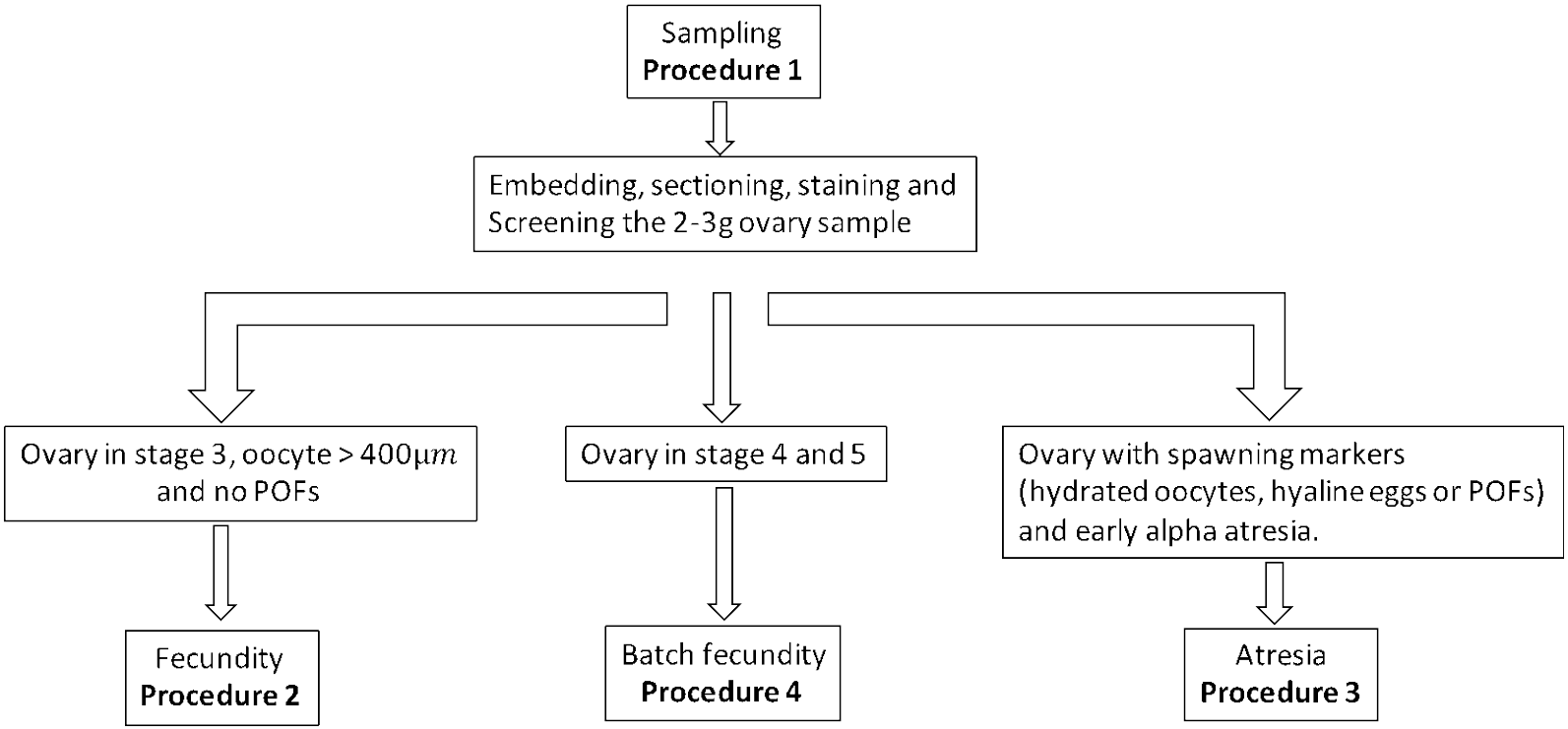
2 Standard and Walsh maturity scale for mackerel and horse mackerel maturity staging

Standard	Walsh	Mature/ Immature	State	Female	Male
1	1	Immature	Immature	Gonads small. Ovaries wine red, clear, and torpedo shaped.	Gonads small. Males pale, flattened, and transparent.
2	2	Mature	Developing	Gonads occupying 1/4 to 3/4 body cavity. Opaque eggs visible in ovaries giving pale pink to yellowish colouration, largest eggs without oil globule.	Gonads occupying 1/4 to 3/4 body cavity. Testes off-white, milt not running.
	3	Mature	Developing	Gonads occupying 3/4 to almost filing body cavity. Ovaries yellow to orange. Largest eggs may have oil globules.	Gonads occupying 3/4 to almost filing body cavity. Testes creamy white.
3	4	Mature	Spawning	Ovaries characterized by externally visible hyaline eggs no matter how few or how early the stage of hydration. Ovary size variable from full to 1/4.	Testes filling body cavity, milt freely running.

Standard	Walsh	Mature/ Immature	State	Female	Male
	5	Mature	Spawning	Gonads occupying 3/4 to < 1/4 body cavity. Ovaries slacker than in Stage 3 and often bloodshot.	Gonads occupying 3/4 to < 1/4 body cavity. Testes with free running milt and shrivelled at anus end.
4	6	Mature	Regressing Regenera-ting	Gonads occupying 1/4 or less of body cavity. Ovaries reddish and often murky in appearance, sometimes with a scattering or patch of opaque eggs.	Gonads occupying 1/4 or less of body cavity. Testes opaque with brownish tint and no trace of milt.
5	-		Omitted spawning	No evidence of omitted spawning	No evidence of omitted spawning
6	-		Abnormal	Hard parts (connective tissue), only one lobe developed, intersex, or similar. Fecundity at least partly reduced.	Hard parts (connective tissue), only one lobe developed, intersex, or similar.

Standard scale as proposed by the WKMSMAC2 2015. See also Annex 1 for pictures of the different stages.

MACKEREL PROCEDURES



3 Procedure 1: Mackerel sampling procedure at sea, AEPM

3.1 Before the cruise

Procure **25–50 µl capillary pipettes** (Wiretrol II 25–50 µl, Cat. Number 5-000-2050 (VWR). Extra plungers can be ordered from the same supplier; be sure to order the long plungers). Test performance of the pipette by practice, taking 25 µl fresh gonad or water samples.

Buffered formaldehyde: 3.6% buffered ($NaH_2PO_4*H_2O$: 29.48 mM, $Na_2HPO_4*2H_2O$: 46.01 mM) formaldehyde (see also excel-file on the IMR ftp-sever: "Buffered formaldehyde").

Labels: IMR and IMARES will send around labels to all the institutes participating in the survey to use on the Nunc and scintillation tubes. Each institute will get its own code with a letter and a colour (Table 3.1.1). Each institute has a land code on the labels to show who has collected the samples (letter) and a colour to which the collected sample has to be sent. Note that some institutes do not have colour, i.e., the institutes collecting but not analysing the samples. Norway has a colour but no letter; this lab will not collect but only process samples.

Nunc and scintillation tubes: fill the labelled 2.5 ml Nunc tubes (with screw on lids) with 1.2 ml of 3.6% buffered formaldehyde. Also fill the labelled 20 ml scintillation tube with 15 ml of 3.6% buffered formaldehyde.

Bottles: these will be labelled and filled with formaldehyde during the cruise.

IMR ftp-server: make sure each institute has access to the sharing point in order to upload/download information.

Table 3.1.1. Land and sending code for each institute used on the fecundity and atresia samples. Along with the sending code responsible person contact details are provided.

Land code	Sending code	Sample	Country	Institute and address	Responsible person	Lab code for ImageJ
-	Blue	a, b, c, d, e	Norway	Nordnesgaten 50 Postboks 1870 Nordnes 5817 Bergen Norway	Merete Fonn	IMR
A, B	Red	a, b, c, d	Ireland	MI, Rinville, Oranmore, Co. Galway, Ireland	Brendan O`Hea	MII
C, D	Yellow	a, b, c, d	Scotland	Marine Scotland Science, Marine Laboratory, Victoria Road, Torry, Aberdeen, AB11 9DB, Scotland	Finlay Burns	MSS
M	White-Even numbers White	a, b, c, d e	Spain	IEO, Subida A Radio Faro 50-52, 36390 Vigo, Spain	Antonio Solla	IEO
K	White-Uneven numbers Orange	a, b, c, d e	Spain	AZTI, Herrera Kaia, Portualdez/ g 20110 Pasaia, Basque Country, Spain	Paula Alvarez / Maria Korta	AZT
I, J	Green	a, b, c, d, e	Netherlands	IMARES, Postbus 68 1970 AB IJmuiden Netherlands	Cindy van Damme	IMA
G	-	-	Germany			
O	-	-	Portugal			
Q	-	-	Faroe			
S	-	-	Iceland			
U	-	-	Denmark			DEN
W	-	-	UK (SCO2)			

3.2 During the cruise

Measure the weight of the whole catch, randomly select a subsample of 100 fish, and measure the total weight of the subsample.

Measure total length, weight, assess maturity (Walsh scale), and sex of each fish in the subsample.

Select females in maturity stages 3–6 (Walsh scale) from the subsample of 100 (if less than 100 fish are in the catch, sample all the mackerel) for fecundity and atresia analysis. If possible, divide the total quota of females equally into the four weight categories: < 250g, 251–400g, 401–550g and > 550g. If the size range of fish is restricted in the catch, the remaining sample quota should be taken from the more abundant classes to fill the weight classes.

Measurements:

- Total length (nearest mm)
- Total weight (nearest gram)
- Sex
- Maturity (Walsh scale)
- Otoliths
- Weight of ovary (nearest 0.1 gram. If it is not possible to measure the ovary weight at sea, take out the ovary and weigh the fish without the ovary. Then, take the pipette and atresia samples and fix the remainder of the ovary and weigh the ovary in the lab. The fixed and frozen weights should be corrected to fresh weights.)

Screening sampling (e):

- From one ovary lobe, take a small (2–3 g) sample (Figure 3.2.1), with a spoon or cut with a scalpel, and immediately put this sample into a prefilled individually coded 20 ml scintillation tube. Make sure the sample is covered with 3.6% buffered formaldehyde solution (one part ovary and nine parts formaldehyde).

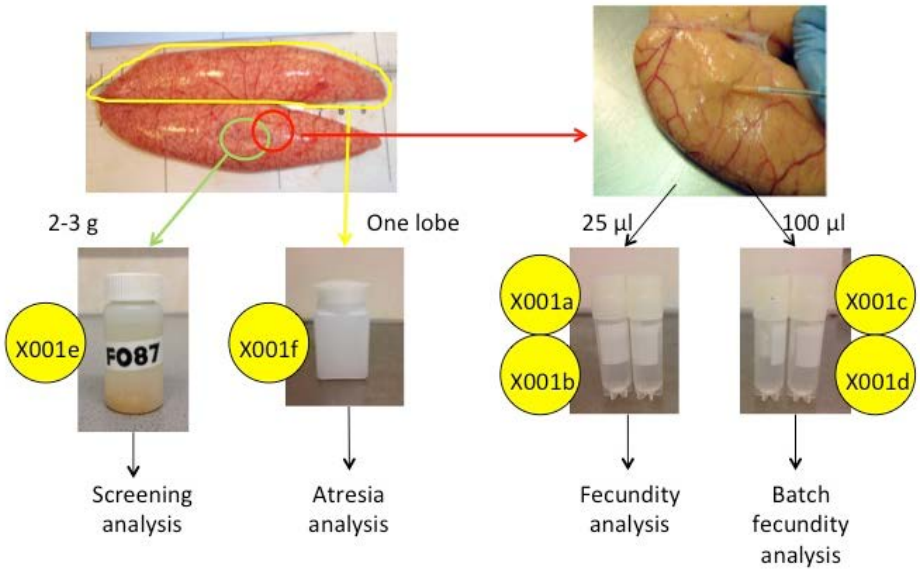


Figure 3.2.1. Subsampling of mature females (stage 3–6, Walsh scale) ovaries at sea.

Ring test sampling:

- Each institute should additionally collect ring test samples for fecundity (for the early cruises, Walsh Stage 3 or 4) or batch fecundity (for the late cruises, Walsh Stage 4 or 5). Take 18 samples from one suitable fish (not used for AEPM or DEPM) and mark them with land code and number (e.g. A01, A02,… A18) using the provided larger white labels.
- 18 samples will allow each institute to have a ring test analysis of the same individual for the following three surveys. Thus, it will be useful to test the consistency of the fecundity analysis procedure used internally in each institute.
- Send out three samples to each institute involved in the fecundity analysis.

Fecundity sampling (a, b, c, d):

- From the same lobe of the ovary (where the screening and ring test samples above where taken from), take two samples of 25 µl (a, b) and two samples of 100 µl (c, d) with a pipette (Figure 3.2.1 and Figure 3.2.2) and immediately put each sample in its own individually coded Nunc tube. Take in a bit more sample than you need and press the plunger until it reaches the line (25 or 50 µl) and blot off any oocyte that is outside the tip, using your hand or a piece of paper. Ensure all oocytes are immersed in 3.6% buffered formaldehyde solution. For the 100 µl samples, take two times 50 µl with the pipette. Rinse the pipette with water and dry it with a paper towel prior to sampling another fish. The reason to obtain two samples of 25 µl and 100 µl respectively is to guarantee samples, in case a sample is lost during the processing. Send out the samples coded as (a, b) and (c, d) to the analysing institutes, following the colour sending code as indicated by the label.

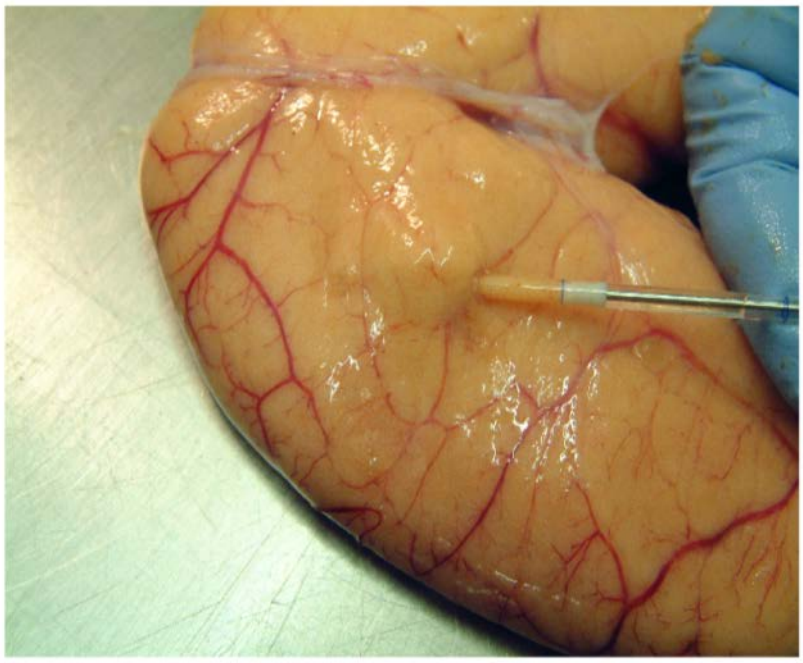


Figure 3.2.2. Method to use a capillary pipette to remove an ovary sample.

Atresia sampling (f):

- For atresia: Cut off both ends (1–2 cm, depending on the size of the ovary) of the other ovary lobe, and place the remaining part in a bottle (100–250 ml with wide opening), and fill it with 3.6% buffered formaldehyde (Figure 3.2.1). Label (f) the bottle with coded label with the sample reference number. Make sure that the bottle is completely filled with formaldehyde and that the tissue is not more than 50% of the volume of the formaldehyde.

3.3 After the cruise

Immediately after the cruise:

- Screening samples in the scintillation tubes should be sent to the analysing institutes (AZTI, IEO, IMARES, and IMR, Table 3.2.1).
- Also send out Nunc tubes for the fecundity and batch fecundity samples (AZTI, IEO, IMARES, IMR, MI, and MSS).

Pack the consignments for each country with a maximum volume of 1000 ml solution in each package. On the outer cover of the package, indicate the volume of fixative and that it is within the limits for unclassified transport. Add safety sheets.

Once results of the screening are obtained, the adult sampling coordinators will divide the samples between the analysing institutes.

All the ovary samples should remain fixed in 3.6% formaldehyde for at least two weeks before whole mount analysis or the sections for the atresia analysis are taken. From the fixed ovary, cut two 5 mm thick slices and put them in a coded histology cassette. Write the code with a wooden pencil on the outside of the cassette. If the ovary is very big, you may have to use two cassettes. Separate the cassettes into four colour-coded (AZTI; IEO, IMARES, and IMR) leak proof bottles, filled with 70% ethanol. Send the cassettes for analysis to the different institutes, based on the list provided by the sampling coordinators.

4 Screening before analysis

4.1 Oocyte development stage, spawning markers and atretic oocytes

Process histologically the 2–3 g samples in the scintillation tubes. Histological procedures (embedding, sectioning, and staining) are described in Procedure 3 (Chapter 6).

Screen the histological slides under the microscope, looking for the most advanced oocyte stage (Tables 4.1.1–4.1.2, and Figures 4.1.1–4.1.3), POFs, hyaline eggs (Table 4.1.2 and Figure 4.1.3), early alpha atresia, massive atresia, if it is spent (Figure 4.1.4) and if it should be discarded.

A hyaline egg is fully ovulated and the follicle layer is not around the cell anymore.

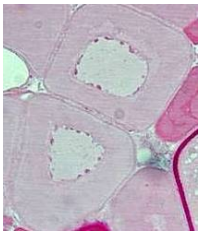
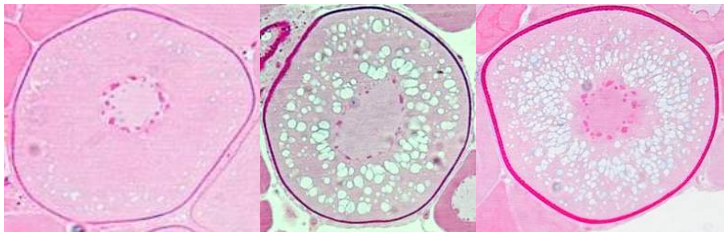
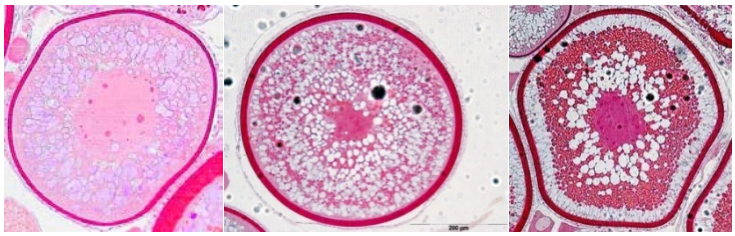
A spent ovary is characterised by the absence of normal vitellogenic oocytes at the end of the spawning period for that individual. Atretic vitellogenic oocytes and POFs may be visible.

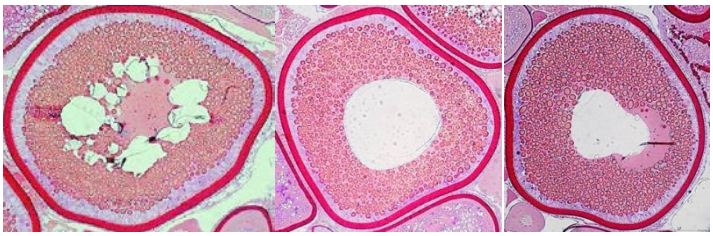
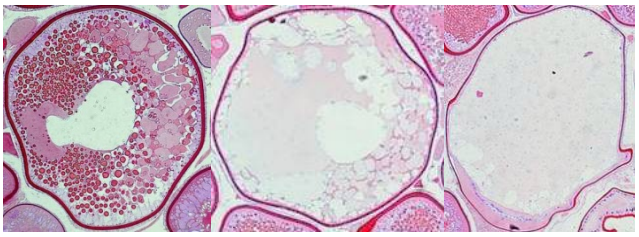
Massive atresia is classed as when 90% or more of the vitellogenic oocytes in the ovary are atretic. In case of massive atresia, the fish will not spawn anymore that season.

Residual eggs are hyaline eggs that remain in the ovary after a batch is spawned and ovulated eggs remain in the ovary and are resorbed.

In cases where an ovary is not characterised by any category mentioned above, but does not look like it should be used in the analyses, there is a column for discarding such samples. Always write a comment if you discard a sample.

Table 4.1.1. Oocyte development stages.

Number	Development stage	Description
1	Pre vitellogenic	No white vacuoles (cortical alveoli) visible.
		
2	Early vitellogenic (< 400 μm)	Smallest vitellogenic stage. White vacuoles visible. Yolk can be visible as well. Oocytes < 400 μm.
		
3	Vitellogenic (400–800 μm)	White vacuoles and yolk granules visible. It is not necessary to see vacuoles and granules at one time. Oocytes are between 400 and 800 μm.
		
4	Migratory nucleus	Nucleus is migrating from the middle of the oocyte towards the side of the cell. The envelope of the nucleus breaks down, and the nuclear contents blends with the surrounding cytoplasm.

Number	Development stage	Description
		
		Lipids are concentrated in one unique drop. (Note that in the central picture, the nucleus is not present, but the fact of seen the lipid big drop means that the oocyte is in the migratory nucleus stage).
5	Hydrated	Yolk granules are fusing together and water is taken up by the oocyte.
		
		Right picture shows the starting of hydration. You can also see the nucleus in the animal pole (end of migration).

The sample stage is based on the most advanced oocyte development stage (Table 4.1.3.).

Table 4.1.2. Hyaline eggs

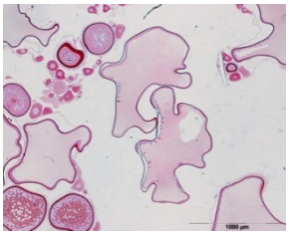
	Hyaline eggs	**Complete fusion and hydration of the oocyte. The follicle layer has disappeared.**
		
	Note that histological processes distort the shape of the hyaline oocytes.	

Table 4.1.3. Microscopic oocyte stages based on the most advanced oocyte development stage.

Number	Development stage	Description
1	Pre vitellogenic	The most advanced oocytes are in pre vitellogenic stage
2	Early vitellogenic	The most advanced oocytes are in early vitellogenic stage
3	Vitellogenic	The most advanced oocytes are in vitellogenic stage
4	Migratory nucleus	The most advanced oocytes are in migratory nucleus stage. Not to be used for whole mount evaluation
5	Hydrated	The most advanced oocytes are in hydrated stage

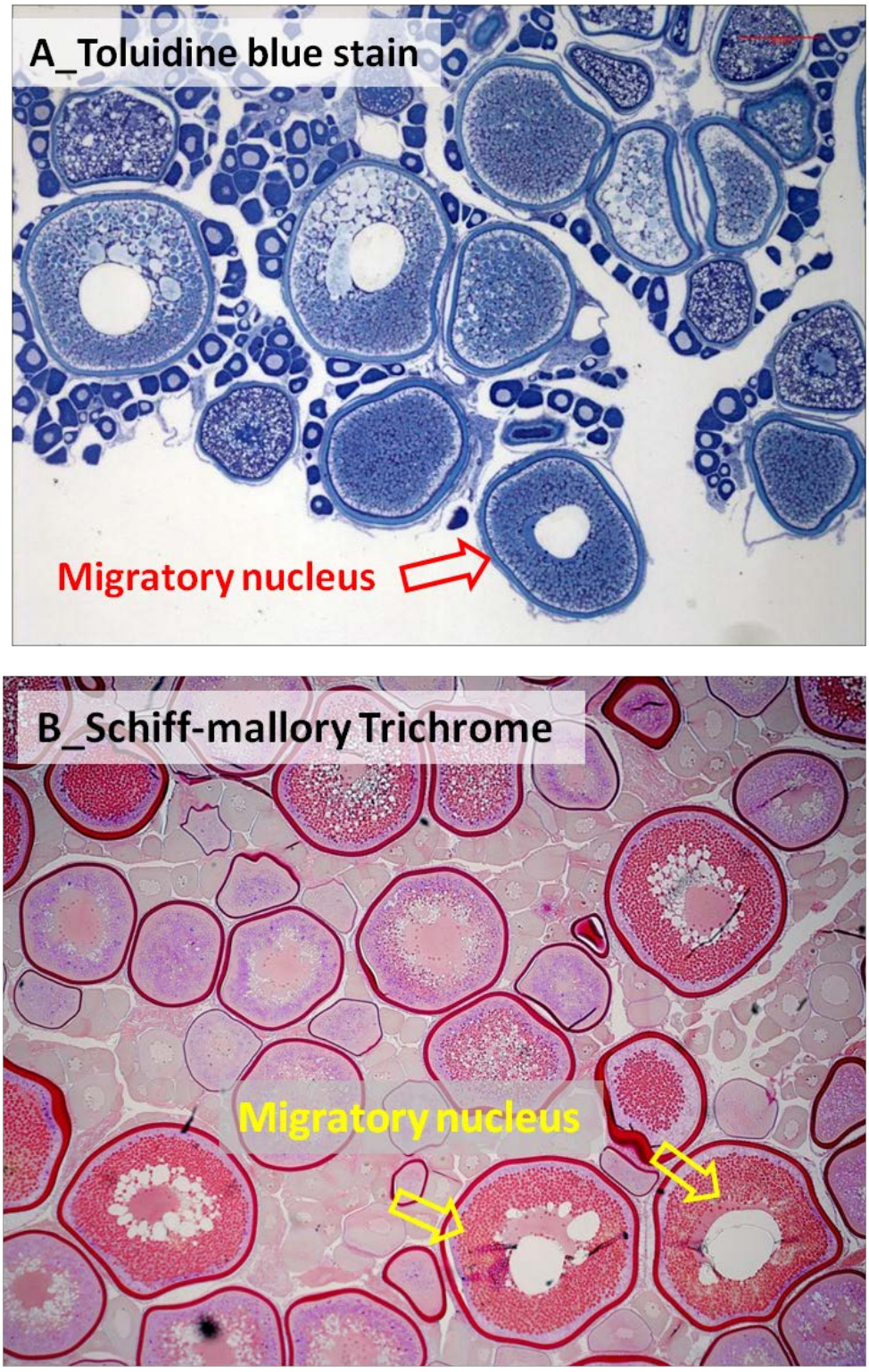


Figure 4.1.1. Migratory nucleus stage. A. Toluidine blue stain. B. Schiff-Mallory trichrome stain.

