

**An Histological and Stereological Approach to the
Bluefin Tuna Fecundity.**

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Organized by IEO-UMA to FAO-COPEMED

Schedule

Tuesday (19/10/99)

9:00. Dehidratation and paraffin embedding of specimens

Theory: Ovary structure. Different type of ovocytes. Follicular atresy (signs of degeneration). Postovulatory folliculi.

Practical session: Determination of previously described structures in the ovaries. This practical lesson consists of the microscopical analysis of diverse histological sections. Forms will be filled in with the information about the presence or absence of the previously studied structures.

11:30 Coffe break.

12:00 We will continue with the embedding of the specimens
Slides revision and discussion.

Theory: Determination of females reproductive status according the structural characteristics of the ovary.

14: 00 Lunch.

15:30 Practical session. We will clasify the ovaries according the data we collected in the morning. **Make paraffin blocks.**

Wednesday (20/10/99)

9:00 Microtome room. We will section and prepare the previously embedded specimens (I).

11:30 Coffe break.

12:00 We will section and prepare the previously embedded specimens (II).

14:00 Lunch

15: 30 Practical session: Take photographs of the sections.

Thursday (21/10.99)

9:00 Staining of sections (Mallory's trichrome, PAS)

11:30 Coffe break.

12:00 Dehidratation, cleaning and mounting the sections

14:00 Lunch.

15:30 Review and revision of the method.

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I. Protocols:

I.1. Sample collection for histological studies on fecundity and sexual maturity in bluefin tuna.

In order to study the absolute fecundity of a species by means of histological studies and stereological methods it is necessary to consider several factors:

- 1.-It is necessary to discard the variability in the composition and state of maturity of the oocytes that integrate each ovarium.
- 2.-To discard the variability in the size and development of the oocytes throughout the ovary.
- 3.-Usually, a sample that totally cross the entire thickness of the ovary is taken. This analysis has demonstrated that the size and maturity state of the oocytes varies radially in the distance to the center of the ovary in several teleostean fish species.
- 4.- Most authors agree that the best sample would be a slide of the central portion of the ovarium. At the time of including the samples for the histological study, to take a sample that includes all the thickness of the ovarium will be tried. The histological procedure entails the risk of a variation in the volume of the oocytes, as well as its deformation. These variations usually increase as the follicular development progresses. As far as possible, in order to avoid these slants, isosmotic formaldehyde solution (4%) will be used. A good option is the phosphate buffer or the sea water.

Sample collection protocol

In spite of previous considerations, we have performed the following collection protocol for blue fin tuna ovaries samples.

- 1.-Measurement the animal length according to the conventional protocols of measures (scheme).
- 2.- To weight the complete animal.
- 3.- Extraction of the ovaries. (scheme).
- 4.- To weight each ovary. To measure the volume of each ovary (if it is not possible to perform the calculation of the density of the ovary by means of the weight and volume, a portion of the same one would be necessary). It is necessary to obtain a central slide of one of the ovaries. (Scheme)
- 5. - The pieces are placed in a container with, at least, the triple of fixative solution volume that the one of the weight. (fixative solution: Formalin 4% in sea water or phosphate buffer -isosmotic solution-)
- 6. - To label the containers properly. The label must inform on the number of identification of each animal, the size, sex, the weight of the complete ovarium, its volume the fixative solution used for its conservation.

I.2. Paraffin embedding protocol:

Histologic preparation of the samples studied

- 1.-To get the ovary sample, to dry the excess of fixative solution, to grief it and to calculate the volume by immersion.
- 2.- To take a sample from 0,5 cm of thickness. To wash, to dehydrate in a series of ethanol of increasing gradation, n-butyl alcohol and to include the sample in paraffin.

Paraffin embedding protocol:

- Wash the sample in phosphate buffer. (three washes of 20 min. each one)
 - Dehydrate the samples in a series of ethanol of increasing gradation:
 - Ethanol 50°-----1 hour
 - Ethanol 70° -----1 hour
 - Ethanol 80°-----1 hour
 - Ethanol 90°-----1 hour
 - Ethanol 96°-----1 hour
 - Ethanol 100°----- 1 hour
 - Ethanol 100°-----1 hour
 - n-butyl alcohol -----1 hour
 - n-butyl alcohol -----1 hour
 - Take in a 60°C paraffin stove. To drain the n-butyl alcohol excess.
 - Submerge the piece in a pot full of liquid paraffin (60°C) during 2 hours.
 - Put the piece in another one pot with new paraffin and to leave 2 hours. Then, to pass the piece to a last paraffin bath and to let shrink during other 2 hours.
- Make the blocks (with this last paraffin) and to cut in the microtome.