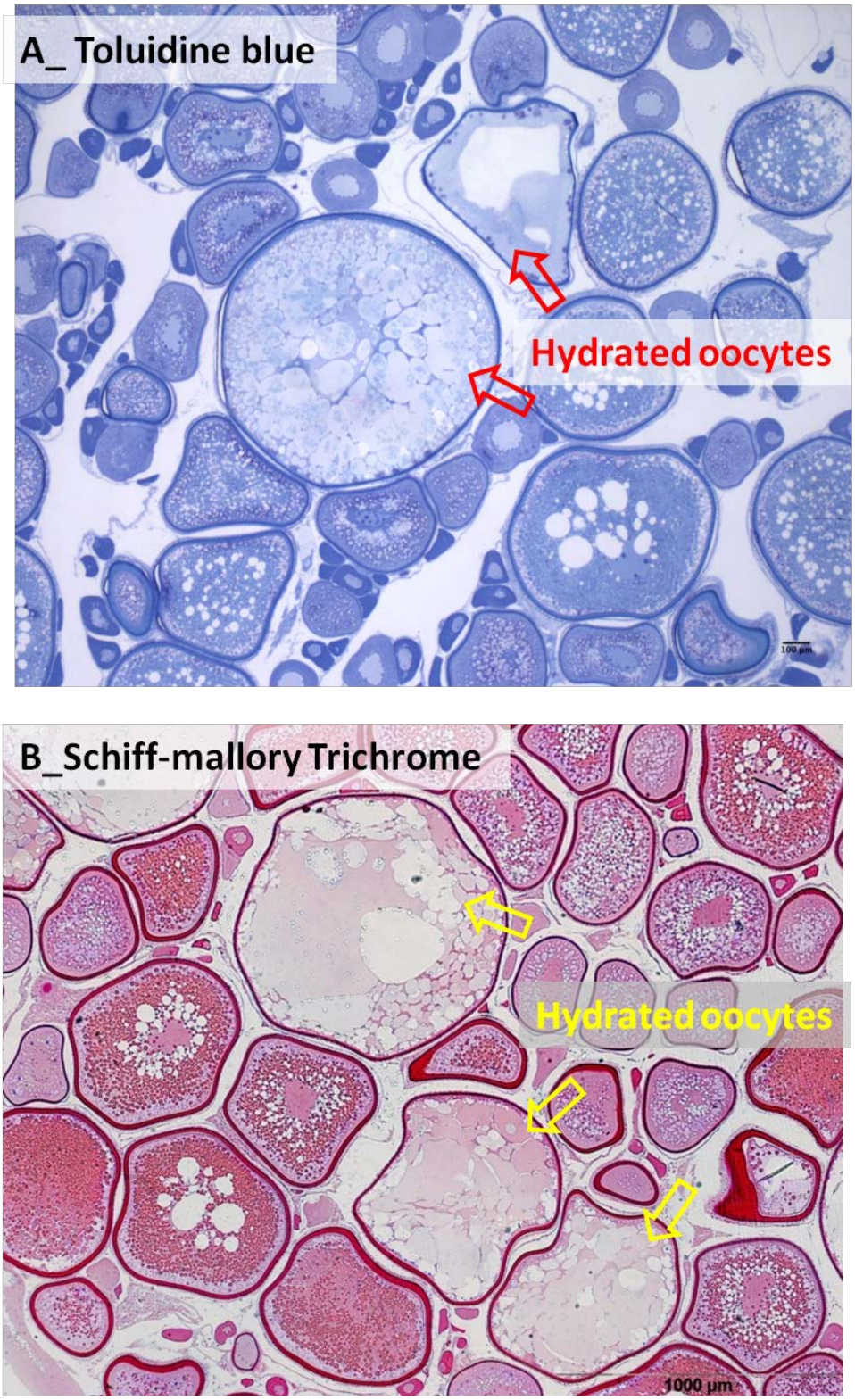


Figure 4.1.2. Hydrated oocyte stage. A. Toluidine blue stain. B. Schiff-Mallory trichrome stain.

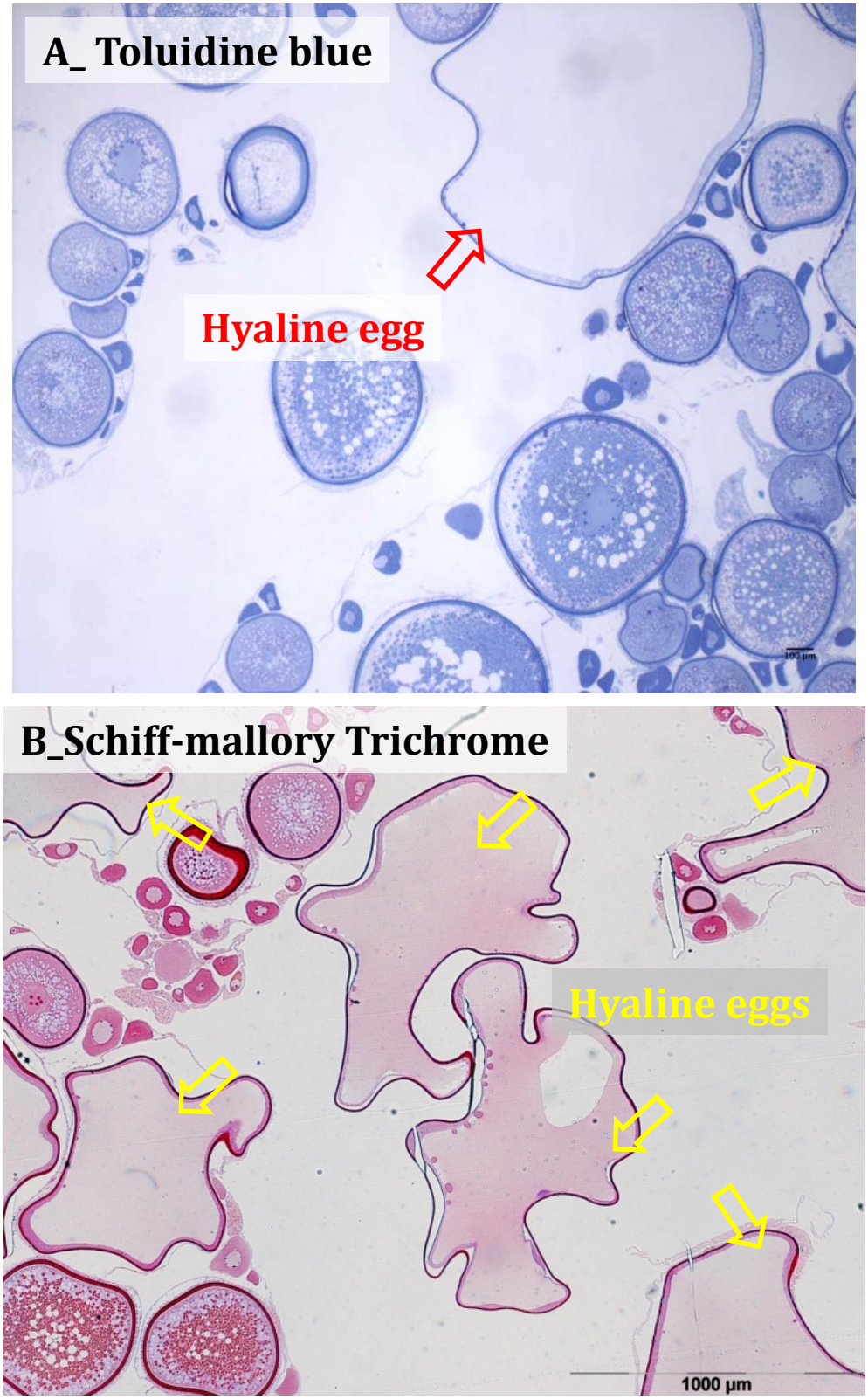


Figure 4.1.3. Hyaline eggs. A. Toluidine blue stain. B. Schiff-Mallory trichrome stain.

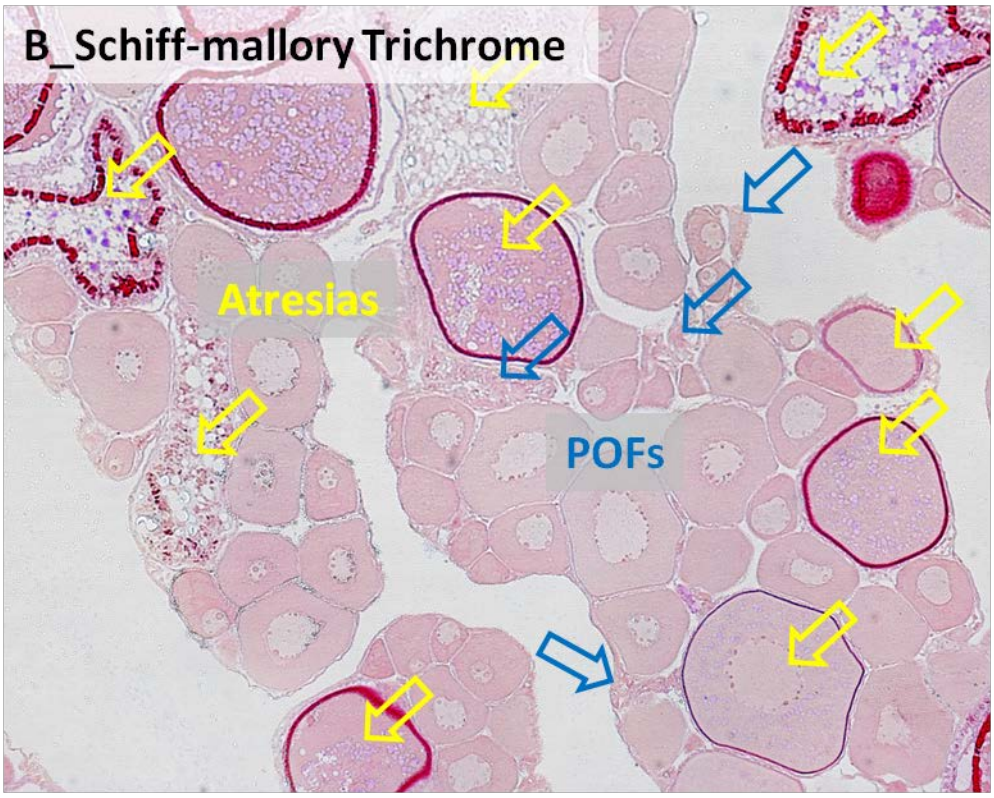


Figure 4.1.4. Spent.

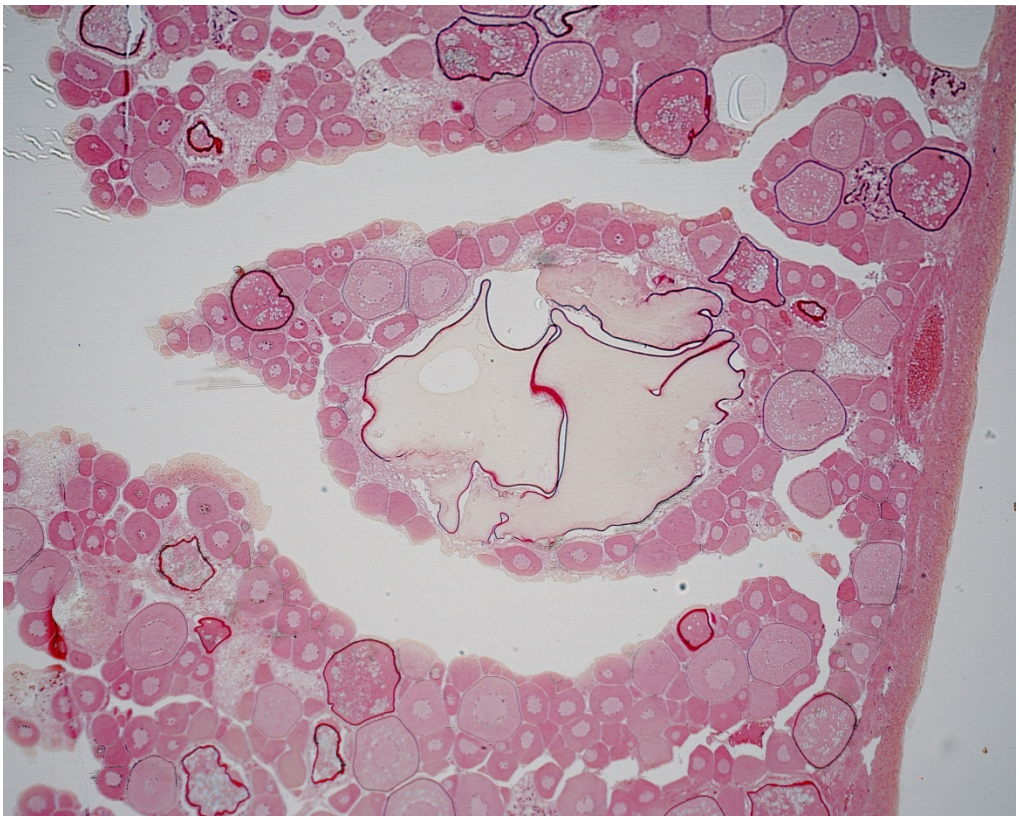


Figure 4.1.5. Residual eggs.

The ovary stage of each sample should be entered in the excel sheet "Screening_histology_form" (Annex 2) and saved on the ftp server.

4.2 Instructions for the samples after the screening analysis

Samples in microscopic most advanced oocyte stage 'previtellogenic' and 'early vitellogenic' (Stages 1, 2) will not be used for any analysis (Figure 4.2.1).

The ovary samples with oocyte stage, vitellogenic > 400 µm (Stage 3, Figure 4.2.1), will be analysed for potential fecundity as described in Chapter 5.

Ovary samples containing early alpha atresia will be analysed for atresia (Figure 4.2.1) following Chapter 6. Not all the early alpha atresia samples will be analysed for atresia. A number of them will be select at random based on the results of the screening analyses.

Ovary samples containing the oocyte stages migratory nucleus or hydrated (Stage 4 and 5) will be used for batch fecundity (Figure 4.2.1).

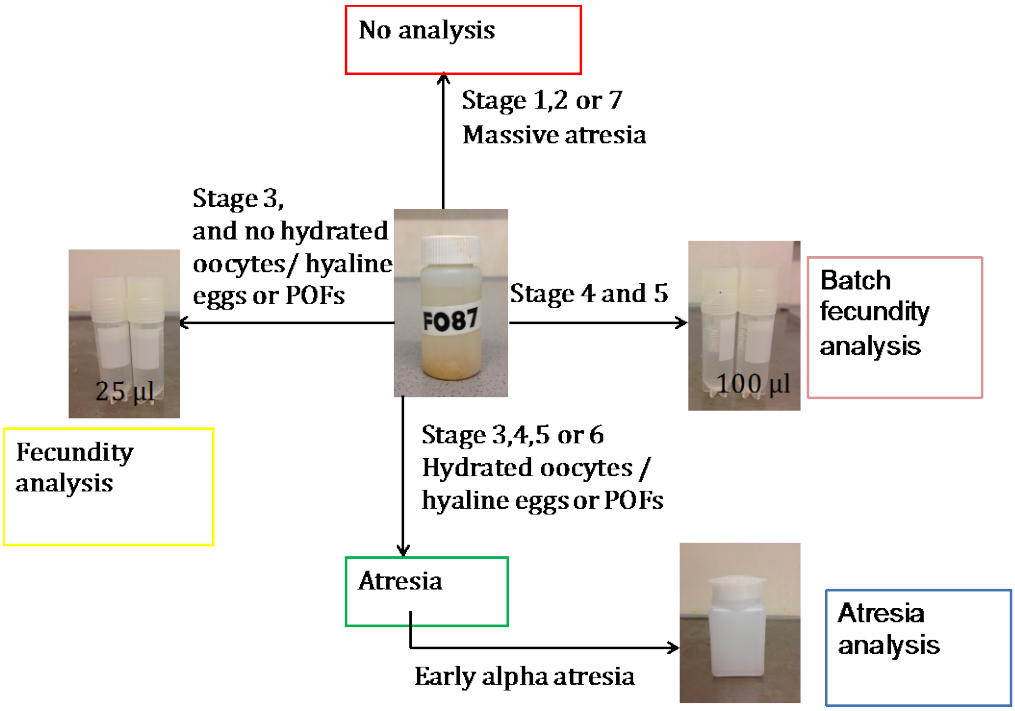


Figure 4.2.1. Microscopic ovary stages within screening samples and consecutive analysis.

5 Procedure 2: Fecundity whole mount analysis in the lab for AEPM mackerel

5.1 Potential fecundity

Samples containing the oocytes stage, vitellogenic > 400 μm (Stage 3), will be analysed for potential fecundity. Distribute the 25 μl sample from the Nunc tube evenly in the tray. If it is not possible to separate the oocytes, exclude the sample for fecundity analysis.

Measure the oocyte diameters automatically using ImageJ software provided for the fecundity analysis. See Annex 3 for image analysis manual.

Count all the oocytes > 185 μm in the sample. The oocyte should completely fill the floating circle of 185 μm to be included in the manual count. Also, advanced atretic oocytes should be included because the number of atretic cells will be subtracted in the calculations later on.

Fill in the "Fecundity template" (Annex 4) with the results obtained from ImageJ.

Whole mount evaluation: when processing samples for fecundity, it is important to check the screening results afterwards. Mainly because a hydrated sample may be not identified during the screening; hydrated oocytes may not have been not collected with the pipette. Other errors like mixing samples may also occur that should be corrected.

Since Stage 4 cannot be detected in whole mount, it is included in Stage 3. Stage 4 is then not used in this form (whole mount evaluation).

5.2 Calculation of potential fecundity

Potential fecundity

$$F_p = \frac{N}{W_s} * OW$$

F_p = potential fecundity

N = number of oocytes

W_s = weight of the pipette sample (0.026 g)

OW = fresh ovary weight (g)

Relative potential fecundity

$$F_r = \frac{F_p}{W}$$

F_r = relative potential fecundity

W = total fish weight (g)

6 Procedure 3: Atresia analysis in the lab for AEPM mackerel

6.1 Embedding, sectioning, and staining

6.1.1 Preparing resin blocks

Using the two 5 mm sections in the cassettes, the different laboratories should follow the relevant steps below:

Table 6.1.1. Procedure used by IMR and IMARES.

Step	Infiltration solution	Duration	Temperature
1	90% ethanol	2 hours	Room temperature
2	Pour out the liquid and add fresh 90% ethanol	1 hour	Room temperature
3	Pour out the liquid and add fresh 96% ethanol	1 hour	Room temperature
4	96% ethanol + Technovit 7100 (1:1 ratio) prepared by diluting Technovit 7100 (from used in steps 4).	overnight	Store cool (+5°C) after the orbital shaker
5	Replace the liquid with Technovit 7100 (from step 5).	3 days	Store cool (+5°C) after the orbital shaker
6	Replace the liquid with freshly prepared Technovit 7100.	2 days	Store cool (+5°C) after the orbital shaker
7	Transfer the sections from the cassettes to the moulds.		Cooling plate (-5°C)
8	Polymerise by adding Technovit 7100: hardener (15:1) at cooling plate (-5°C).	6 hours	Cooling plate (-5°C)
9	Leave overnight	overnight	Store cool (+5°C)
10	Block up using Technovit universal	15 minutes	Room temperature
11	Store the blocks in a box containing 70% glycerol		

Table 6.1.2. Procedure used by IEO.

Step	Infiltration solution	Duration	Temperature
1	70% ethanol 70%	1 day	Room temperature
2	90% ethanol 90%	1 day	Room temperature
3	96% ethanol 96%	1 day	Room temperature
4	96% ethanol 96% + activated resin (technovit 7100) (1:1)	2 days	Store cool (+5°C) with several slight shakes
5	100% activated resin (technovit 7100)	2.5 days	Store cool (+5°C) with several slight shakes

Table 6.1.3. Procedure used by AZTI.

Step	Infiltration solution	Duration	Temperature
1	70% ethanol	32 hours	Room temperature
2	90% ethanol	16 hours	Room temperature
3	96% ethanol	8 hours	Room temperature
4	96% ethanol + Resin activated (1:1 ratio)	2 days	Store cool (+5°C)
5	Resin activated	2–3 days	Store cool (+5°C)
6	Transfer the tissue from the cassettes to the moulds.		Store cool (+5°C) after the orbital shaker
7	Cover the tissue with resin activated and hardener (15:1) and put the block	1 day	Room temperature

6.1.2 Disposal of waste resin (in the fume cupboard)

After step 3 (Tables 6.1.1–6.1.3), the 1:1 resin mix should be put in an aluminium tray and left in the fume cupboard over a few days to allow the ethanol to evaporate from the resin. Use about 1 g hardener to 100 g resin to polymerise and wrap the block in a poly bag for disposal. Caution the reaction is exothermic and potentially hazardous if too much hardener is added.

6.1.3 Sectioning the blocks

The block needs to be trimmed until you get a section with the part of the sample needed. Use a microtome to cut 5 μm sections. Put the section in water containing a drop of ammoniac. Pick the section from the water with an object glass. The section should be completely flat on the glass. Dry the object glass on a heating plate at 100°C. Write the sample number on the object glass.

If the ovary is small, one section may not be enough to get the correct area for analysis (see Table 6.3.2). Trim the block until the next section does not contain the same oocytes as the first one. (In other words, the distance between each section should be greater than the oocyte size.)

6.1.4 Staining the sections

Recipe 2% Toluidine blue as used by IMR and IMARES

2% Toluidine blue and 1% Sodium tetraborate (Borax). The Borax is dissolved in the distilled water and then the dye added under constant stirring. Filter the solution before use.

For individual slides: Cover the section with a few drops of 2% Toluidine blue and pour the excess back in the bottle and rinse the section with hot (60°C) tap water for 20 seconds. Dry on a 60°C hot plate. Cover the section with a cover slip using two drops of Mountex.

Schiff-Mallory Trichrome used by IEO

Step	Reagent	Time (min:sec)	Exact
1	5% Periodic acid	4:30	Yes
2	Distilled water	00:10	No
3	Schiff's	60:00	Yes
4	Tap water	10:00	No
5	1%Acid Fuchsine	1:00	Yes
6	Distilled water	00:30	Yes
7	Distilled water	00:30	Yes
8	1% Phospho Molybdic acid	1:00	Yes
9	Distilled water	00:10	Yes
10	Mallory Trichrome	00:15	Yes
11	Distilled water	00:10	Yes
12	90% Ethanol	00:05	Yes
13	100% Ethanol	00:05	Yes
14	100% Ethanol	00:05	Yes
15	1:1 100% Ethanol – OTTIXCLEAR*	00:05	Yes
16	OTTIXCLEAR*	00:05	Yes
17	OTTIXCLEAR*	00:05	Yes
Exit	Exit	.	

Haematoxylin and Eosin (H&E) as used by AZTI

Cover the sections following the protocol:

- 5 minutes in Haematoxylin
- 5 minutes in running tap water
- 5 minutes in 1% eosin (1 gr/100 ml)
- Clean the rest of eosin with running water

6.2 Atresia analysis

All atresia measurements in this manual are based in an early alpha atretic stage, defined to easily quantify atresia by stereological methods (ICES, 1993).

All oocyte classification should be done in at least 100% view. Classification of atretic oocytes is based mainly on the breakdown of the chorion layers, but other changes also occur. Subdivision of the alpha stage into early alpha and late alpha atresia is based on the size of breaks and position of the chorion layer.

Differentiation between early and late alpha atresia.

If any perforation or breakdown in the chorion layer is observed and if the breaks are smaller than twice the width of the chorion thickness, the oocyte is classed as early alpha atretic. If the outer chorion layer has breaks more than twice its width and the fragments are displaced inwards from the outer follicle boundary the oocyte is classed as late alpha.

Practical remarks.

When the section of the oocyte is towards the edge or in small oocytes, it can be difficult to see the difference between the inner and outer chorion. However, when breaks are visible in a part of the chorion the oocyte is atretic. As stated before, if any breaks or cuts are visible in the chorion, the oocyte is considered atretic. Care should be taken when the chorion is damaged due to preparation of the sample. If the chorion is damaged the oocyte is not necessarily atretic. Alpha atresia begins when there is a cut in the inner chorion. During the analyses, one should not mix up the vitelline membrane and the inner chorion.

When in doubt about the initial phase of late alpha atresia, the chorion thickness should be measured to estimate the size of the break. The width of the chorion should always be measured at the thinnest part because the chorion expands during the atretic process.

Beta atresia

After the chorion has disappeared, the breakdown progresses from the alpha into the beta stage and the oocyte is now much reduced in size, highly vacuolated, and with no yolk contents visible.

The edge of the oocyte in early alpha atretic stage is smooth, while in later atretic stage the edge is jagged.

For mackerel we only score vitellogenic oocytes at early alpha atretic stage.

The vitellogenic oocytes are divided into three different stages (Table 6.2.1):

YV (yolk vesicle stage): arises from the smallest vitellogenic oocytes making up the potential fecundity ranging in size from 175 (appearance of cortical alveoli) to 325 μm when a complete ring of vacuoles extends throughout the oocyte cytoplasm.

YV-YG (yolk vesicle to yolk granule stage): the oocytes range in size from 325 to 525 μm and contain yolk granules that slowly enlarge and start to fill the cytoplasm.

YG (yolk granules): yolk granules occur throughout the full depth of the cytoplasm. This stage also includes the largest oocytes making up the potential fecundity up to oil droplet formation and the migratory nucleus stage.

Atresia analyses are performed in part of the ovary area. All oocytes classification should be done in at least 100% view in order to get sufficient detail and following the indications in the Image Analysis Manual (Annex 3).

Table 6.2.1. Atretic oocyte stage classification.

Acronym	Development stage	Description
YV	Yolk vesicle stage	Smallest vitellogenic stage. White vacuoles visible, ranging in size from very small to relatively large. Oocyte size varies from 185–325 µm.
YV–YG	Yolk vesicle – Yolk granule stage	White vacuoles still present. Yolk granules (blue particles in Toluidine blue) begin to enlarge throughout the oocytes. Oocyte size varies from 325–525 µm.
YG	Yolk granule stage	Yolk granules begin to fill whole cytoplasm. In the late YG stage, oil droplets will appear. And in the late YG stage, migratory nucleus is also present > 525 µm.

Pictures of the three different stages in normal oocytes stained with toluidine blue.

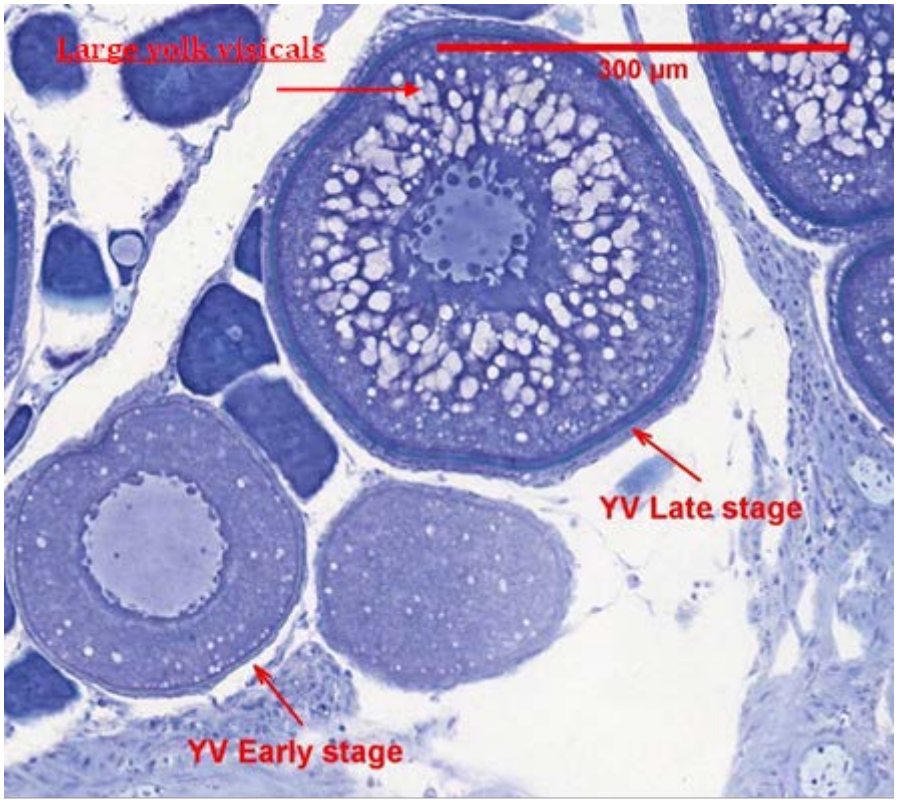


Figure 6.2.1. YV stage.

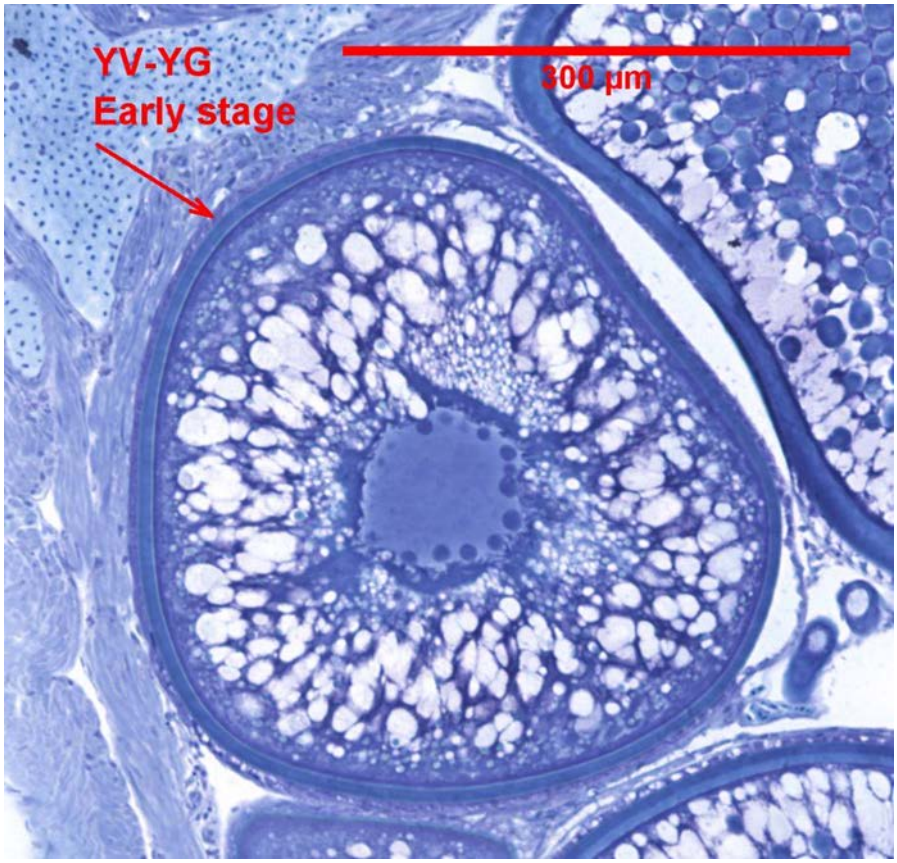


Figure 6.2.2. YV-YG early stage.

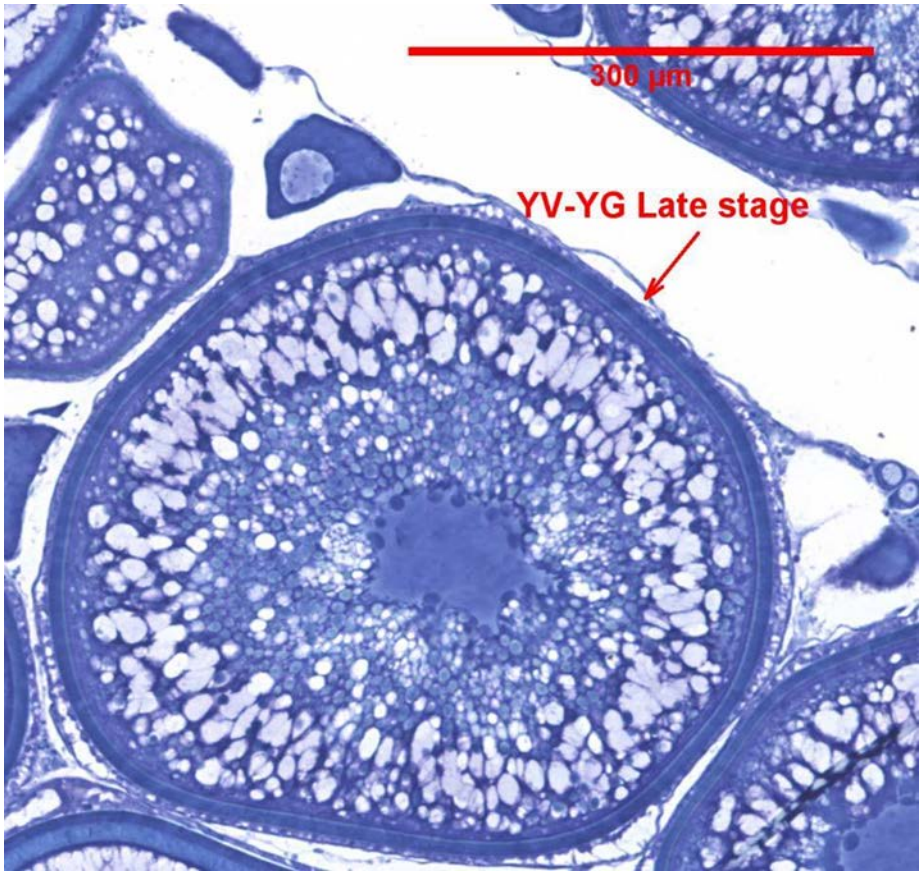


Figure 6.2.3. YV-YG late stage.

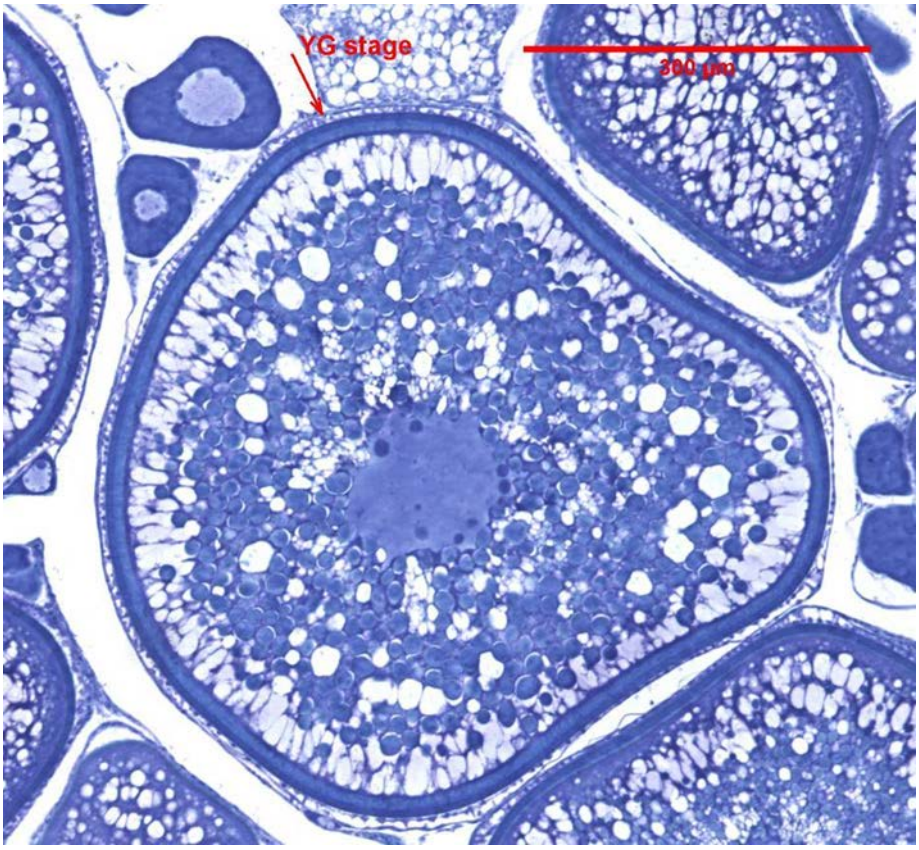


Figure 6.2.4. YG stage.

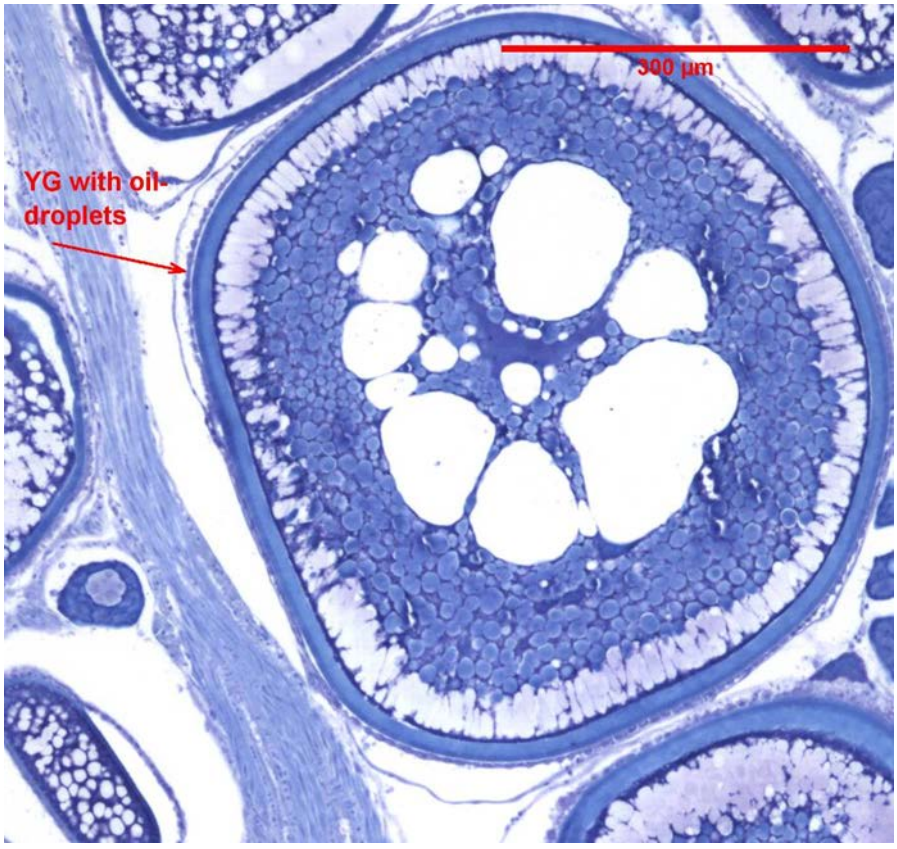


Figure 6.2.5. YG stage with oil-droplets.

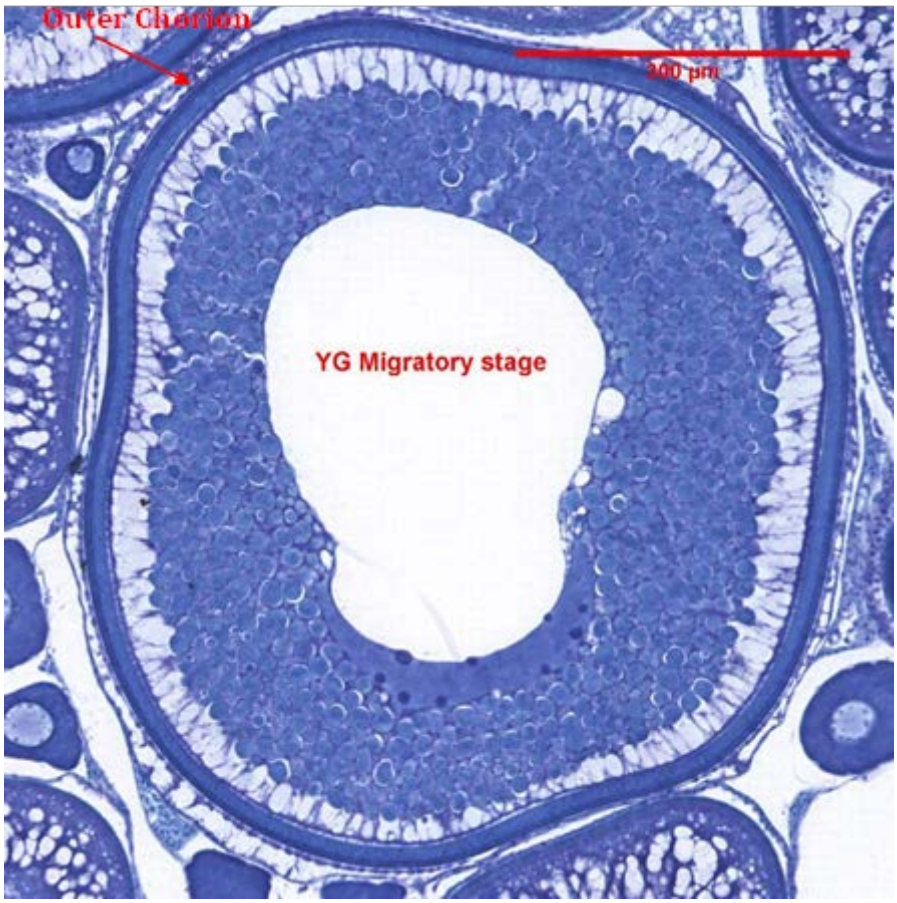


Figure 6.2.6. YG and migratory nucleus stage.

Pictures of the three different stages in early alpha atretic oocytes stained with toluidine blue.

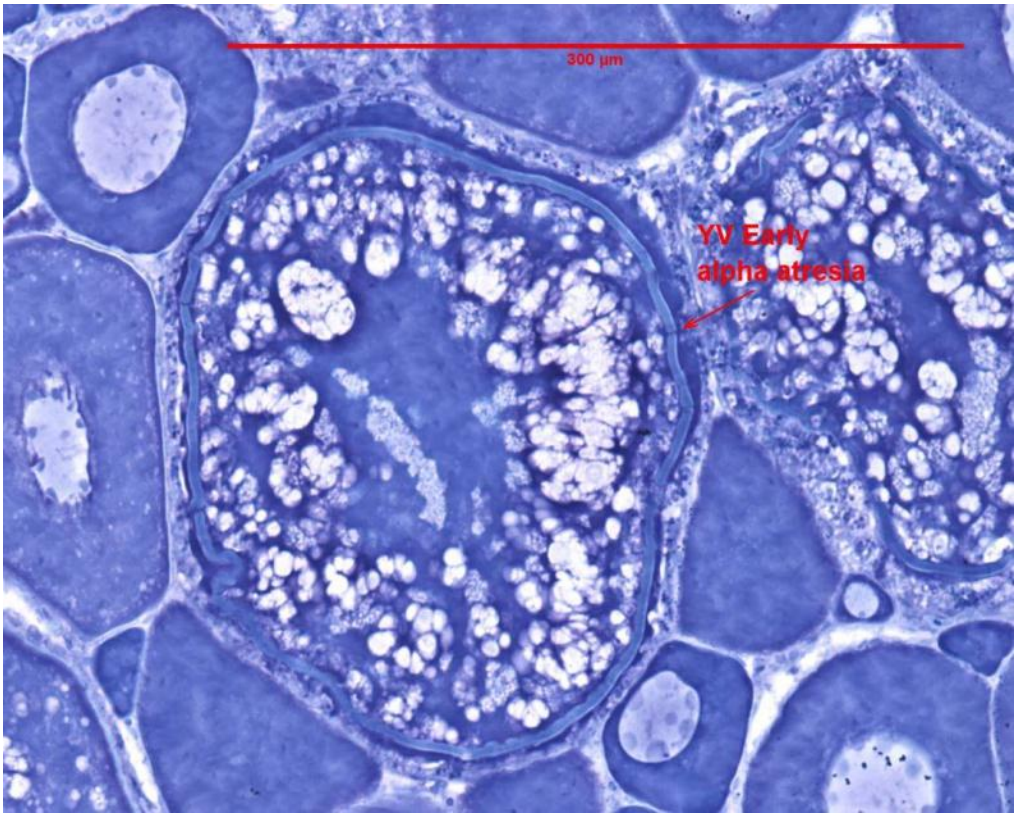


Figure 6.2.7. YV early alpha atresia stage.

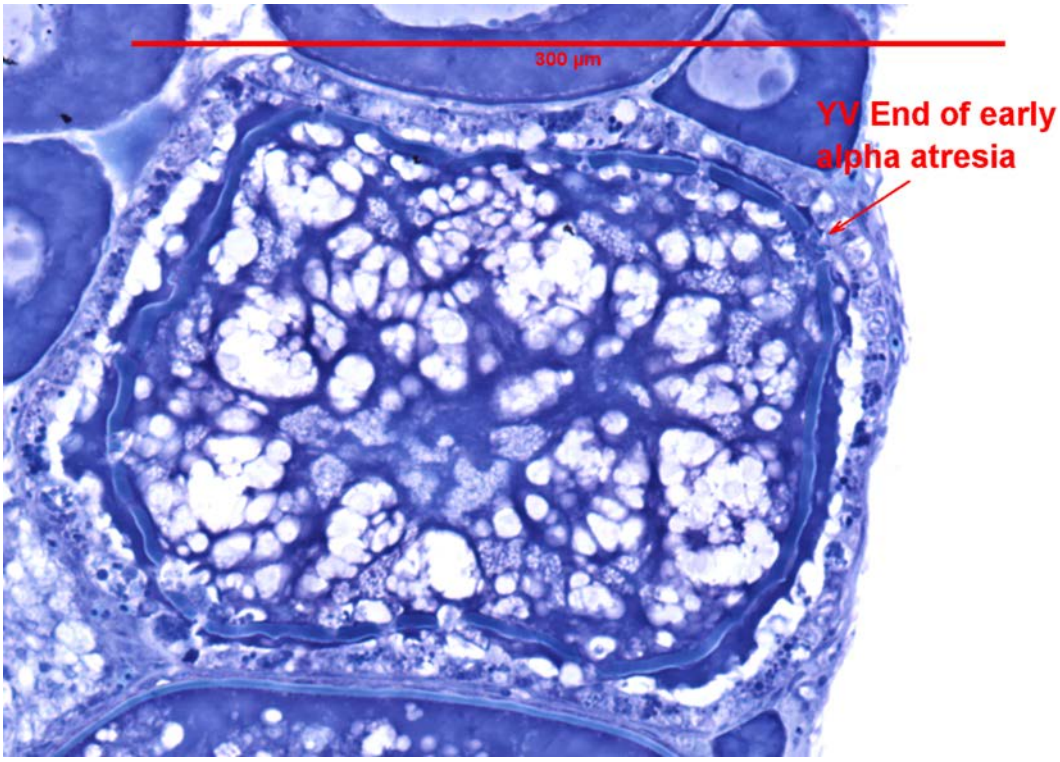


Figure 6.2.8. YV end of early alpha atresia stage.

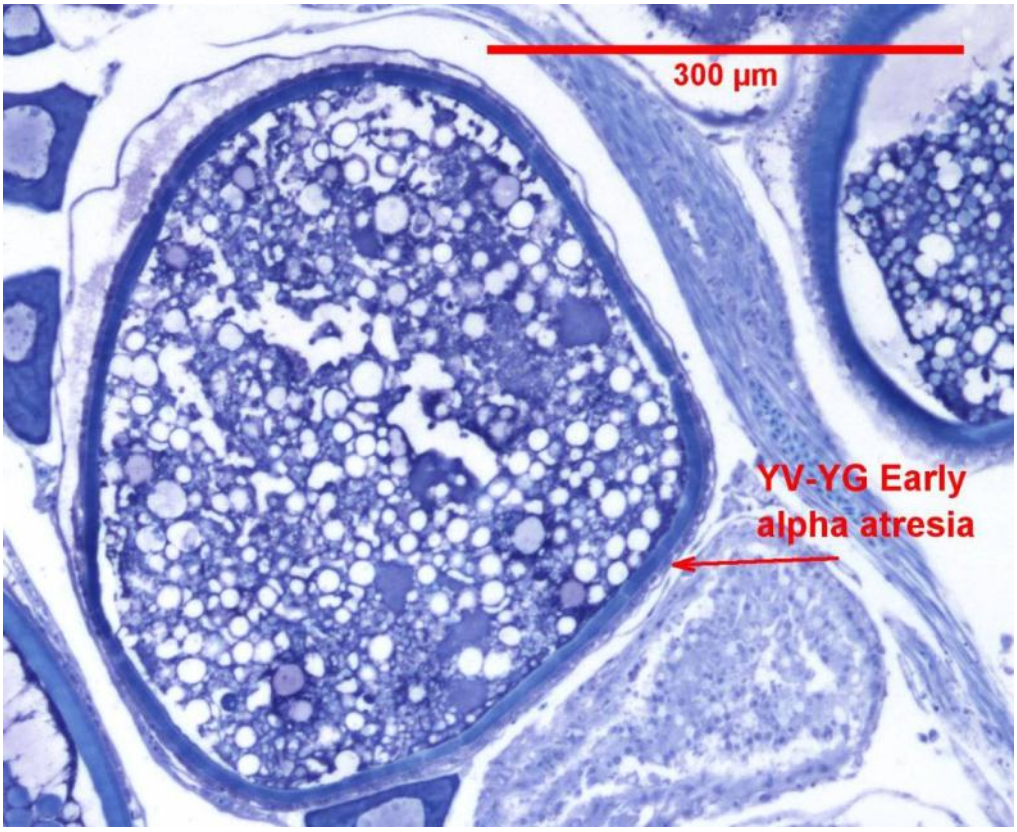


Figure 6.2.9. YV-YG early alpha atresia stage.

Figuer 6.2.10. YV-YG end of early alpha atresia stage.