I.3. Protocol to obtain histologic sections.

Take at least, two separated samples (the minimum distance has to be at an equal one to the diameter of an oocyte of the greatest size class). Obtaining sections of 5-10µm in thickness.

1- Carve the paraffin block in a truncated pyramid form so that in the sectioning the order of the same series can be clearly recognizable (ilustrative image).

2- Put the block on the microtome so that the resulting sections include the total ovarian thickness (ilustrative image).

3- Select the section's thickness (between 5 and 10 µm), so that the possible finest sections can be obtained keeping a good quality. It is necessary to reach a "compromise" between the section thickness and the quality of those sections.

4- Eliminate the initial portion of wax until the complete sections appear.

5- Take between ten and twenty sections of the samples and to eliminate portions between 800 and 1000 µm among them. (care should be taken to obtain

2 samples as minimum). In order to avoid problems it is a good idea to take at least five sections from each weave sample. To take the maximum number of sections that fit in the glass for each sample is advisable.

6- Stretch the sections in a bath with distilled water to (40°C). Once stretched, to gather with the glass -properly labeled- and dealt with an adherent solution (Poly-l-lysine, 0.02% in distilled water).

7-Dry at least during 24 hours in a 37°C stove to 1hour in stove at 60°C.

I.4. Staining procedures:

Dye half of the sections with a PAS and the rest with a Mallory's trichrome.

Staining protocol of the PAS (periyodic acid stain). (the PAS stain dyes of red color the cortical alveolii mucopolycaccharides but only during vitelogenic stages, Khoo, 1979). The PAS's technique recognizes the presence of carbohydrates in the tissue. The recognition takes place by means of an oxidation of aldehydes, what allows to distinguish the presence of these glucidic groups. The result is an intense a pink color.

- Dewax and hydrate.
- Rinse in distilled Water-----5 min.
- Peryodic acid (0.5 %)-----30 min.
- Rinse in distilled Water fast step
- Schiff's reactive-----15 min.
- Distilled Water fast step

Mallory's Trichrome Stain Protocol. (Insertar gónada image with of dyed red tuna with mallory)

- Dewax and hydrate.
- Rinse in distilled Water----- 5 min.
- Corrosive sublimate -----30 min.
- Distilled Water fast step
- Acid Fucsin (1 %)----- 15-25 sg.
- Distilled Water fast step
- Phosphomolibdic acid (1 %) (dark passage)-----75 sg.
- Distilled Water fast step
- Mallory's liquid----- 85 sg.
- Distilled Water fast step.

-Dehydrate the sections after the staining by a 96° ethanol immersion for 5 minutes and a later passage of equal duration in absolute ethanol (100°).
- Fix the stain with eucalyptol and to clean with xylene. To mount the slides with special glue (DPX). To photograph the sections.

APENDIX 1:

Composition of buffers, stains and reagents used for optical microscopy.

Phosphomolibdic acid (1 %).

Phosphomolibdic acid ----1 g.
distilled water -----100 ml.
Incubation in the darkness. This liquid is photosensitive.

Peryodic acid (0.5 %).

Peryodic acid ----- 0.5 g.
distilled water----- 100 ml.

Acetic Water (1 %).

Acetic acid -----1 ml.
Distilled Water----- 99 ml.

Acetic Water (3 %).

Acetic acid -----3 ml.
Distilled Water----- 97 ml.

Mayer's Albumen.

Dust Albumen -----0.1 g.
Distilled Water----- 10 ml.

Once dissolved add 10ml of glycerine and a small crystal of timol. To preserve in cold. This solution is used to adhere the sections to the glass. A small amount is uniformly distributed on the glass surface and then it is dried in stove to 37°C.

Alcohol-eter (degreaser solution)

Ethanol 96° -----50 ml.
Anaesthetic ethylic-Ether -----50 ml.

Alcian Blue.

Alcian Blue.----- 0.05 g.
Acetic Water (3 %)------ 100 ml.

To fit to pH 5.8 by means of the addition of NaOH. After fitting pH = 5.8, the corresponding amount of Cl₂Mg is added (0.3 %, 0.65 %, 0.9 % or to 1.0 %).