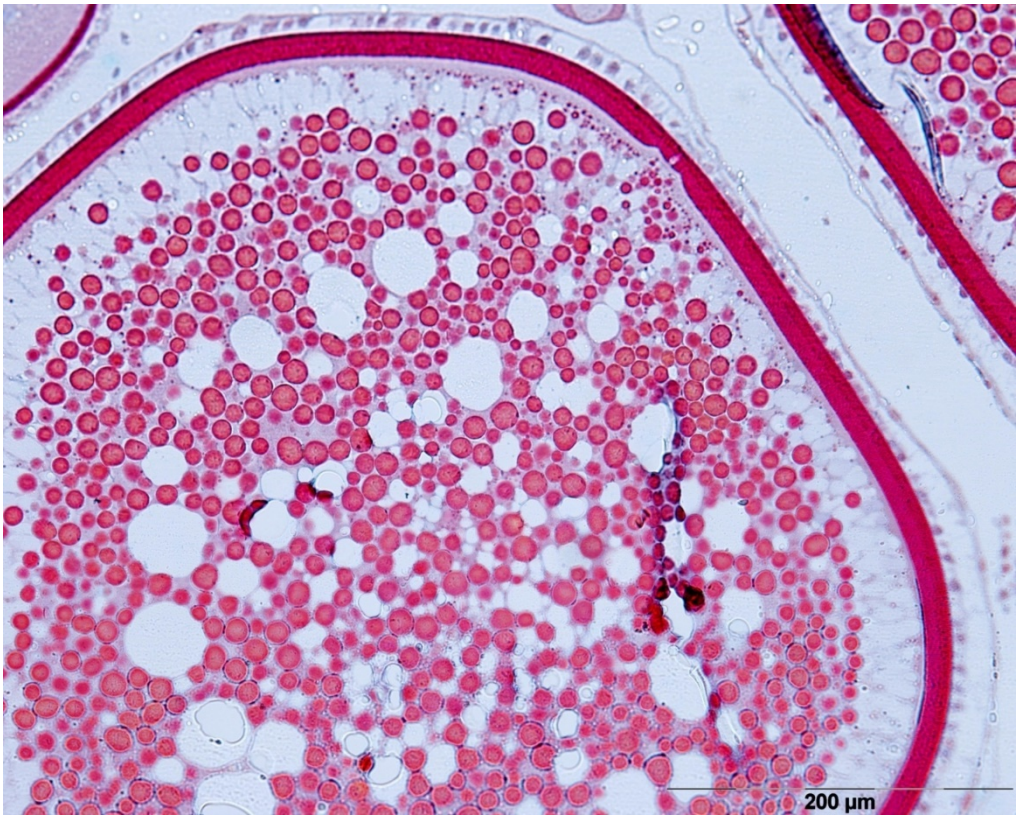


Figure 6.2.11. YG early alpha atresia stage. Note the cut in the inner part of the chorion.

Figure 6.2.12. YG end of early alpha atresia stage.

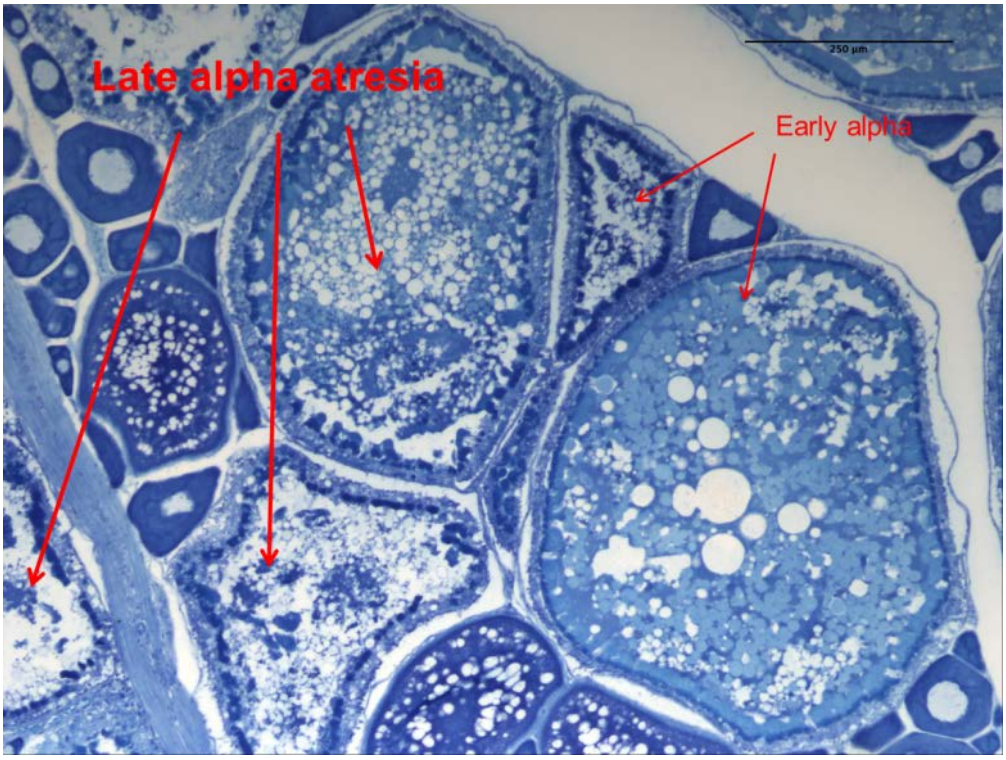


Figure 6.2.13. Early and late alpha atresia stages.

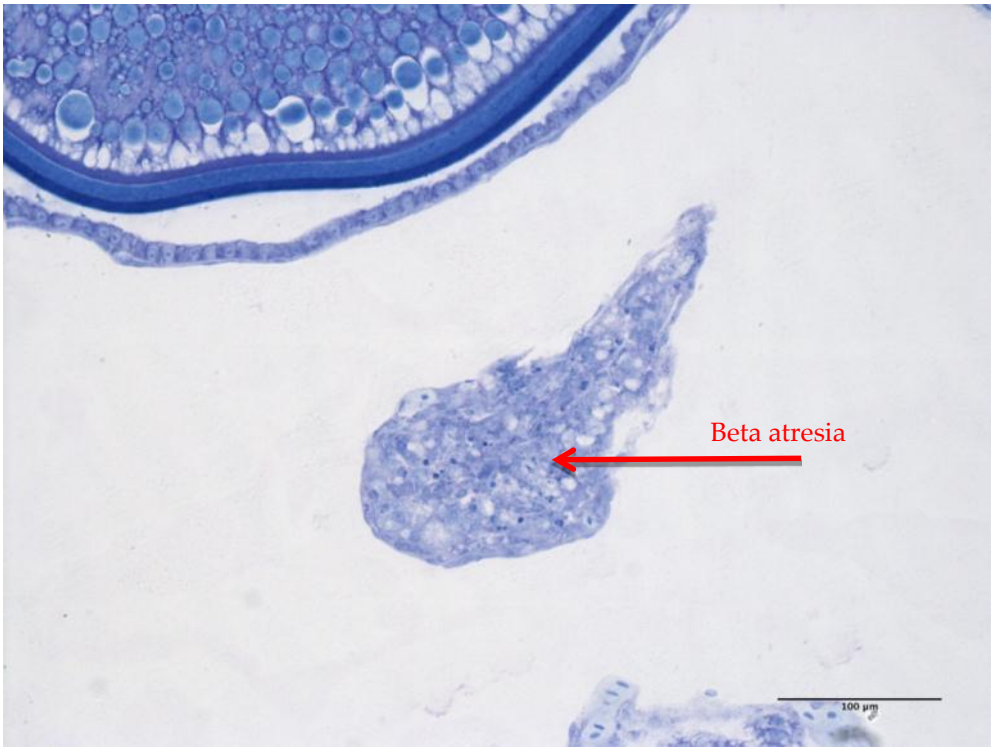


Figure 6.2.14. Beta atresia stage.

6.2.1 Measurement of partial area of atretic oocytes

Frames (Figure 6.2.15) are superimposed across both ovary sections at regular intervals in order to estimate the mean number of vitellogenic atretic oocyte transactions per unit area and the partial area of vitellogenic atretic oocytes in the histological section of the fish. The area analysed should be proportional to the ovary weight (Table 6.2.2).

Table 6.2.2. Area and number of fields to be analysed for different ovary weights.

Ovary weight (g)	Approximate area	Number of fields
2–9	0.3 cm^2	6
10–19	0.4 cm^2	8
20–29	0.6 cm^2	12
30	0.7 cm^2	14

A Weibel grid made up of test points is superimposed on the section (Figure 6.2.16) in order to estimate the partial area of early alpha atretic oocytes as a proportion of the total surface area in the sample frame. The test points are located at the ends of the lines in a grid.

The grid should have about 5000 points per cm^2 to cover the field. In Figure 6.2.16, the area inside the frame is 0.050 cm^2 and there are 256 points, which means that there are 5120 points per cm^2.

Images for atresia analysis are processed using ImageJ (Annex 3).

The outer grids should include area occupied by the ovary tunica (Figure 6.2.15).

Count the point that hit early alpha atretic oocyte in each of the three stages: YV, YV–YG, YG. All points inside and on the follicle and theca layer should be included in the point counts. Points lying outside the ovary tunica wall should not be counted, but marked as negative grid.

Calculate the partial area of vitellogenic atretic oocytes in the histological section (V_i) for each stage using the following equation:

$$V_i = {Number\ of\ hits}\Big/{(total\ points - negative\ grid)}$$

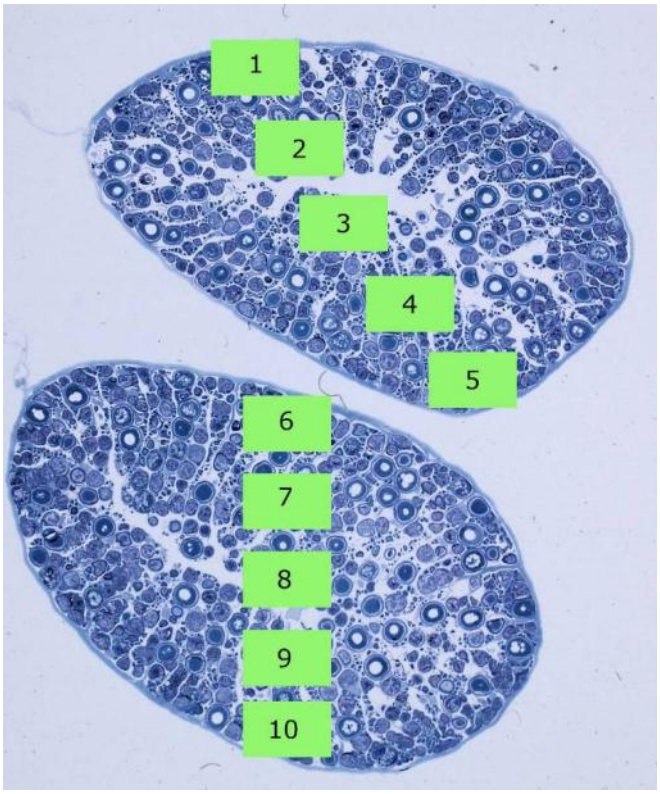


Figure 6.2.15. Frames superimposed on the ovary sections.

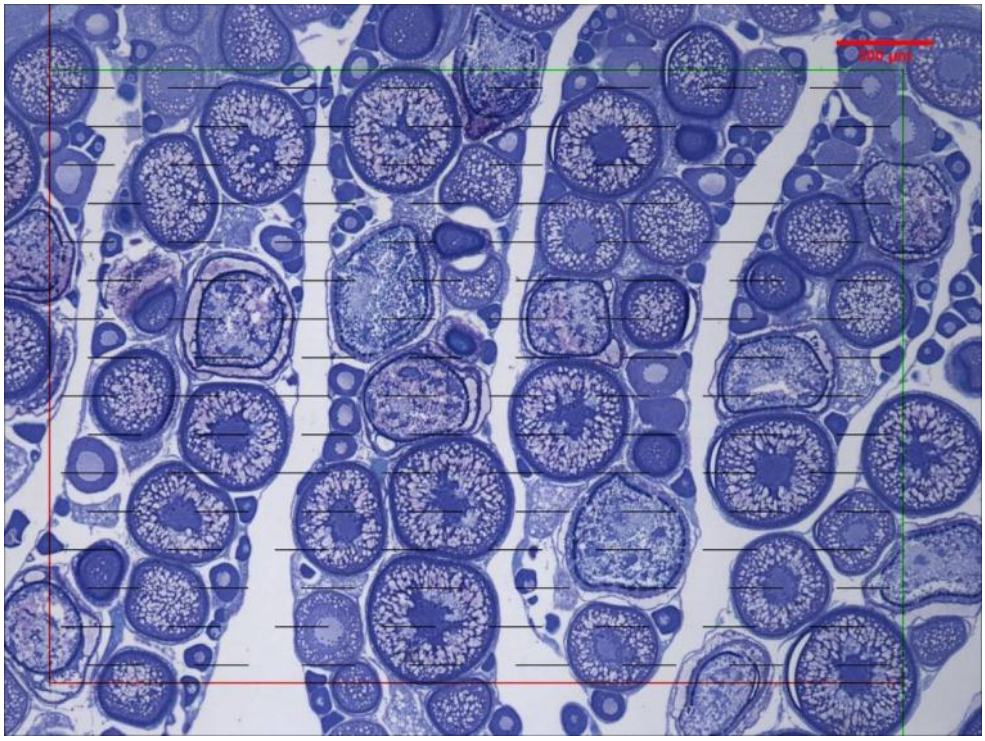


Figure 6.2.16. Weibel grid superimposed on the sample.

6.2.2 Measurement of the number of vitellogenic atretic oocytes

A frame is superimposed over the section and the number of early alpha atretic cells in each class of oocyte counted using the rules shown in Figure 6.2.17. Oocytes touching the forbidden line (red) or extended red line will not be counted (N). Oocytes inside the frame or touching only the green line should be counted (Y).

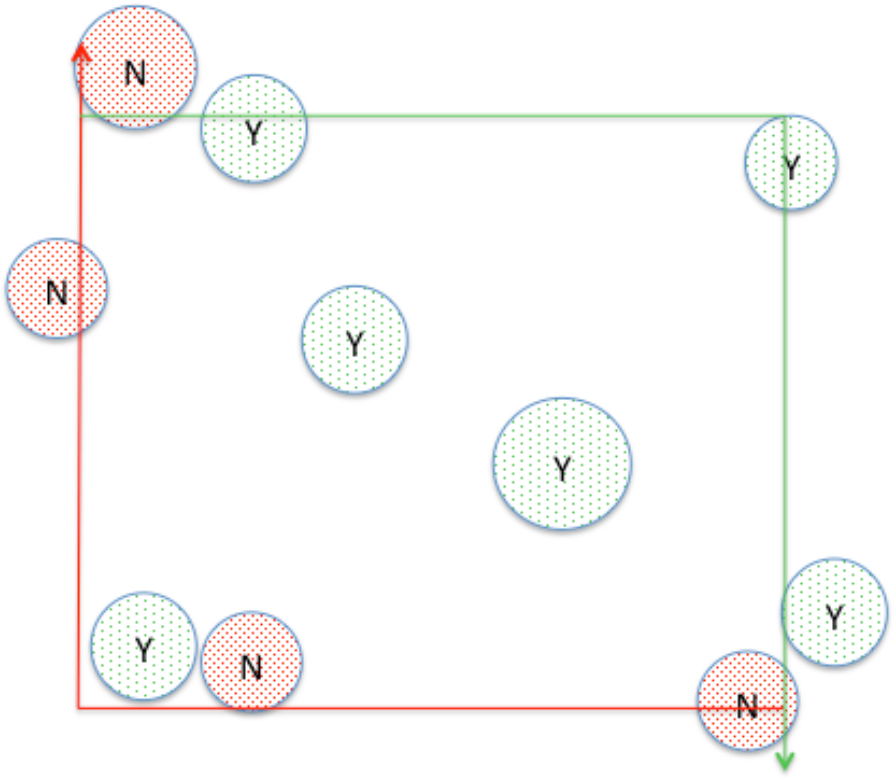


Figure 6.3.17. Frame superimposed on the sample to count the number of atretic oocytes.

Calculate Na for each stage using the following equation:

$$N_a = {}^{number\ of\ profiles}/_{field\ area}$$

In Figure 6.2.18, early alpha atresia cells in the stage (YV-YG) are counted. The area inside the frame is 0.053 cm², Na for YV-YG will be 4 / 0.053 = 75.5 profiles / cm².

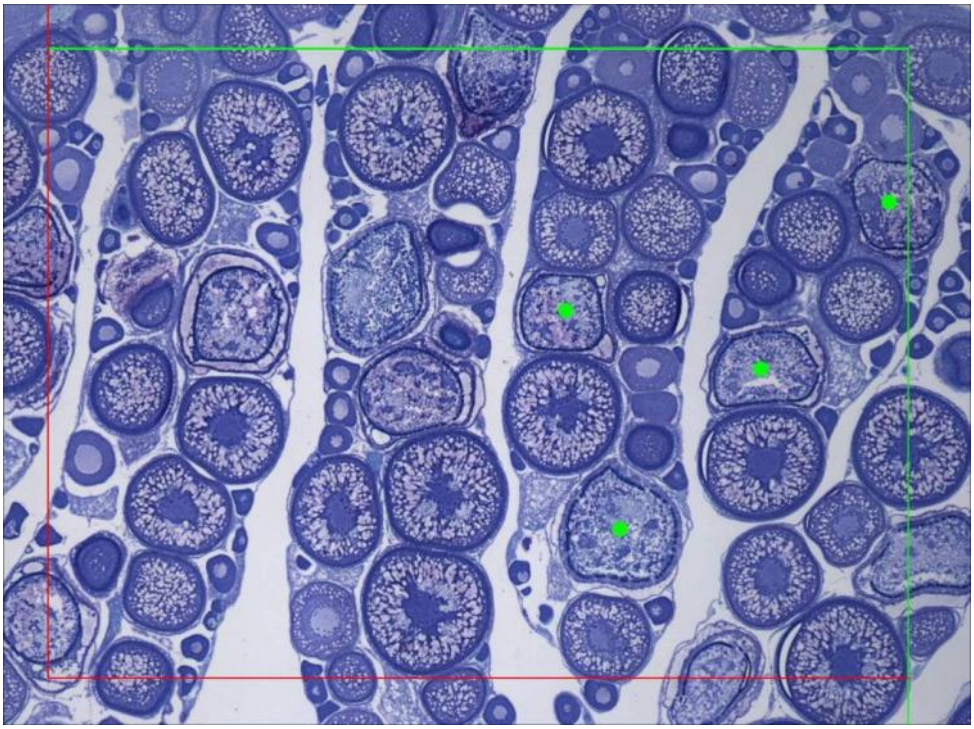


Figure 6.2.18. Example of counting the number of atretic oocytes.

6.2.3 Saving of results and pictures

For each fish, create a separate folder, containing the ObjectJ (J000.ojj) file and the pictures for the fish J000. Save the pictures using the standard code: e.g. J000_A_2013_IMR, built up as: Sample code_number for the pictures_year of the survey_institute initials (three letters). There will be an example of the folder on the ftp-site.

Fill in the "Atresia template" (Annex 5) with the results from the image analysis (Annex 3).

6.3 Calculations

6.3.1 Calculation of intensity of atresia

To estimate the number of atretic oocytes in the ovary, we use the following equation:

$$F_{atr} = \frac{OW * B * K * \ N_a{}^{3/2}}{V_i{}^{1/2}} = \frac{OW * 0.72 * \ N_a{}^{3/2}}{V_i{}^{1/2}}$$

F_{atr} =number of atretic oocytes
OW=Ovary weight
B = 0,72 (constant value, ratio between the longest and shortest axis of the oocytes transected)
K = 1 (constant value for atretic oocytes)
Na= no. profiles/field area
Vi= no. of hits/(total points-negative grid)

Summarize F_{atr} for the 3 stages and calculate the mean atresia from all the fish examined.

Calculate relative atresia using the equation:

$$F_{atr,r} = \frac{F_{atr}}{W}$$

$F_{atr,r}$ = Relative number of atretic oocytes
$F_{atr,r}$ this is the number that should be entered into the datasheet.

6.3.2 Calculation of mean atretic loss

To estimate the mean atretic loss, we use the following equation:

$$A_{avg} = \frac{F_{atr,r} * SD}{D}$$

Mean atr. loss = mean atresia * spawning duration / duration of early alpha atresia
A_{avg} = mean atretic loss
SD = spawning duration (60 days)
D = duration of early alpha atresia (7.5 days)

SD and D were set in the Mackerel/Horse Mackerel Egg Production Workshop (ICES, 1993)

7 Procedure 4: AEPM sampling at sea procedure for Mackerel (all periods) and DEPM sampling for mackerel (Periods 2–3) and Horse mackerel (Periods 6–7)

7.1 Before the cruise

Procure 25–50 µl capillary pipettes (Wiretrol II 25–50 µl, Cat. Number 5-000-2050 (VWR). Extra plungers can be ordered from the same supplier; be sure to order the long plungers!). Test the performance of the pipette by practice, taking 25 µl fresh gonad or water samples.

Buffered formaldehyde: 3.6% buffered ($NaH_2PO_4*H_2O$: 29.48 mM, $Na_2HPO_4*2H_2O$: 46.01 mM) formaldehyde (see also excel-file on the IMR ftp-sever: "Buffered formaldehyde").

IMR and IMARES will send around labels to all the institutes participating in the survey to use on the Nunc tubes. Each institute will get its own code (Table 3.2.1). Fill the labelled 2.5 ml Nunc tubes (with screw-on lids) with 1.2 ml of buffered formaldehyde. Also, fill the labelled 20 ml scintillation tube with 15 ml of buffered formaldehyde.

7.2 During the cruise

Fishing hauls

Surveying for adult fish will take place simultaneously with the ichthyoplankton sampling. Over the whole survey area, one fishing haul per transect should be carried out. If possible, adult samples should also be obtained at night. Good spatial and temporal coverage is essential to reduce bias in batch fecundity and spawning fraction estimations.

Measure the weight of the total catch. Then, randomly select a subsample of 100 fish, and measure the total weight of the subsample. For all the 100 fish in the subsample, measure total length, total weight, maturity (Walsh scale), and take otoliths for age reading.

Female sampling

For the 100 fish in the subsample, select the first 30 females in maturity stages 3–6 for the full biological sampling or stages 2–6 for DEPM sampling.

If fewer than 30 females are in the hydrated stage, collect additional females from the remaining catch. For these extra samples, select females with macroscopically visible hydrated oocytes, but without running eggs. For each extra female, do the full biological sampling, and take samples for screening analysis (2–3 g in scintillation tube) and batch fecundity (two samples of 100 µl) These females will be numbered 101, 102, 103... . If after a total of 100 extra individuals you do not obtain a total of 30 females with hydrated oocytes, the sampling of the haul is finished.

Full biological sampling
Measurements:

- Total length
- Total weight
- Maturity
- Otoliths
- Weight of ovary (If it is not possible to measure the ovary weight at sea, take out the ovary and weigh the fish without the ovary. Then, take the pipette

and atresia samples and fix the remainder of the ovary, and weigh the ovary once back in the lab. The fixed and frozen weights should be corrected to fresh weights.)

Screening sampling:

- From one ovary lobe, take a small sample (2–3 g) for screening (Figure 3.2.1) with a spoon or cut with a scalpel, and immediately put this sample into a prefilled, individually-coded 20 ml scintillation tube. Make sure the sample is covered with 3.6% buffered formaldehyde solution.

Fecundity sampling:

- From the same lobe of the ovary take 2 pipette samples of 25 µl and 2 samples of 100 µl (Figure 3.2.1 and Figure 3.2.2) and immediately put each sample in its own individually-coded Nunc tube. Ensure all oocytes are immersed in 3.6% buffered formaldehyde solution. Rinse the pipette with water, and dry it with a paper towel prior to sampling another fish.

Atresia sampling:

- For atresia: Puncture the other ovary lobe with a fine needle, without breaking the lobe. Place the lobe of the ovary in a labelled bottle (100–250 ml with wide opening), filled with 3.6% buffered formaldehyde (Figure 3.2.1).
- Make sure that all the ovary samples are completely covered with formaldehyde.

7.3 After the cruise

Immediately after the cruise, the screening samples in the scintillation tubes should be sent to the analysing institutes (Table 3.2.1).

All the ovary samples should remain fixed in 3.6% formaldehyde for at least two weeks, before whole mount analysis or the sections for the atresia analysis are taken. From the fixed ovary lobe, cut two 5 mm thick slices and put them in a coded histology cassette. Write the code with a wooden pencil on the outside of the cassette. If the ovary is very big, you may have to use two cassettes. Separate the cassettes into four colour-coded, leakproof bottles, filled with 70% ethanol. Pack the consignments for each country with a maximum volume of 1000 ml solution in each package. On the outer cover of the package, indicate the volume of fixative and that it is within the limits for unclassified transport.

After the screening, the adult sampling coordinators will divide the samples between the analysing institutes. Send the cassettes and Nunc samples for analysis to the different institutes, based on the list provided by the sampling coordinators.

7.4 Screening before analysis

7.4.1 Spawning markers and atretic oocytes

Process histologically the 2–3 g samples in the scintillation tubes. Histological procedures (embedding, sectioning, and staining) are described in Procedure 3 (Chapter 6).

Screen the histological slides under the microscope looking for the most advanced oocyte stage (Tables 4.1.1 and 4.1.2, and Figures 4.1.1–4.1.3), POFs (Table 7.7.1 and Figures 7.7.1–7.7.7), hyaline eggs (Table 4.1.2 and Figure 4.1.3), early alpha atresia,

massive atresia, if it is spent (Figure 4.1.4) and if it should be discarded. Take pictures of all samples for identification and staging of POFs (See Section 7.7 and Table 7.7.1).

The results should be entered in the excel sheet "Screening_histology_DEPM_form" (Annex 6) and saved on the ftp server.

7.5 Batch fecundity whole mount analysis in the laboratory

7.5.1 Batch fecundity

Samples containing the oocytes stages, migratory nucleus or hydrated (Stages 4 and 5), will be analysed for batch fecundity. Distribute the 100 µl sample from the Nunc tube evenly in the tray.

Measure the oocyte diameters automatically, using ImageJ software provided for the fecundity analysis (Ensure the correct ojj file is being used). See Annex 3 for image analysis manual.

Count and measure all the oocytes > 500 µm in the sample.

Based on the length, frequency distribution of the oocyte sizes the batch sizes will be estimated.

7.5.2 Calculation of batch fecundity

Batch fecundity

$$F_b = \frac{N_b}{W_s} * OW$$

F_b = batch fecundity

N_b = number of oocytes in the batch

W_s = weight of the pipette sample (0.100 g)

Relative batch fecundity

$$F_{rb} = \frac{F_b}{W}$$

F_{rb} = relative batch fecundity

7.6 Sex ratio

Sex ratio is estimated in weight, from all the adult samples taken during the peak spawning period, using the following equation:

$$R = \frac{W_f}{W_{(f+m)}}$$

R = Sex ratio
W_f = mean weight of mature females
W_m = mean weight of mature males

The mean weight is calculated as the mean fish weight per haul and weighted by the number of hauls.

7.7 Spawning Fraction

7.7.1 Embedding, sectioning, and staining

The procedure is described in procedure 3 (Chapter 6).

7.7.2 Spawning fraction analysis

For the spawning fraction estimation, we will use the POF method. In the histological sections, all POFs will be counted and staged. Staging of POFs will be done using morphological criteria (Table 7.7.1 and Figures 7.7.1–7.77). The results of the POF staging should be entered in the excel sheet "POF staging form" (Annex 7) and should be saved on the ftp server.

First, take a general overview of the whole sample to assess which stages of oocytes and how many different stages of POFs are present. Be aware that, like with the vitellogenic and atretic oocytes, if you cut the POFs close to the edge, the appearance of the POF can be small, but look carefully at the size of the cells in the theca and follicle layer and the size of the lumen.

Table 7.7.1. Post ovulatory follicle (POF) stages.

Development stage	Description
1	**Horse mackerel** Immediately after spawning. POFs usually surrounded by hydrated oocytes. Exhibit large dimensions. Granulosa cells large, well defined, with columnar or cuboidal shape and nuclei prominent in a characteristic linear arrangement. Granulosa cells arranged in an orderly manner. **Mackerel** Newly-formed POF, often still in simultaneous with hydrated oocytes. Large POFs. With cord-like structure, extended or folded with large loops and folds. It's easy to follow the line of cells. Large lumen (area where the oocyte used to be). Granulosa cells are arranged in narrow lines, and most of them have the nucleus in an apical position. Cell boundaries are quite clear. The theca is still much stretched. Separated from the granulose, thin and not very clearly distinguishable at first, being clearer as the POF advance toward Stage 2. No signs of degeneration.
2	**Horse mackerel** POFs structure more irregular, but still convoluted shape. Linear arrangement of columnar epithelial cells was still evident. Cells of granulosa become hypertrophied and are columnar in shape. The nuclei of the granulosa cells are spherical and have a basal location. **Mackerel** It's unusual to observe hydrated oocytes. Large POF, with evident loops. More folded than Stage 1. Large lumen. Granulosa cells still arranged in lines, but these are wider. More nuclei in a basal position than in stage 1. Slightly hypertrophied cells, with a column or cubical aspect. It's possible that in some part of the POF the line of cells is difficult to follow. Theca is clearer.
3	**Horse mackerel** POFs with a more irregular structure. Loss of linear arrangement of granulosa cells. Breakdown of granulosa cell walls. Nuclei of granulosa cells can appear pycnotic, spherical and still had basal location.

Development stage	Description
	Mackerel Granulosa folds are still clearly recognized. Granulosa cells are still aligned, but less ordered and in thicker lines. Less obvious cell boundaries. Lumen clearly reduced in size with respect to Stage 2. Evident signs of degeneration: Pycnotic nuclei (darker pigmentation of the nucleus) in the granulosa cells. First vacuoles in granulosa cells. Theca is closer to granulosa and frame it.
4	**Horse mackerel** POFs decrease in size to about one-half. Marked degeneration of the columnar epithelial cell lining. Granulosa cells become indistinct. **Mackerel** The POFs are still large, but smaller than in Stage 3. Compact POFs in which folds can very seldom be distinguished in the granulosa. The lumen is small, and sometimes can't be seen. If it is visible it is a white line, more or less wider, than drawn granulose folds. Granulosa cells are more disordered and their limits are not apparent. Pycnotic nuclei and vacuoles are more frequent. Theca is closer to granulosa. It´s more difficult to see than in stage 3.
5	**Horse mackerel** The convoluted structure no longer distinct. Lumen much reduced or absent. Granulosa cells walls absent and a few "vacuoles" or pycnotic nuclei may be seen. **Mackerel** Strong decrease in size with respect to the previous stage. POF without ordering patterns, except some short alignment of nuclei. Folds aren't visible, and POF looks quite degenerate, with a more regular shape than previous stages. Lumen isn't visible. Granulosa presents numerous pycnotic nuclei and vacuoles. Only few cells are intact. It's possible to see large white areas of vacuoles that can lead to confusion with the lumen. Follicle perimeter layer is made wider. This layer, which closely frames the follicle, is considered to be the theca layer. However Alday *et al.* (2010) considered it as stromal connective tissue.
6	**Horse mackerel** POFs degeneration clearly more advanced. Distinguishing them from old atretic follicles could be a problem. Lumen typically occluded. Granulosa cells present, irregular shape with pycnotic nuclei. **Mackerel** POF of reduced size and shows a polyedric shape, frequently triangular. The lumen is absent. Granulosa is reduced to a few remaining cells, normally with pycnotic nuclei and vacuoles. Peripheral tissue (theca or stroma tissue) is proportionally a higher fraction of the POF. Possible confusion with ß-stage atresia.
7	**Mackerel** Very small POF. Difficult to see with 4x magnification. Their number in the sample is very low. POF reduced to almost only the peripheral tissue. Granulosa, if present, is residual.

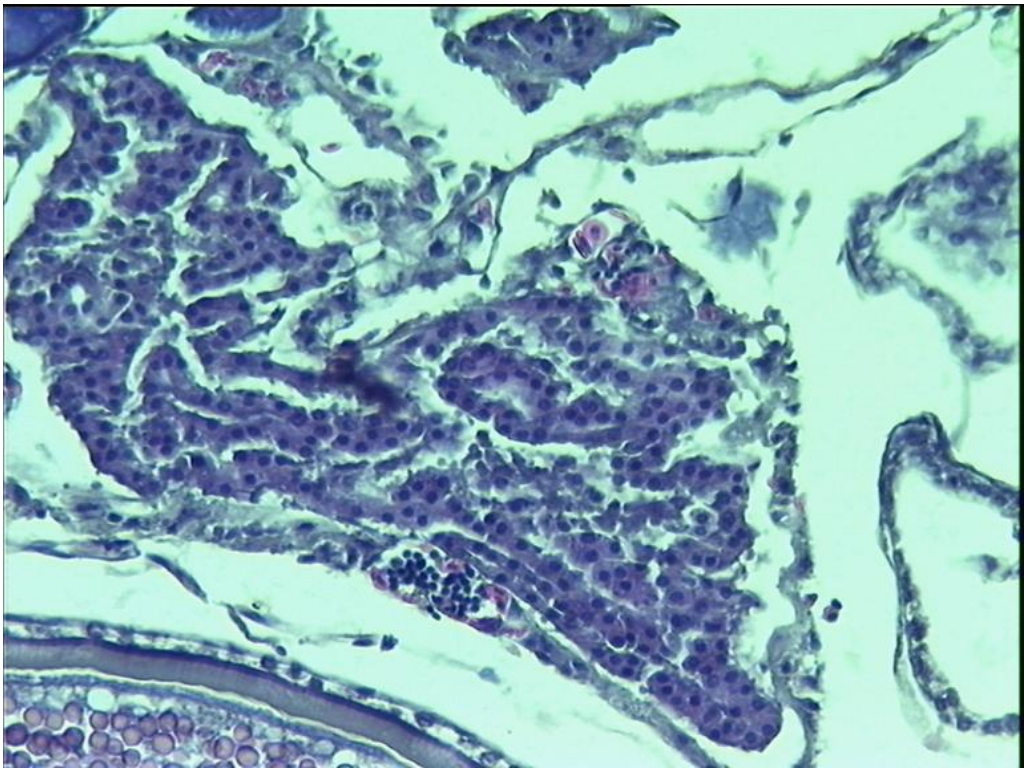


Figure 7.7.1a. POF Stage 1 in horse mackerel.

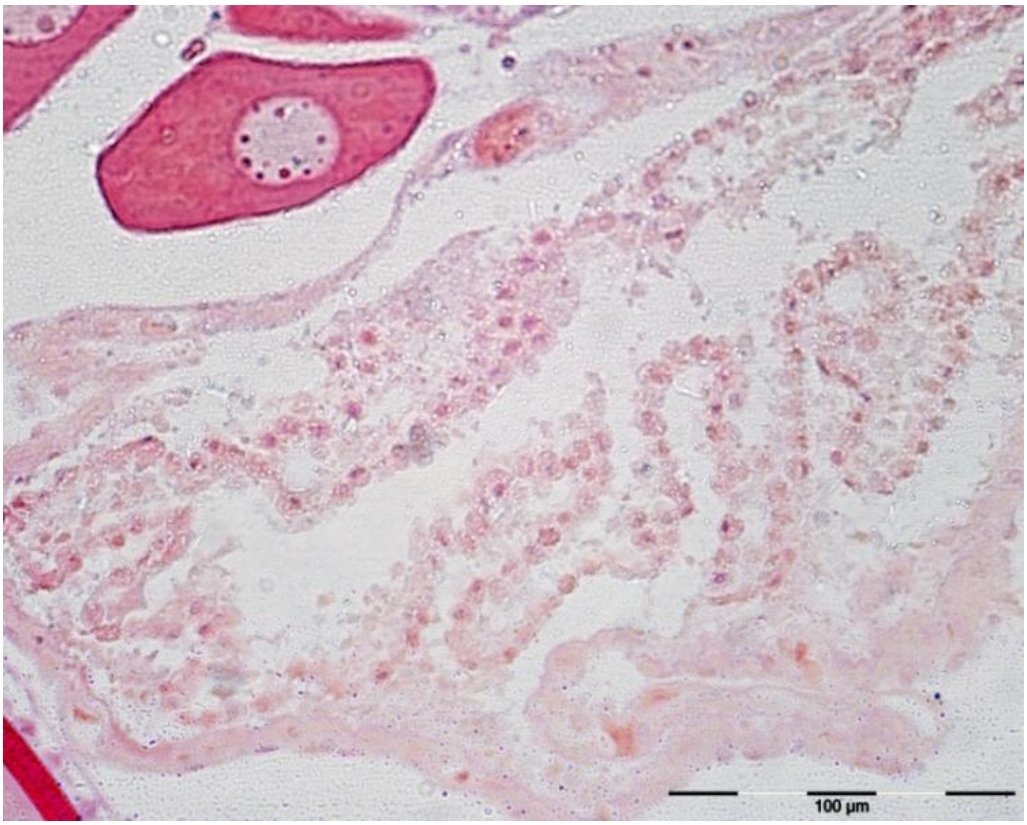


Figure 7.7.1b. POF Stage 1 in mackerel.

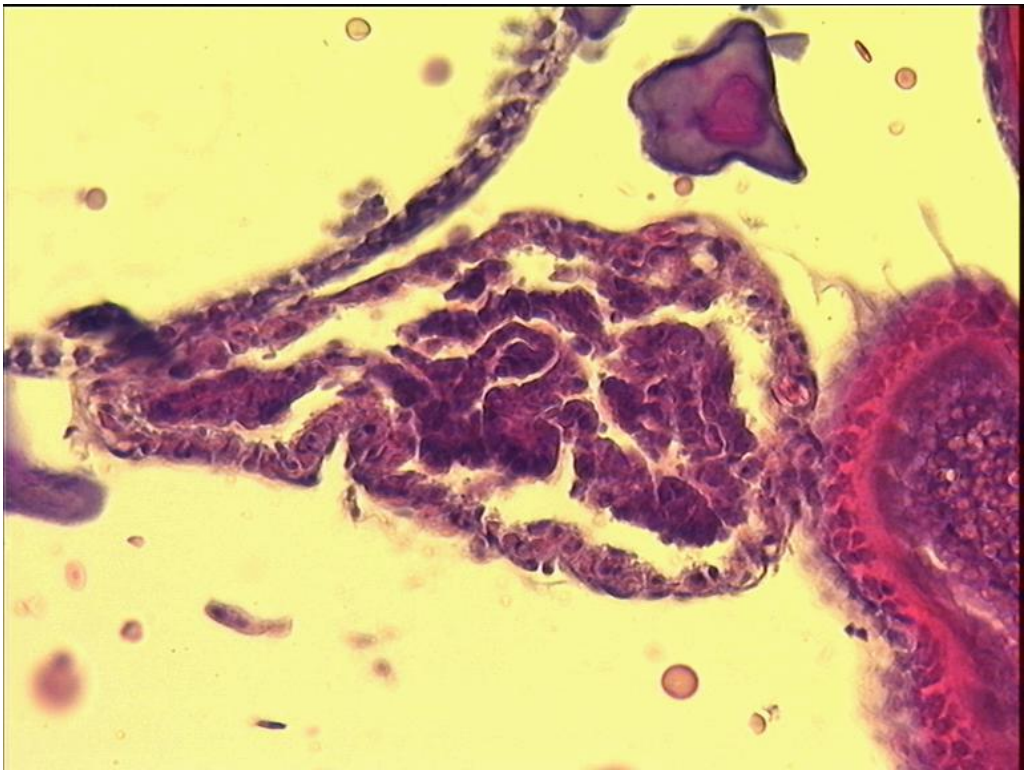


Figure 7.7.2a. POF Stage 2 in horse mackerel.

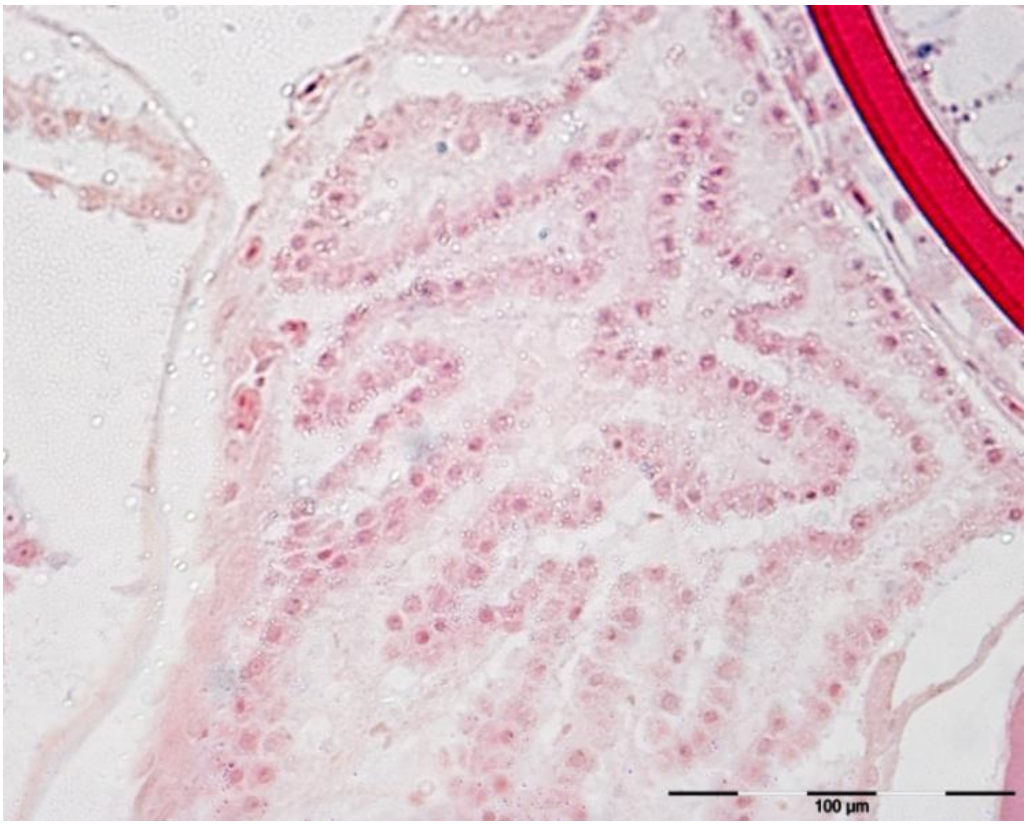


Figure 7.7.2b. POF Stage 2 in mackerel.

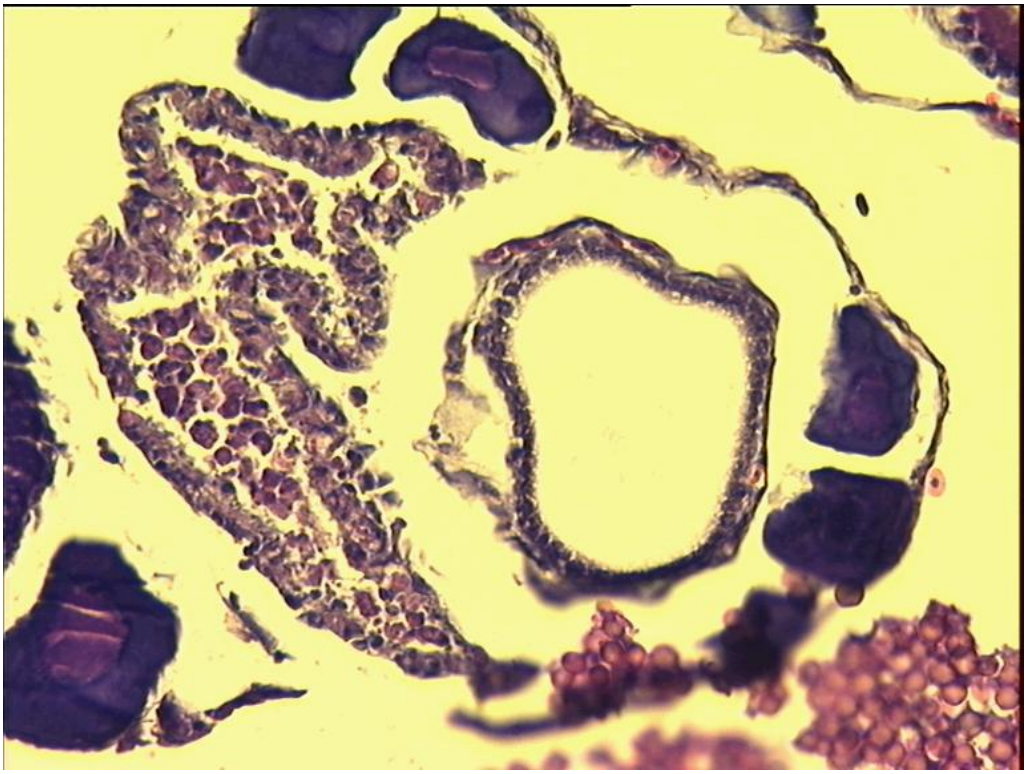


Figure 7.7.3a. POF Stage 3 in horse mackerel.

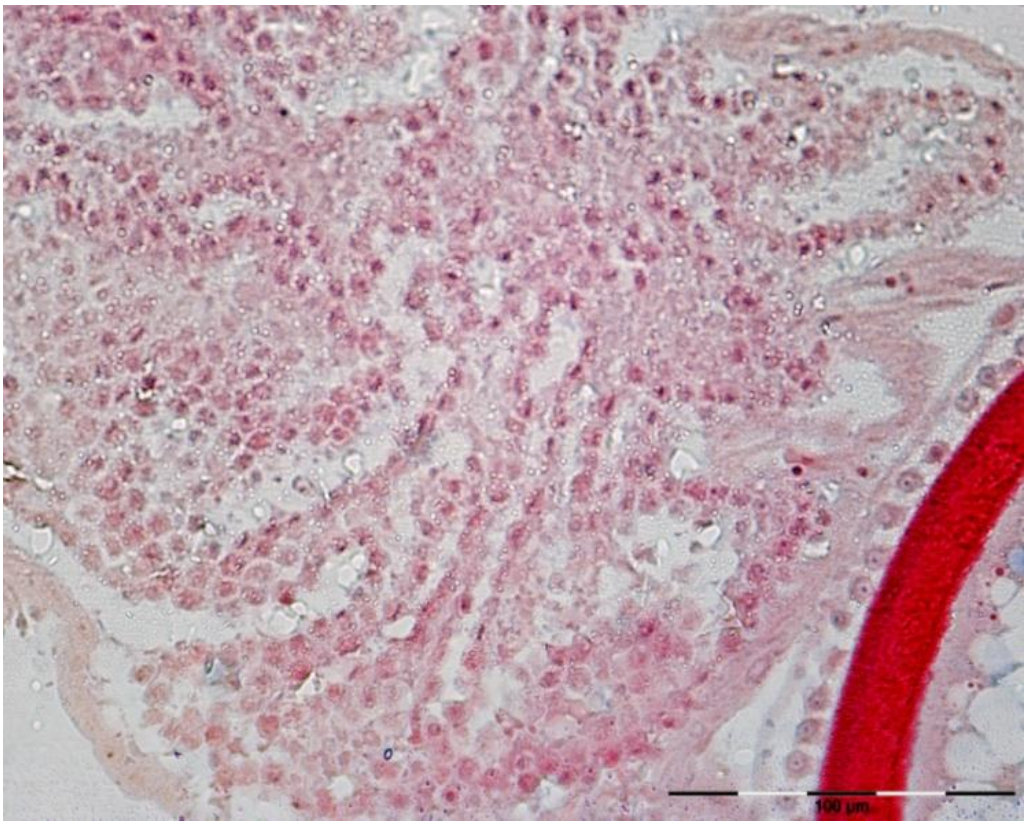


Figure 7.7.3b. POF Stage 3 in mackerel.

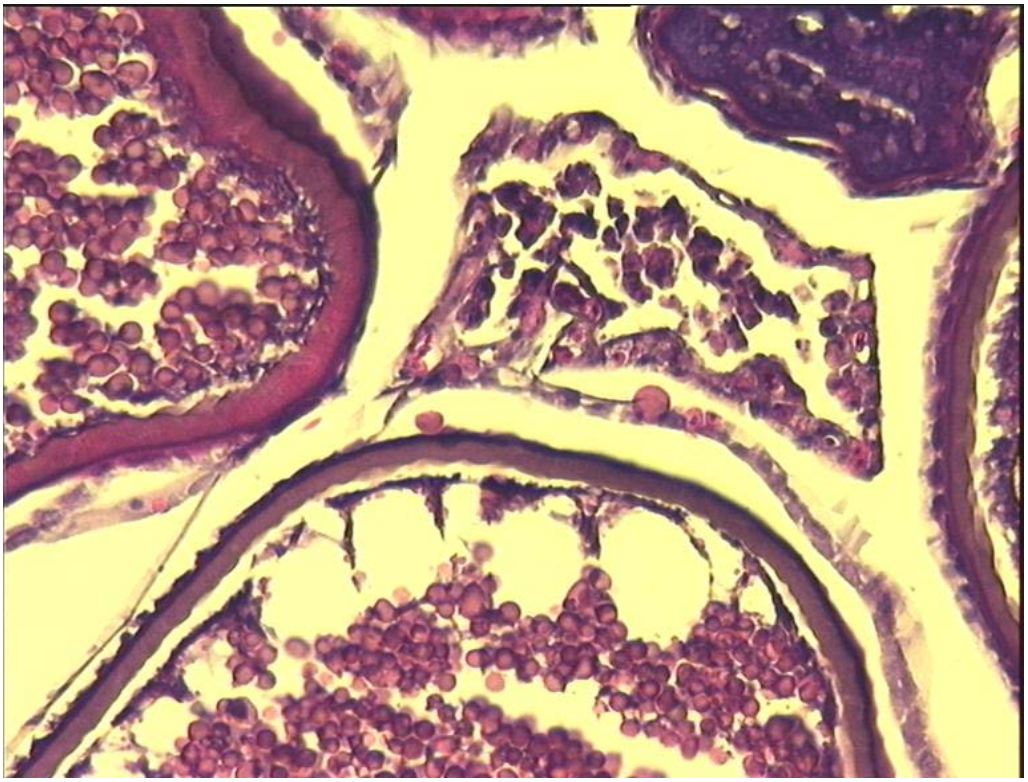


Figure 7.7.4a. POF Stage 4 in horse mackerel.

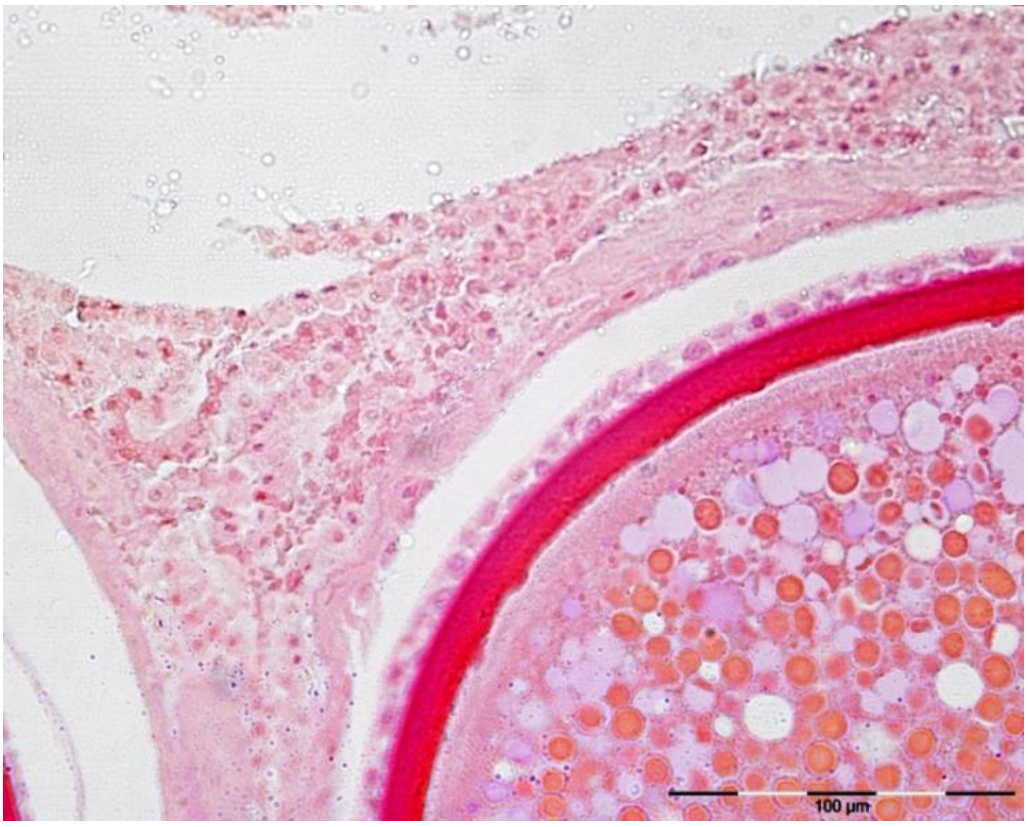


Figure 7.7.4b. POF Stage 4 in mackerel.

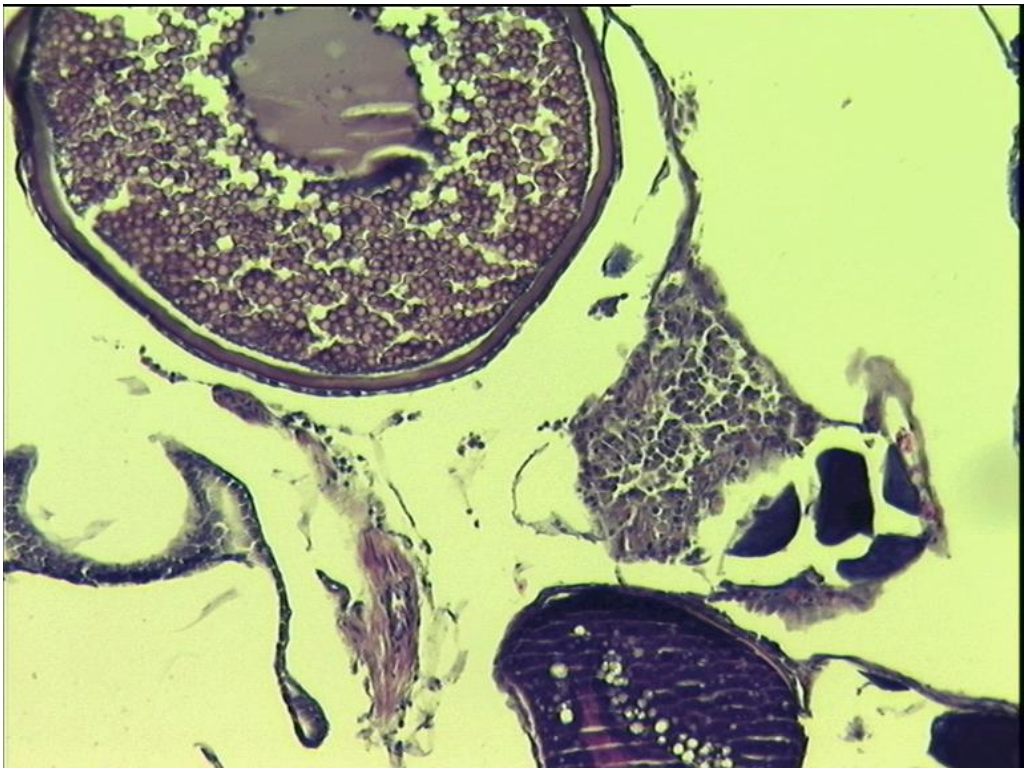


Figure 7.7.5a. POF Stage 5 in horse mackerel.

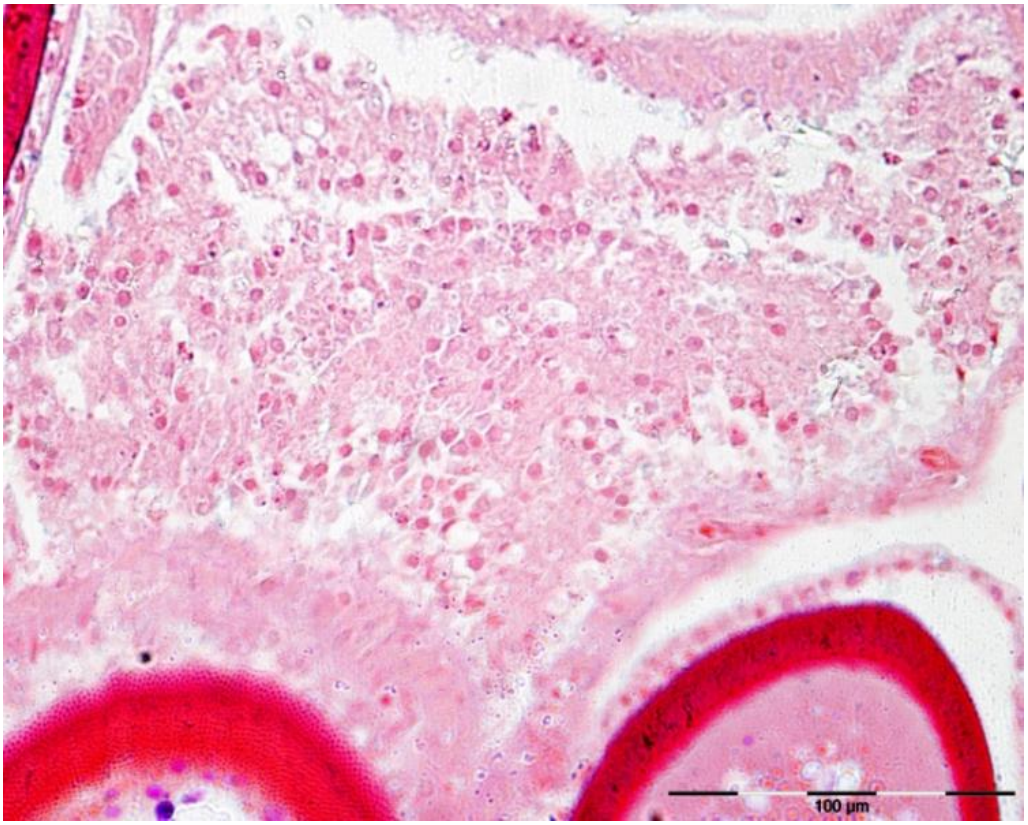


Figure 7.7.5b. POF Stage 5 in mackerel.

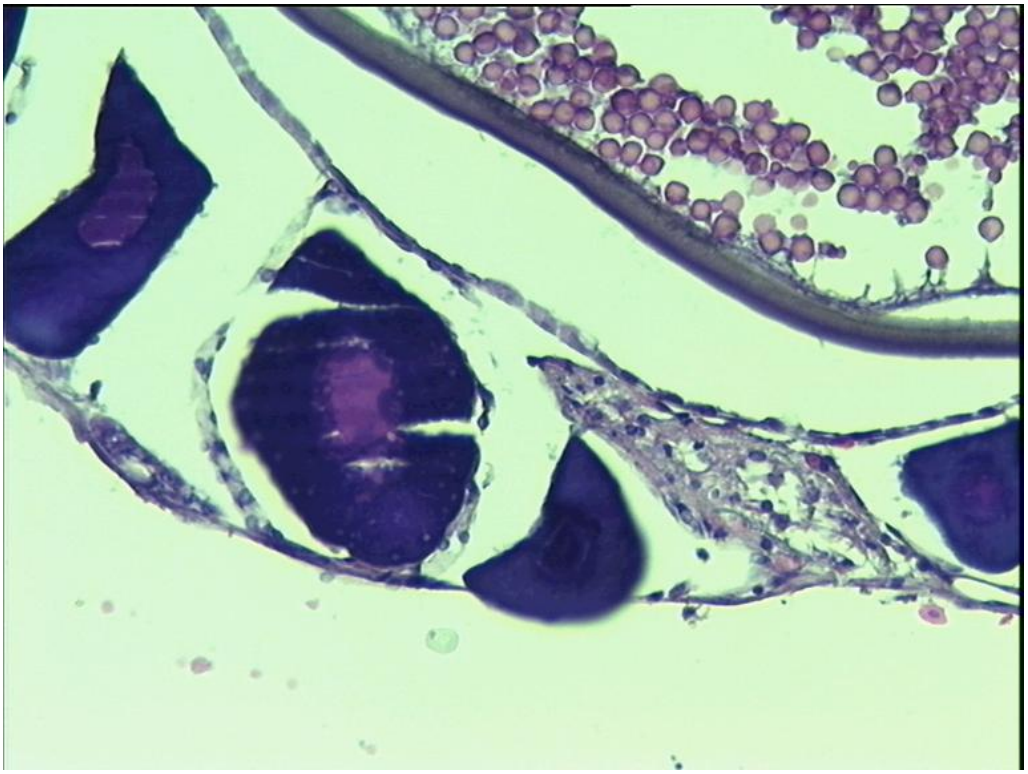


Figure 7.7.6a. POF Stage 6 in horse mackerel.

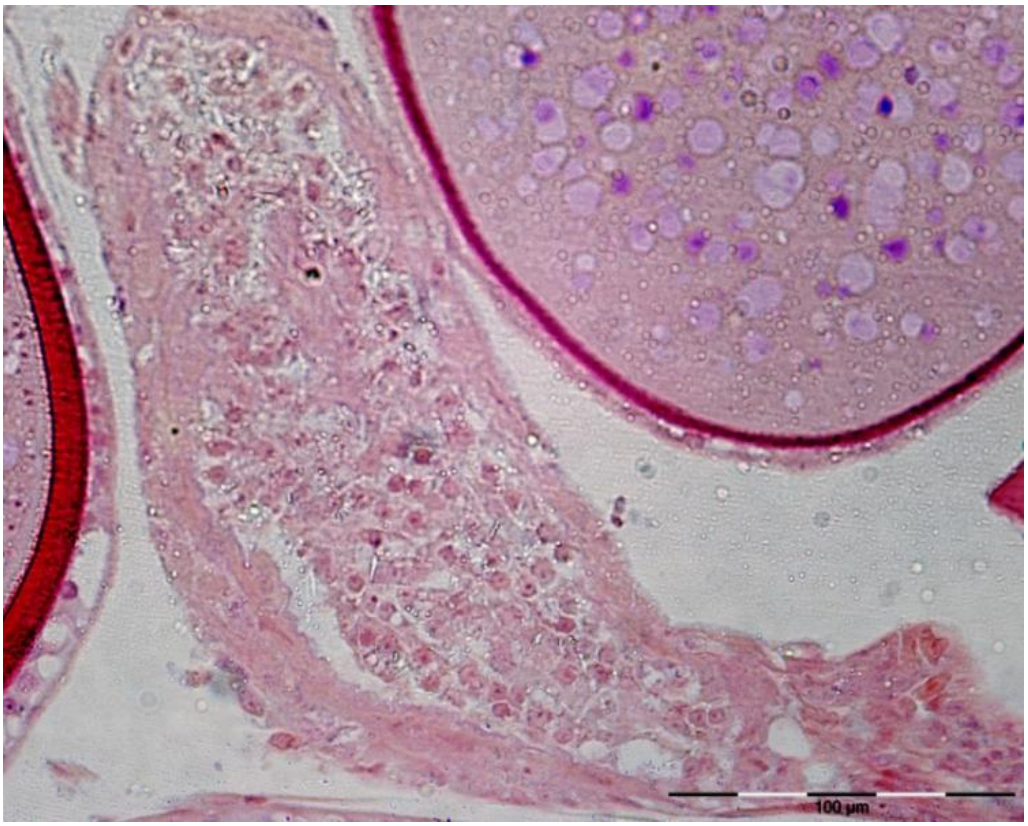


Figure 7.7.6b. POF Stage 6 in mackerel.

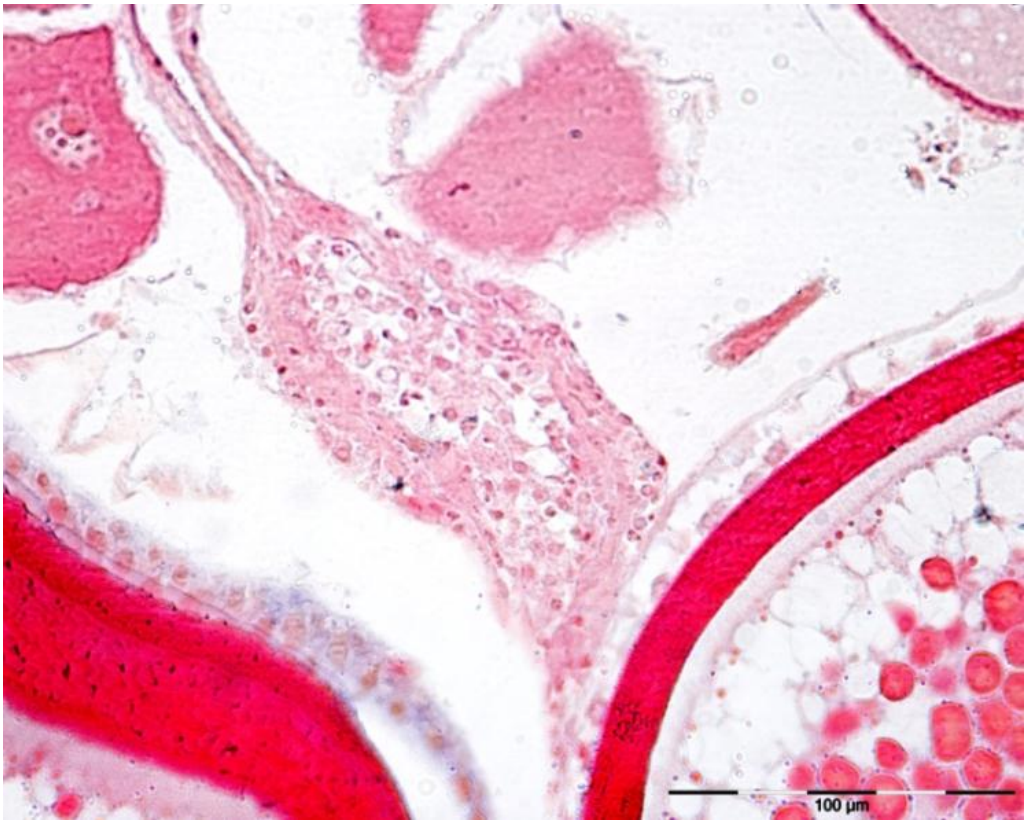


Figure 7.7.7. POF Stage 7 in mackerel.

Since no data is available at the moment on the stage durations in mackerel and horse mackerel, we will use the stage duration of sardine for these species. Day 0 POFs are POFs in stages 1, 2, and 3; Day 1 POFs are stages 4 and 5; Day 2 POFs are in stage 6; and, stage 7 are older POFs.

7.7.3 Spawning fraction estimation

Spawning fraction will be estimated using the following formula:

$$S = \frac{(n_1 + n_2)}{2(n_0 + n_1 + n_2 + n_{\geq 3} + n_{noPOF})}$$

S = Spawning fraction

n_n = number of females with day n POFs

n_{noPOF} = number of mature females with no POFs

$$n_0 = \frac{(n_1 + n_2)}{2}$$

7.7.4 Hydrated female weight correction

In order to correct the extra weight of the ovary due to the hydration process, a correction of the ovary weight should be applied. For this, the random weight of the spawning females (all stages except hydrated stage) is multiplied to assess the total weight (W_t) and ovary free weight (W_{fov}) for each fish by mean a linear regression.

$$W_t = a + b * W_{fov}$$

8 Southern stock horse-mackerel DEPM survey (Period 1)

Adult Surveying

Surveying for adult horse mackerel will take place simultaneously with the ichthyoplankton sampling. An average of two fishing hauls, with bottom-trawl, are aimed to be conducted per day, along the whole survey area. Fish samples are to be representative of the population demography and distribution, and thus a good spatial and temporal coverage is essential for avoiding bias on the DEPM parameters estimation.

Sampling will be complemented with fish from commercial vessels, obtained at four or five ports along the coast during the period of the campaign.

8.1 Fish biological sampling during the survey

From each trawl, a sample of 60 fish will be randomly selected and sampled biologically onboard. Reserve some extra fish, in case it is necessary to complete the sample with additional fish (see below).

If fewer than 60, but more than 30 fish are caught, conduct full biological sampling with all fish caught. For hauls with less than 30 fish, fish will only be sampled for batch fecundity in case hydrated females (stage 4 ovaries, but without running oocytes) are present: in this case, conduct full biological sampling only with these females, remove the otoliths, and collect and preserve hydrated ovaries in formaldehyde solution.

Record individual biological data for the 60 fish sampled: length, total weight, gutted weight, sex, macroscopic maturity stage (Walsh scale, 1990; and possibly level of fat and stomach fullness). Remove the otoliths of the 60 fish for ageing.

For the first 30 females encountered (of all macroscopic maturity stages), immediately collect the gonads and preserve them in formaldehyde solution (4% formaldehyde solution buffered with Sodium phosphate salts, diluted in distilled water). The flask with the gonad sample should be labelled with the following information: code number of the survey, haul number, fish number, species FAO code (HOM) and maturity stage (e.g. F3). The volume of formaldehyde solution in the flask must be at least 3 times the volume of the gonad, and the latter must be completely immersed.

Important: the total weight of the gonads will be subsequently obtained in laboratory from the preserved material. Gonads have a delicate envelope; it is important during the sampling to handle the gonad carefully, in order not to lose material before preservation in the formaldehyde solution.

Extra effort should also be taken to obtain females with hydrated ovaries, since the number of the latter is usually low in samples. If possible, search for additional hydrated females from the remaining fish of the catch, and if present, perform the full biological sampling on these females, including the removal of the otoliths and the preservation of the gonads in formaldehyde solution. Ideally, 150 females (but no more than 30 fish per trawl) should be obtained along the coast.

If the random sample of 60 fish contains fewer than 30 females, continue collecting and sampling fish from the catch until this number is reached. For these extra fish, carry out the following procedure: open the fish, if the individual is a male, do not sample it; if it is a female, conduct full biological sampling, including the removal of the otoliths and the preservation of the gonads in formaldehyde solution. If, after a total

number of 100 fish have been sampled, 30 females have not been obtained, the sampling for this haul is considered finished.

The extra fish sampled (either to complete 30 females or to obtain additional hydrated females), as well as their corresponding otoliths and ovaries, will be labelled with the numbers 61, 62, 63,… .

The sampling procedure for the samples coming from the bottom-trawling commercial vessels will be adjusted, depending on the fishing operation (time before the vessel reaches the port) and facilities on board and at the ports, but in case fish collected by the fleet need to be frozen for subsequent biological sampling, gonads should always be preserved previously as fresh material. In any case, it is recommended that:

- the fish samples are obtained during the period of the survey, and ideally within 1–2 weeks of the surveying of the area by the RV
- information of the time and location of the fishing haul is obtained
- if possible, the fish sample is obtained from the last fishing haul of the trip, to reduce the elapsed time between fish capture and the ovaries preservation.

Mackerel sampling will be carried out whenever possible to support the EPM estimation undertaken by the WGMEGS, following the Procedure 1 (Chapter 3).

8.2 Laboratory work after the survey

Otoliths and preserved gonads will be returned to the institute after the survey. Otoliths will be processed and analysed to determine fish age. Gonad samples should remain fixed in the formaldehyde solution for at least two weeks before being processed.

Ovaries total weight:

Before being processed for histology, all ovaries should be first weighed, with a precision weighing balance (0.01 g), taking care that before being weighed, each ovary is first wiped gently with tissue to remove excess formaldehyde solution.

The ovary weight data obtained is then converted to total fresh gonad weights (Wgon), using a conversion factor fixed/fresh obtained previously.

Histology:

All preserved ovaries (from the survey and commercial samples) will be processed for histological analysis: 1) to confirm microscopically the maturity stage, 2) to be used in the estimation of the spawning fraction, and 3) to check for the presence of POFs) in the hydrated ovaries (not to underestimate batch fecundity).

For each ovary, 2–3 slices are cut at the middle area of one lobe and put in a labelled cassette for subsequent histological processing. The gonad tissue samples in the cassettes are dehydrated with successive alcohol solutions (ethanol of increasing grade and finally butanol), and then embedded in paraffin (56–58°C). The resulting blocks are sectioned (3–5 μm thick), stained according to Harris' Haematoxylin and Eosin Procedure after rehydration, and finally mounted with Entellan®.

8.3 Analysis – Estimation of the DEPM adult parameters

In the DEPM, the spawning biomass is estimated based on the following equation:

$$SSB = \frac{A \cdot P \cdot W_f}{R \cdot S \cdot F}$$

where P is the **daily egg production** (number of eggs produced per day per unit area), A is the total **surveyed area**, W is the **average body mass of mature females**, R is the **fraction of the mature population** that are females (by mass), F is the **batch fecundity** (number of eggs spawned per mature female per batch), and S is the **spawning fraction** (fraction of mature females spawning per day).

W, R, F, and S are estimated from the adult fish sampling. The sex ratio (R) and female mean weight (W) are obtained from the biological data collected during the survey, and from the commercial samples, whereas the preserved gonads from the hydrated ovaries are used to estimate batch fecundity (F) and the gonads from the 30 females used to estimate spawning fraction (S).

Additionally, age reading from the otoliths allows for the construction of a microscopic maturity ogive to be used in assessment estimations.

The adult parameters (W, R, F, and S) are estimated independently for each fishing haul, using only the **mature fraction of the population** (determined by the fish macroscopic maturity data, Costa, 2009): only fish with maturity stage ≥ 2 are included in the calculations.

Then, for the whole survey, and for each parameter, weighed means and variances are calculated using the methodology from Picquelle and Stauffer (1985). In case samples from hauls would be of unequal size, for parameters estimation, each haul is thus weighed by the number of mature fish/females in the sample:

$$\bar{\bar{y}} = \frac{\sum_{i=1}^{n} m_i \bar{y}_i}{\sum_{i=1}^{n} m_i} \quad \text{and} \quad \hat{Var}(\bar{\bar{y}}) = \frac{\sum_{i=1}^{n} m_i^2 (\bar{y}_i - \bar{\bar{y}})^2}{\left(\sum_{i=1}^{n} \frac{m_i}{n}\right)^2 n\,(n-1)}$$

where $\bar{\bar{y}}$ = the estimate of the population mean,

n = the number of stations,

$\bar{y}_i = \sum_{j=1}^{m_i} \frac{y_{ij}}{m_i}$ = the mean of the ith station, and

m_i = the number of fish subsampled from the ith catch.

The coefficients of variation (CV) for each parameter y are calculated as:

$$CV = \frac{\sqrt{Var(\bar{\bar{y}})}}{\bar{\bar{y}}}$$

The coefficient of variation of the SSB is obtained from the following equation:

$$CV_{SSB} = \sqrt{CV_{Po}^2 + CV_W^2 + CV_S^2 + CV_F^2 + CV_R^2}$$

A major assumption in DEPM is that all parameters are constant over the geographical range and duration of the survey. When this assumption is violated, i.e. when the adult

parameters estimated per haul present a significant spatial variability over the area surveyed, Picquelle and Stauffer (1985) recommend a post-stratification: the parameters means and variances are re-estimated separately for a certain number of geographical areas defined a posteriori.

Female mean weight (W):

Female mean weight per haul is estimated based on the observed female total weight data obtained from the fish sampling. However, before the estimation, it is necessary to take into account the extra weight that the hydrated females sampled have acquired due to the hydration process of the ovary. In this case, two options are possible:

1) the hydrated females are excluded from the calculation of the mean female weight;
2) the total weight of the hydrated females sampled is first corrected by applying to the latter a linear regression between the total weight of the sampled non-hydrated females and their corresponding gonad-free weight (Wnov).

Sex ratio (R):

The sex ratio in weight per haul is obtained as the quotient between the total weight of females in the random haul sample on the total weight of males and females:

$$R = \frac{\sum_{1}^{i} W_{fi}}{\sum_{1}^{i} W_{f_i} + \sum_{1}^{j} W_{mj}}$$

where W_{fi} is the weight of each female (i) in the haul, and W_{mj} the weight of each male (j) in the haul.

Batch fecundity (F):

The individual observed batch fecundity (F_{obs}) is measured in hydrated females, by means of the gravimetric method applied to the hydrated oocytes (Hunter *et al*., 1985): 3 subsamples of ovary weighing ~ 100 mg are collected from one lobe of the ovary, images of the whole mount subsamples are taken under a stereomicroscope, and the number of hydrated oocytes (HO) in the subsamples are counted using ImageJ automated routines (Gonçalves *et al*., 2012). This procedure assumes that there are no significant differences in the number of hydrated oocytes per unit weight between the left and the right ovary lobes. Individual observed batch fecundity is obtained according to:

$$F = \frac{Wgon \times n}{Wsub}$$

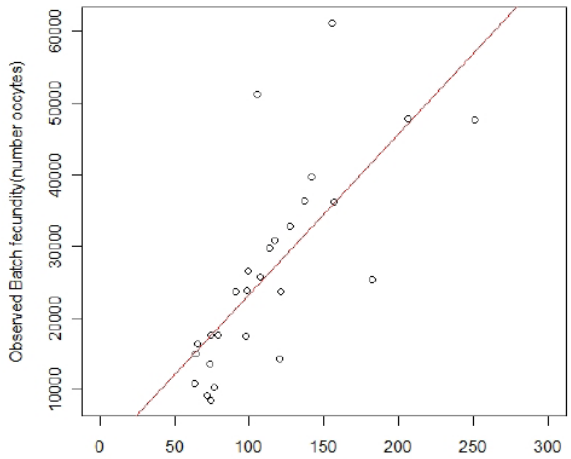
where F is the number of HO in the ovary (from the batch to be spawned), Wgon is the total weight of the ovary, n is the sum of the number of HO counted in the subsamples, and Wsub is the sum of the weights of the subsamples.

Note: As the subsamples have been weighed in already preserved tissue, the ovary total weight (Wgon) to be used in this calculation is also the preserved measured (and not the fresh converted) weight.

As hydrated females are often scarce in the samples, the gravimetric method can also be applied to migratory nucleus stage oocytes, using the same procedure described above but for females with ovaries whose most advanced oocyte batch is at the migratory nucleus stage (Ganias *et al*., 2010, Gonçalves *et al*., 2012)

In both cases, ovaries should be first analysed histologically: in case hydrated ovaries contain recently formed POFs or the ovaries with the oocyte spawning batch at the migratory nucleus stage is affected by alpha atresia, these females cannot be included in the calculations as batch fecundity would be underestimated.

To obtain the expected individual batch fecundity (Fexp) for all mature females (hydrated and non-hydrated) sampled per haul, the individual observed batch fecundities (Fobs) are first modelled against the corresponding female gonad-free weights (Wnov) by means of a Generalized Linear Model (GLM;it is assumed that the relationship is linear, i.e. that relative fecundity is constant throughout horse-mackerel life). This model is subsequently applied to all mature females in the sample to obtain the expected individual batch fecundity (Fexp), i.e. the batch fecundity that these sampled females (with a given body weight) would have if they were hydrated at the time of capture.



Spawning fraction (S):

The spawning fraction per haul is calculated from the fraction in the random sample of the mature females which have a given spawning marker, i.e. any sign of previous or imminent spawning (oocytes at the migratory nucleus MN or hydrated HO stage, or POFs). For horse-mackerel, the MN oocyte stage is currently the only one with a known duration (~ 24h: Eltink, 1991), but MN and HO stage ovaries are often scarce in fish samples (due to aggregative behaviour of actively spawning fish and/or to a different selectivity of the gear for these individuals) and S estimates can be biased.

The POFs method is the most common used method to estimate S, and provides less biased estimates, but the degeneration rate of POFs is still unknown for horse-mackerel (Gonçalves *et al*., 2009).

Until no further information is available, the spawning fraction per haul will be estimated based on the fraction of mature females containing POFs belonging to different daily classes. It is estimated as the average number of females with Day-1 or Day-2 POF, divided by the total number of mature females in the sample. Due to

possible over- or under-sampling of active spawning females, the hydrated females are not included in the calculation and the number of females with Day-0 POFs is corrected by the average number of females with Day-1 or Day-2 POFs, according to the formula:

$$S = \frac{1/2 \cdot (\text{day 1} + \text{day 2})}{1/2 \cdot (\text{day 1} + \text{day 2}) + \text{day 1} + \text{day 2} + \text{day 2}^{+} + \text{No POFs}}$$

Staging of POFs will be based on both histomorphological (see 7-stages classification presented by Solla and Garabana, 2015, and Figure 8.2) and biometrical criteria (cross-sectional area, Figure 8.1, Ganias *et al*., 2007), those criteria being then related to the time of capture in order to attempt assigning each POF to a daily cohort. The overall size of the POFs in the histological slide can indeed be useful to help assessing its stage/age, as the size of POFs decreases throughout the degeneration process (Ganias *et al*., 2007). After a quick scanning of the whole slide, measure the cross-sectional area of the relatively largest POF present at the edge of an ovarian lamella (using ImageJ: Figure 8.1), and by comparison to the area values obtained for the other slides in the random sample, it can help inferring the daily class of the POFs. This method applies better to species exhibiting a daily spawning synchronicity; recent results (eggs and adults data) argue in favour of the existence of such a daily limited period of time when most individuals spawn (ICES, 2015).

Notes:

- Depending on the embedding medium used in histology, the areas of POFs may vary considerably (paraffin embedding produces a considerable shrinkage of the tissues and for the same POF age, the area is significantly lower compared to resin embedding).
- Depending on how the POFs were cut, POFs will present different sizes and aspects throughout the histological slide, and this is the reason for which is it important to consider the relatively largest POF in the slide for the cross-sectional area measure.

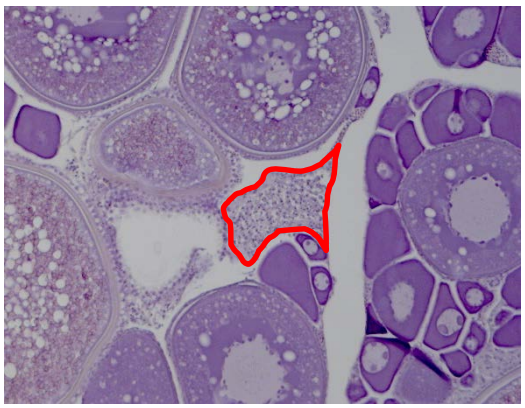


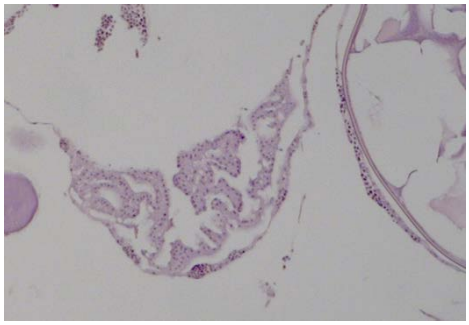
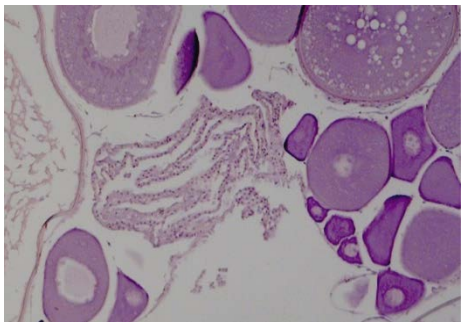
Figure 8.1: Measure of the cross-sectional area of a POF at the edge of an ovarian lamella (x100).

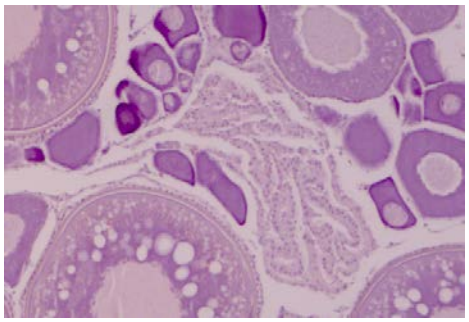
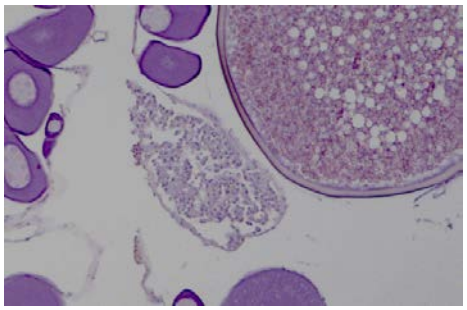
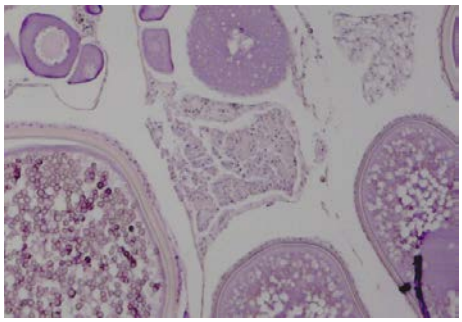
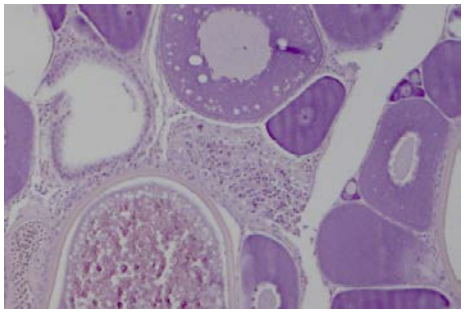
Notes for the histological analysis of POFs:

- POFs present slightly different aspects depending on whether the ovary tissue was embedded in resin of paraffin.
- Unless horse mackerel has a very short inter spawning interval (high spawning frequency), the POFs present in the histological slide of an individual have likely been produced at the same spawning event, and

should thus be more or less at the same histomorphological stage and have more or less the same age.

- One major problem in POFs method is the possible misidentification of POFs with atresia. Tips to deal with this issue:
  - Analyse the whole histological slide: when doubts if a given structure is a POF or atresia, it is unlikely that that all structures would appear doubtful.
  - It helps to look for POFs at the edge of ovarian lamellae because atretic follicles are never "opened" to the lumen of the ovary, they always lie within the lamellae; whereas POFs, in theory, are always "opened" to the lumen of the ovary as they result from the release— ovulation—of the mature oocyte into the ovarian lumen.
  - In POFs, the theca layer remains conspicuous during all its degeneration process; whereas in atresia, the theca is hardly visible; moreover, late atretic stages may contain yellow/brown pigments whereas POFs not.
  - In POFs, "empty vacuoles" are usually of small and identical sizes (they are said to correspond to what remains from the granulosa cells after these degenerate); whereas in (late) atresia, the "empty vacuoles" are of varying sizes and often large (some of these correspond to the lipid droplets of the former oocyte, lipids which take longer than the yolk granules to go through the process of digestion and absorption by the granulosa cells).

Time sample	Picture histological slide	Description
15:00	 x 100 – PEN1-6	**Ovary:** still abundant number of hydrated oocytes in the lumen, but already POFs present **POFs:** newly formed, large, irregular, convoluted (loops clearly delineated), large lumen, no signs of degeneration **Granulosa:** nuclei clearly visible and linearly organized **Theca:** separated from the granulosa, thin (stretched)
16:00	 x100 - MAT1-72	**Ovary:** a very large and empty central lumen, with some remnant hydrated oocytes and many POFs present **POFs:** recently formed, large, irregular, convoluted (loops clearly delineated), large lumen, no signs of degeneration **Granulosa:** nuclei clearly visible and linearly organized **Theca:** separated from the granulosa, thin (stretched)

00:30	 x100 – POR3-20	**Ovary:** no longer present a large and empty lumen, a few remnant hydrated oocytes **POFs:** recently formed, large, irregular, convoluted, but thicker loops, lumen visible, no signs of degeneration **Granulosa:** nuclei linearly organized, located more basally **Theca:** separated from the granulosa, less stretched
09:00	 x100 – PEN3-16	**POFs:** large, irregular, loops thicker, lumen still visible, first signs of degeneration **Granulosa:** most nuclei still linearly organized, first pycnotic nuclei and vacuoles **Theca:** separated from the granulosa
16:30	 x100 – PEN6-21	**POFs:** still large, but less irregular in shape, loops less distinguishable, lumen less visible **Granulosa:** nuclei less organized, pycnotic nuclei and vacuoles increase in number **Theca:** separated from the granulosa, thicker
11:30	 x100 – MAT4-28	**POFs:** reduced in size, more rectangular shape, lumen and convolutions no longer visible **Granulosa:** nuclei disorganized, forming a compact mass, most nuclei are pycnotic, vacuoles numerous **Theca:** thicker and more attached to the granulosa

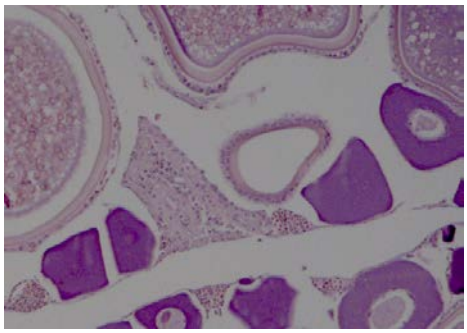
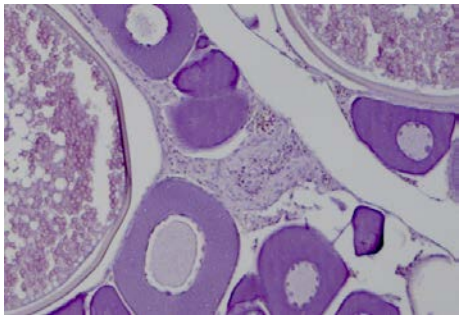
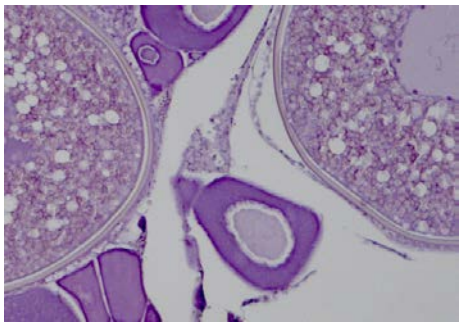
17:30	 x100 – MAT3-45	**POFs:** more reduced in size, more triangular shape **Granulosa:** granulosa reduced, few or none healthy nuclei, vacuoles present **Theca:** thick and attached to the granulosa
09:00	 x100 – PEN3-23	**POFs:** reduced size, triangular shape **Granulosa:** granulosa very reduced, no healthy nuclei, vacuoles less visible **Theca:** becomes dominant in the POF
09:00	 x100 – PEN3-14	**POFs:** very reduced size, triangular shape **Granulosa:** almost inexistent **Theca:** represents almost all the POF

Figure 8.2: Horse mackerel–Histomorphological description of POFs stages from females collected daily at different y times during the 2013 DEPM survey.

9 Data submission deadlines

Step (analyses and text for WD)	Person in charge	Coordinator	Deadline for sending the raw data for the preliminary estimate	Deadline for results of analyses and WD WGWIDE	Deadline for the raw data of remainder of the samples	Deadline for results and text for WD WGMEGS 2017
Mackerel						
Screening	Anders Thorsen Merete Fonn	Anders Thorsen Merete Fonn	As soon as possible	When cruises are finished	31-12-2016	April 2017
Potential fecundity	Paula Alvarez Maria Korta	Anders Thorsen Merete Fonn	1-8-2016	24-8-2016	31-12-2016	April 2017
Atresia estimation	Paula Alvarez Maria Korta	Anders Thorsen Merete Fonn	1-8-2016	24-8-2016	31-12-2016	April 2017
Realised fecundity	Anders Thorsen Merete Fonn	Anders Thorsen Merete Fonn		24-8-2016	31-12-2016	April 2017
WD WGWIDE		Anders Thorsen Merete Fonn		31-8-2016	31-12-2016	April 2017
WG WGMEGS		Anders Thorsen Merete Fonn			31-12-2016	April 2017
Batch fecundity	Dolores Garabana Antonio Solla	Cindy van Damme			31-12-2016	April 2017
Sex ratio	Dolores Garabana Antonio Solla	Cindy van Damme			31-12-2016	April 2017
Spawning fraction	Dolores Garabana Antonio Solla	Cindy van Damme			31-12-2016	April 2017
SSB	Cindy van Damme	Cindy van Damme			31-12-2016	April 2017
WD WGMEGS		Cindy van Damme			31-12-2016	April 2017
Horse mackerel						
Screening	Cindy van Damme	Cindy van Damme	As soon as possible	When cruises are finished	31-12-2016	April 2017
Batch fecundity	Paula Alvarez Maria Korta	Cindy van Damme			31-12-2016	April 2017
Sex ratio	Paula Alvarez Maria Korta	Cindy van Damme			31-12-2016	April 2017
Spawning fraction	Paula Alvarez Maria Korta	Cindy van Damme			31-12-2016	April 2017
SSB	Cindy van Damme	Cindy van Damme			31-12-2016	April 2017
WD WGMEGS		Cindy van Damme			31-12-2016	April 2017

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Annex 1. Walsh maturity scale (supplied by IEO BIOMAR group)

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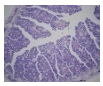
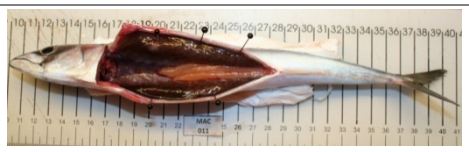
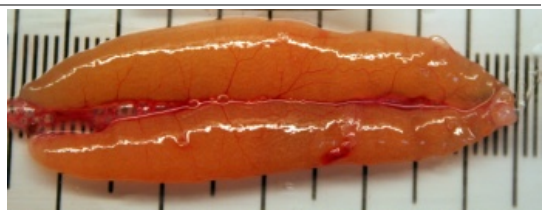
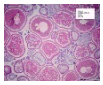
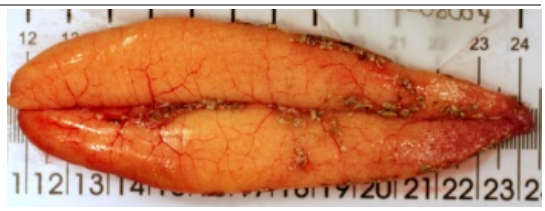
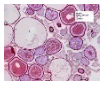
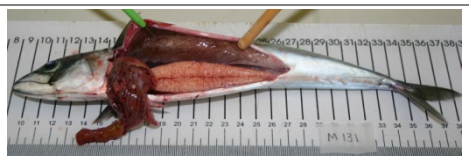
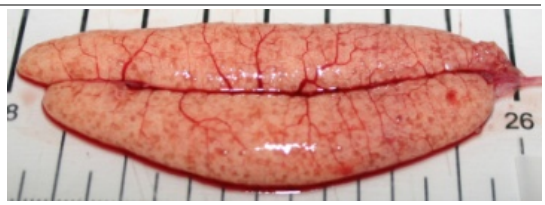
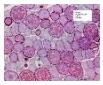
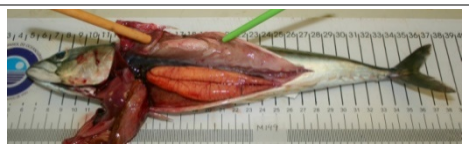
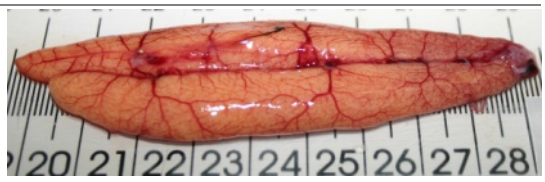
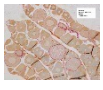
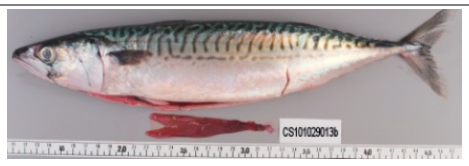
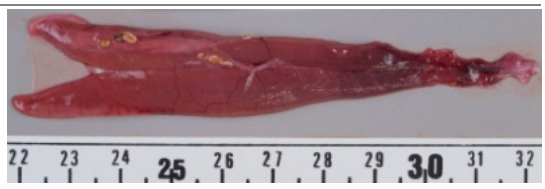
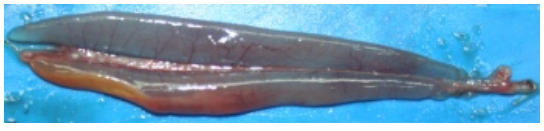
2 		
3 		
4 		
5 		
6  spent		
6 recovery 		

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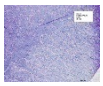
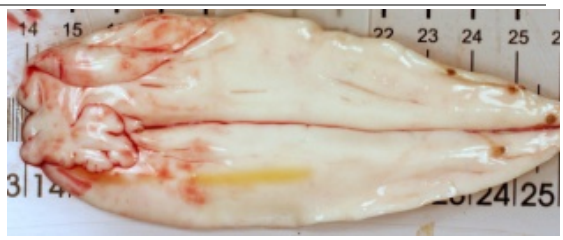
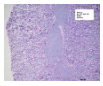
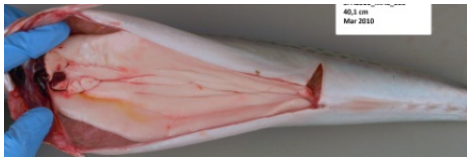
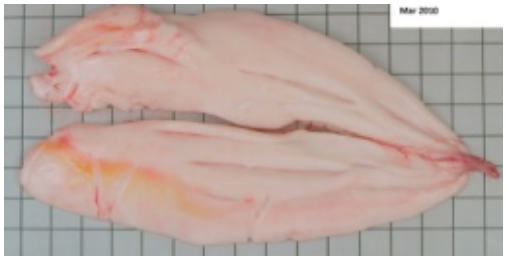
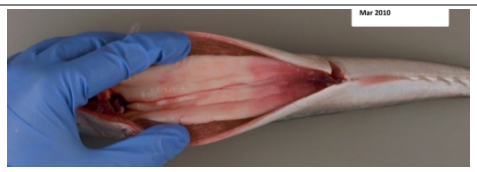
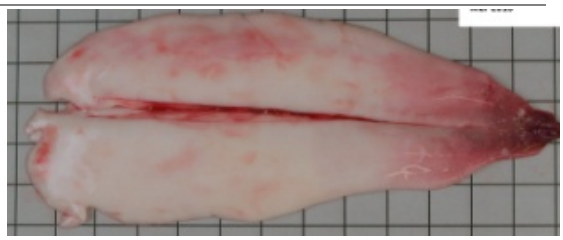
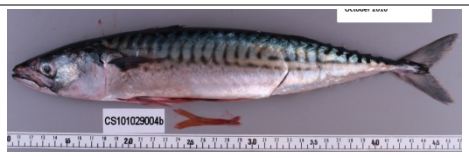
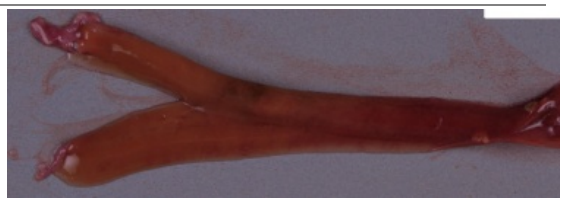
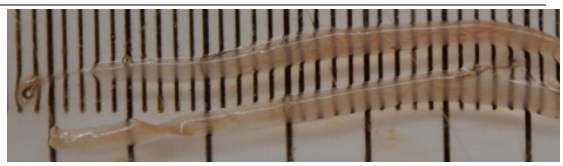
3 		
4 		
5 		
6 spent 		
6 recovery 		

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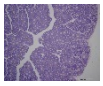
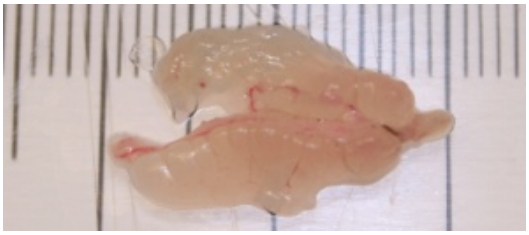
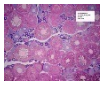
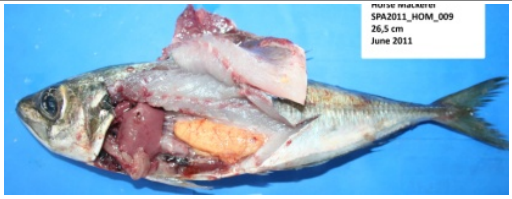
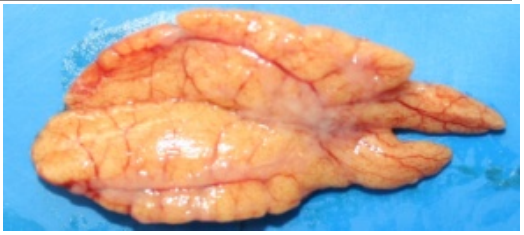
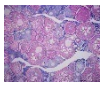
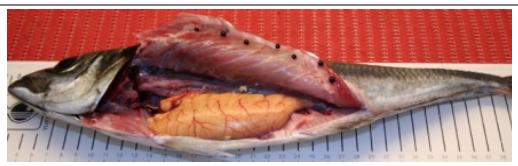
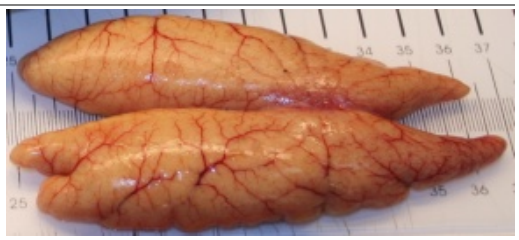
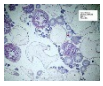
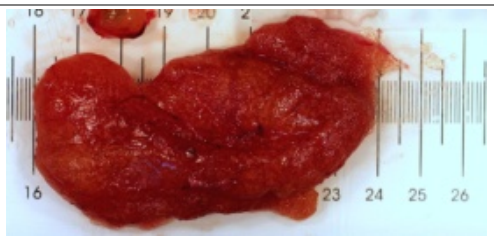
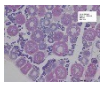
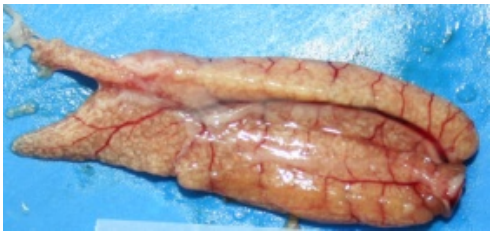
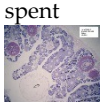
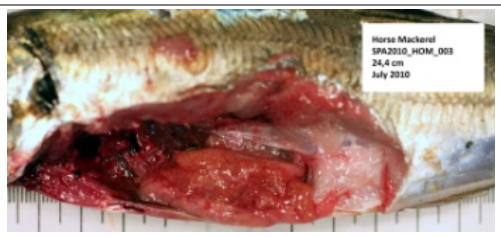
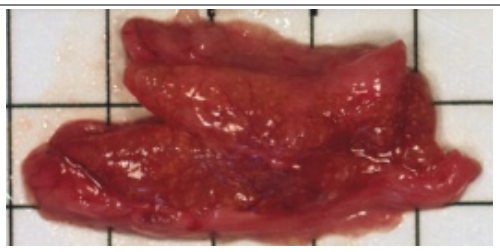
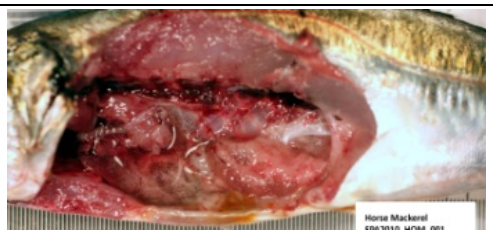
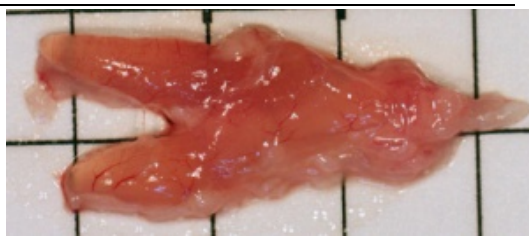
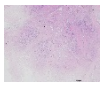
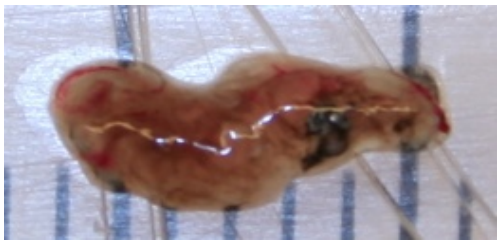
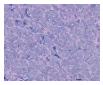
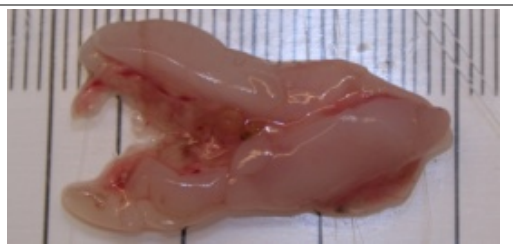
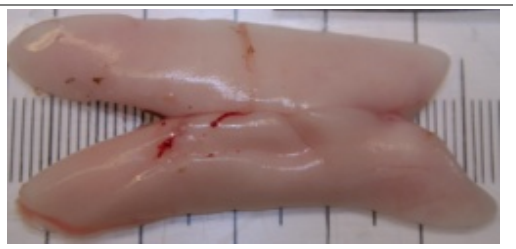
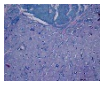
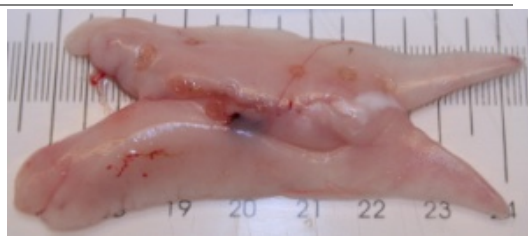
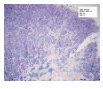
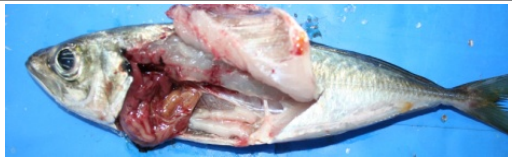
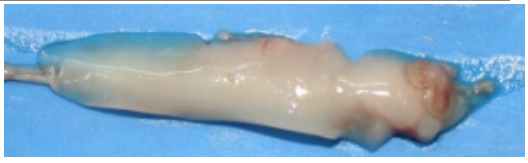
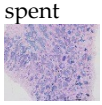
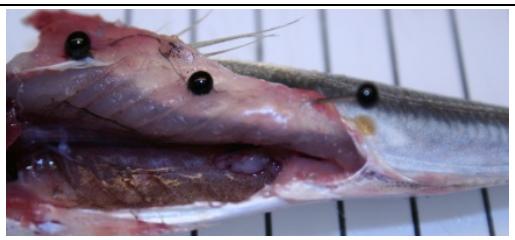
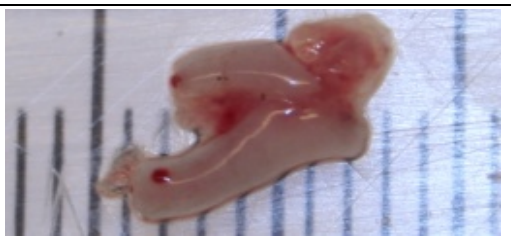
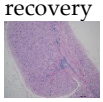
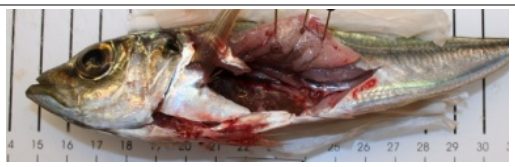
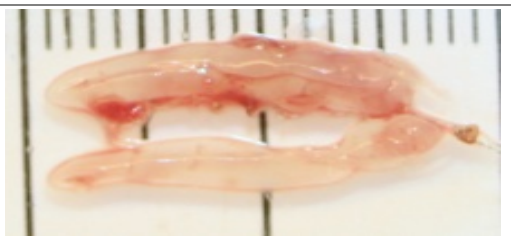
1 	 Note that advanced Stage 6 (recovery) can be easily confused with immature!!!!	
2 		
3 		
4 		
5 		
6 spent 		

6 recovery 		

Walsh		**Horse mackerel MALES**
1 	 Note that advanced Stage 6 (recovery) can be easily confused with immature!!!!	
2 		
3		
4 		
5 		

6 spent 		
6 recovery 		

Annex 2. Excel sheet used for screening analysis

The Excel sheet, below, should be used for the screening analysis of the AEPM samples.

Sample_ref	POF	Oocyte_stage	Early_alpha	Massive_atr	Fecundity	Atresia	Batch_fec	Embedding	Comment	Institute
B003	0	3	0	0	1	0	0	0		IMR

Annex 3. Manual for the image analysis of the whole mount using ImageJ

Image analysis manual for Mackerel fecundity work

Version 2015-1

Introduction

For the fecundity and atresia analysis we use the open source image analysis program ImageJ (http://rsb.info.nih.gov/ij/index.html) with the ObjectJ (https://sils.fnwi.uva.nl/bcb/objectj/) plugin. For general use of ImageJ and ObjectJ, you should read the tutorials and manuals found on their respective homepages.

A Windows and Macintosh version of the ImageJ/ObjectJ can be downloaded from the IMR ftp site (ftp.imr.no/Mackerel 2016/Image analysis software). These versions include the necessary plugins and macros for the Mackerel fecundity and atresia work for 2016. To install, unzip and then move the ImageJ folder to your Applications folder.

At the same location, you can also download ObjectJ project folders. Excel data templates for the image analysis work can be found at: ftp.imr.no/Mackerel 2016

For support on the fecundity image analysis software, you may contact Anders Thorsen at Institute of Marine Research, Norway, (E-mail: anders.thorsen@imr.no; Skype name: athorsen63)

ObjectJ project folders:

Oocytes 30-1 Mackerel (for fecundity counting)

Weibel-8-1 Mackerel (for estimation of atresia)

Batch Fecundity-30-1 Mackerel (for batch fecundity counting)

Data templates:

Atresia_v2016_1 mackerel.xlsx

Batch_fecundity_v2016_1 mackerel.xlsx

Fecundity_v2016_1 mackerel.xlsx

Note that these files may be updated in the time period between finalization of the manual and start of the work. Therefor please check that you have the latest versions before you start.

Ftp server:

Address: ftp.imr.no

Username: mackerel

Password: hu89iop

It is recommended to use a dedicated ftp client like FileZilla (filezilla-project.org) to connect to the ftp server.

Fecundity analysis

The project "oocytes 30-1 mackerel.ojj" allows automatic and manual measurements of oocytes. Resulting parameters are cell size and elliptical aspect ratio. Size is visualized by a red line that indicates the diameter of an equivalent circle with same area, as well

as a pink line that shows the best fitting ellipse. A category (1.9) can be added manually, and is visualized by the "hand of a clock".

In this manual, only the steps necessary to perform the Mackerel fecundity analysis for 2016 are described. For complete description of functionality of this project, refer to the Read Me file inside the project folder.

Organizing your project folders

Make sure that the project file (.ojj) and the related images to be analysed are in the same folder—we call it the "project folder." Typically, you would place up to 10–20 images into your project folder. If you put too many pictures in the same project folder, your computer may work more slowly with them. It is best practice to make a copy of the project folder template for each batch of images (each sample) to be analysed.

A. Download Template Project

Download project folder called Oocytes 30-1 Mackerel (ftp.imr.no/Mackerel 2016/ Image analysis software /ObjectJ projects/ Oocytes 30-1 Mackerel).

The folder contains the project file (oocytes 30-1 mackerel.ojj), a sample image and a read me file.

B. Usage

Taking pictures

The resolution of the pictures should be approximately 0.2 px/μm or higher (e.g. 7.5x magnification using 5 Mpx camera). Lower resolution will result in reduced precision, while much larger resolution will cause longer computer processing time, as well as larger demand for storage. Pictures should be stored in tiff format (.tif). When saving pictures, remember to follow the naming convention as described in the main manual (e.g. A034_A_IMR.tif).

If your camera saves pictures of another format than tiff (e.g. jpg), you should convert them to .tiff before analysis. This can be performed in batch mode using the "BatchConvert" macro on the Start-up action bar of ImageJ.

Since we are going to do automatic size measurements of the oocytes based on thresholding, it is important that the illumination of the pictures is optimal and the same illumination is used every time. Therefore, always follow the same procedure when you take your pictures. Use a light source that gives even illumination of your sample.

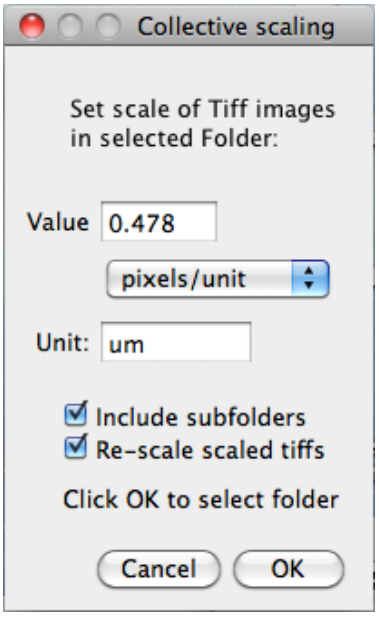
To adjust the illumination to the same level every time, take a photo of your sample tray filled with clean water, and measure the light of a clean area in the centre of the picture. Adjust your lighting or exposure time, so that you get a grey value of 206 ± 2 in the centre. You may have to take several pictures to find the correct settings. Repeat this as a start-up procedure every time you take a series of pictures.

To help with the light measurements, you may want to use the "measure light" button on the Start-up action bar. However, this function has been made for pictures that are 5 Mpx large. If your camera takes pictures with other sizes, you can contact Anders Thorsen at IMR for help to adapt this function to your setup.

Scaling pictures

Before you start to analyse your images, they should be scaled. If your camera software setup gives you the possibility to do so, you may scale your images when you acquire them.

If you do not scale your images while taking them, you will need to scale them afterwards. To do this, you can use the batch scaling macro included in our common ImageJ version for 2016. You will find it on the Start-up action bar ("CollectiveScaling"). The macro will scale all tiff images in a folder selected by you to a specified value.

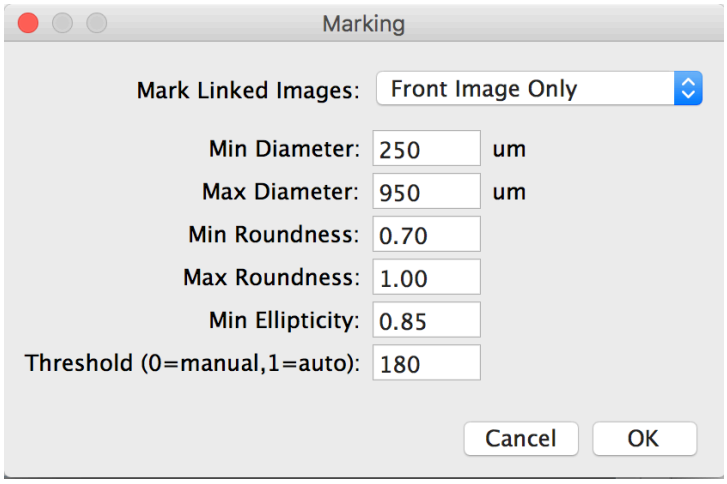


Open your project

In the ImageJ menu select ObjectJ/Open project. Navigate to the project folder and select the project file (with ending .ojj). Then, link the images in the folder to the project by selecting in the menu: ObjectJ/Linked images/Link all images from project folder.

1. Automatic counting and marking of diameters

Chose menu ObjectJ/Mark Oocytes (or use shortcut M). This marks diameters and ellipse perimeters automatically.



You can choose to mark the front image only (default), all unmarked images, or all images. Use the default values for Minimum and Maximum Diameter (250–950 µm). Also use the default values for Minimum Roundness (minor axis/major axis) and Minimum Ellipticity (a perfect ellipse will have a value of 1, less perfect ellipses will have smaller values), 0.7, and 0.8 respectively.

After images have been marked, inspect the image. You should correct automatic measurements when the marking includes more than one oocyte, or that the mark is on something other than an oocyte. Also, oocytes that are measured hugely incorrect should be corrected.

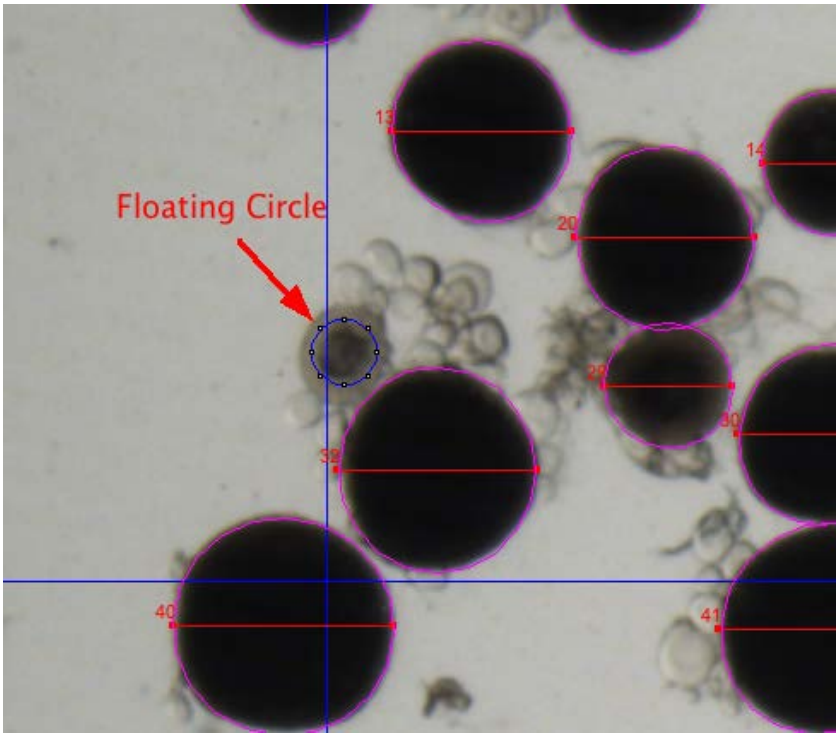
All corrections of automatic measurements are done by deleting the markings, as described below, and then the oocyte is counted (not measured), as described under "Manual counting… (next section).

To remove (kill) a marking, hold your cursor over the marking and press 0. You can also use the Gun tool from the Object tools, which can be activated in the ObjectJ drop down menu.

2. Manual counting of oocytes that have not been marked by the automatic procedure

Use the floating circle to count cells manually. The "BlueGrid" (from Start-up action bar) will help you to work systematically through your images. Always when doing manual measurements and counts use 100 % zoom factor for maximum accuracy.

Activate the Floating Circle in ObjectJ menu, or by using the shortcut "v." The floating circle has a diameter of 185 µm, and will follow your mouse pointer. All cells that fill the floating circle completely can be marked by pressing the number key 2. If the mouse is inactive for 10 seconds, the floating circle will automatically be switched off. Pressing "v" will reactivate it.



3. Results

Choose ObjectJ/Show Project Results to display numeric results. These columns are shown:

Dia equivalent diameter in um

Cat category 1..9, -1 for automatic markings

Roundn. same as Minor/Major (range 0..1)

Ellipt. ellipticity= ellipse perimeter/actual perimeter (range 0..1, or empty for manual marking)

Minor minor axis (μm)

Major major axis (μm)

Area3 area (unit is 10^3 μm ^{2} for convenience)

It might happen that the full results are not shown in the results window when you first open it. If so, then choose "Recalculate" in the ObjectJ menu (ObjectJ/Results/Recalculate).

4. Saving

Periodically save the coloured markers and results via menu ObjectJ/Save project. Note that menu "File/Save" only saves an image, not the markers.

5. Export Results to Excel

In the Results Window, press the "Copy/Export" button. In the dialog, choose to include "All Columns" and "Index column." Deselect "Include Headers" and "Include Statistics". Press the button "Copy" and then go to Excel and paste the data into the "fecundity v2016_1 mackerel.xlsx" data template

Batch fecundity

For estimating batch fecundity, use the ObjectJ project file called, "batch fecundity 30-1 mackerel.ojj" (download from the ftp cite). This project is similar to the "oocytes 30-1 mackerel.ojj," except that it only measures oocytes larger than 500 µm. Also, the moving circle diameter is 500 µm, instead of 185 µm. Initially measure the vitellogenic and hydrating oocytes automatically, the same way as for fecundity counting. When the automatic measurements have been completed, remove errors, being especially aware of markings that cover more than one oocyte or particles that are not oocytes.

Measure the size of the remaining oocytes larger than 500 µm, using the variable ellipse tool (activate using the shortcut "v"). If an unmarked oocyte fill out the 500 µm moving circle, measure it by moving the mouse cursor to the rim of the oocyte, hold down the left mouse button, and drag the circle to the opposite rim, so that the circle cover the whole oocyte. Release the mouse button, and move the cursor into the circle again, to adjust the circularity and orientation of the circle. Press the number key "3" to mark and measure the oocyte.

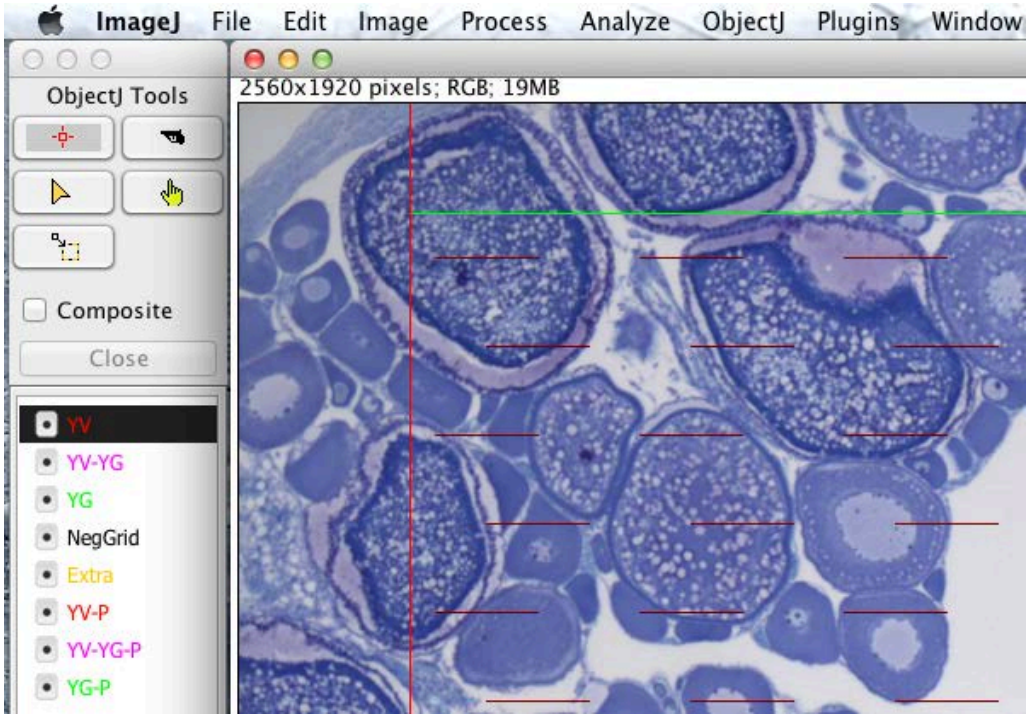
Export the results to the "Batch_fecundity_v2016_1 mackerel.xlsx."

Atresia analysis using grid and profile counting

Pictures used for atresia analysis should have a resolution of about 0.85 px/µm or higher (e.g. 4x magnification using 5 Mpx camera). Pictures should be stored in tiff format (.tif).

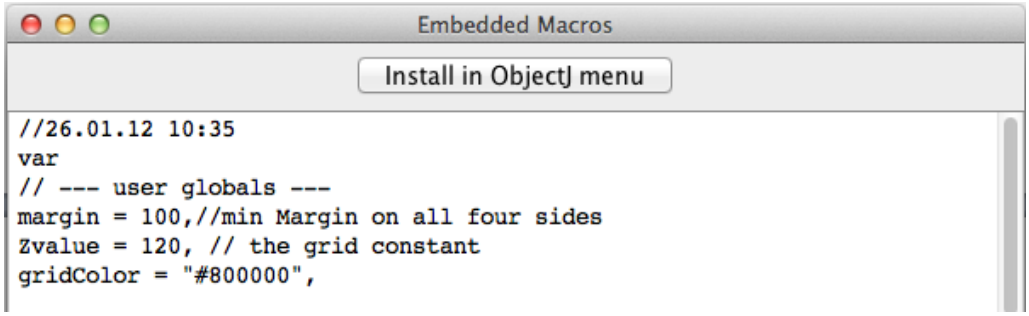
In this analysis you first count atresia using a grid and thereafter count atresia as profile counting.

Use the ObjectJ project file "Weibel 8-1 mackerel.ojj". Add all the histology images that belong to the same fish into the same project folder. Link the images to the project.



Add the Weibel grid to your picture (ObjectJ/Grid [F1]).

The grid should have a density of about 5000 points per cm^2. If the default grid settings do not give the desired density in your picture, you can change the grid settings in the project macro (ObjectJ/Show Embedded Macros).



If you need to increase the grid spacing then increase the zvalue (default value 120). If you need to decrease grid spacing, add a lower value. After the value has been changed, press the button "Install in ObjectJ menu. The value will now be active. When you save your project, this value will be stored in the project as the new default value.

In the Object Tools you will find the oocyte stages that are going to be counted.

YV: Yolk vesicle stage

YV-YG: Transition stage; yolk vesicle –yolk granule stage

YG: Yolk granule stage

NegGrid: Hits outside the ovarian tunica wall

Extra: For special observations. Will not be included in the atresia count

YV-P: Yolk vesicle stage – Profile counting

YV-YG-P: Transition stage; yolk vesicle –yolk granule stage – Profile counting

YG-P: Yolk granule stage – Profile counting

Count the stages according to the main manual; first the grid counting, then the profile counting. Before you start with the profile counting, you should add the frame that has red forbidden lines and green permitted lines (ObjectJ/Frame [F2]).

Finish all pictures in the project. View the results (Menu: ObjectJ/Show Project Results). In the statistics options (header of the left column), select "Count." Enter the counts in the Excel data template for Atresia (Atresia_v2016_1 mackerel.xlsx). Each fish will be represented with one row in the Excel data sheet.

Annex 4. Excel sheet used for whole mount evaluation

The Excel sheet, below, should be used for the whole mount evaluation.

Institute	Sample_ref	Oocyte_ stage	Egg_stage	Spent	Discard	Comment	Fecundity	Spawn_ marker	Atresia	Batch_fec
IMR	A001	3	1	0	0		no	yes	yes	no

Annex 5. Excel sheet used for atresia counting

The Excel sheet, below, should be used for atresia counting.

Institute	Person	Year	Sample_ref	No_Pictures	Grid_points	Field_area	YV	YV_YG	YG	Neg_grid	Extra	YV_P	YV_YG_P	YG_P
IMR	Merete	2012	A001	8	256	0.0502	0	173	0	11	0	0	29	0

Annex 6. Excel sheet used for screening analysis of DEPM samples

The Excel sheet, below, should be used for the screening analysis of the DEPM samples.

Institute	Sample_ref	Oocyte_stage	Egg_stage	POF	Spent	Massive_atr	Early_alpha	Discard	Comment	Fecundity	Spawn_marker	Atresia	Embedding	Batch_fec
IMR	B001	3	0	0	0	0	0	0		yes	no	no	no	no

Annex 7. Excel sheet used for the POF staging

The Excel sheet, below, should be used for the POF staging.

Sample_ref	POF_stage 1	POF_stage 2	POF_stage 3	POF_stage 4	POF_stage 5	POF_stage 6	POF_stage 7	Stage of the whole sample	Comments

Annex 8: Author Contact Information

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