Yellowish Eosin (1 %).

Yellowish Eosin -----1 g.
Distilled Water -----100 ml.
Once dissolved, add 1-2 drops of acetic acid.

Neutralized formaldehyde

Formaldehyde (40 %) (saturated with CO₃Mg and diluted in distilled water 1:10)

Acid Fucsin (1 %).

Acid Fucsin -----1 g.
Distilled Water ----- 100 ml.

Fucsin-ponceau

Acid Fucsin ----- 0.1 g.
Ponceau red ----- 0.2 g.
Distilled Water ----- 300 ml.
After obtaining the dissolution, add acetic acid 0.6 ml.

Delafield's Haematoxin

Solution A:

Haematoxin ----- 4 g.
Absolute Alcohol ----- 25 ml.

Solution B:

Sulphate of aluminium and ammonium (amoniacal alumbre)----40 g.
Water-----400 ml.

To mix the solutions A and B. After 4 days, to leak and add:

Glycerin----- 100 ml.
Methanol -----100 ml.

After 4 days, to leak and keep. For its use, dilute the stock in an equal proportion (1:1) in distilled water.

Groat's Hematoxin

Solution A:

Concentrated Sulfuric Acid----- 0.8 ml.
Sulphate of ammonium and iron (III) ----- 1 g.
Water ----- 50 ml.

Solution B:

Hematoxin----- 0.5 g.
Alcohol 95° -----100 ml.

To mix the solutions A and B. Rest 1 hour and leak. Can be preserved for 3 months.

Weigert's Hematoxin

Solution A:

Ethanol 96°----- 100 ml.
Hematoxin ----- 1 g.

Solución B:

Solution to 29 % of FeCl ----- 4 ml.
Concentrated hydrochloric acid--- 1 ml.
Distilled Water -----95 ml.

To mix solution B on solution A just before its use. Can be preserved for 1-2 semanas.

Mallory's Liquid.

Anilin Blue (sun watery)-----0,5 g.

Orange G ----- 2 g.
Oxalic Acid ----- 2 g.
Distilled water -----100 ml.
To leak before using. No limit of preservation.

Orange G-phosphomolibdate

Orange G -----2 g.
Fosfomolibdic acid ----- 1 g.
Distilled Water ----- 100 ml.
Incubation in darkness. Photosensitive fluid.

Schiff's Reagent.

Solution A:

Basic Fucsin ----- 1 g.
Boiling Distilled Water----- 200 ml.

Solution B:

Sodic Metabisulfite ----- 2 g.
Hydrochloric acid-----10 ml.

Add solution B on solution A. Cool the solution during 24 hours in darkness and it filters. Activated charcoal (0.5 g.) is added. Shake strongly during 1min. and let it settle. Then filter. Keep it is c in darkness (4° C).

Corrosive sublimate.

Mercury dichloride saturated watery Solution.

A distilled water amount is taken, mercury dichloride is added until saturating the dissolution, and heated at a temperature of 60°C during, at least, 12 h. Let it cool and then praised/poured it off.

Physiological serum

Sodic Chloride ----- 0.9 g.
Distilled Water ----- 100 ml.

PBS Buffer (0.02 M) pH = 7,3-7,4

Na₂HPO₄ -----7.572 g.
K₂HPO₄ ----- 1.813 g.
ClNa ----- 3 g.
Distilled Water -----1000 ml.

To fit pH with HCl (1 M) or NaOH (1 M). In the case the salt is an hydrated one (Na₂HPO₄ 12H₂O) add 18,93g.

Phosphate Buffer (0.02 M) pH=7,6

Major Component: Na₂HPO₄ (0,1M)-----870 ml.
Minor Component: NaH₂PO₄ (0,1M)----- 130 ml.

Both components are mixed, the minor on the majorone, until obtaining a pH of 7,6.

Tris-PBS Buffer (0.02 M) pH=7,8

Na₂HPO₄ ----- 1.48g.
K₂HPO₄ ----- 0.43 g

Tris -----5 g.
ClNa ----- 7 g.
Sodic Azide ----- 0.2 g.
Distilled Water -----1000 ml.
To fit pH with HCl (1 M) or NaOH (1 M).

II. Ovary structure.

Introduction.

The different teleostean species show diverse maturation processes and reproductive strategies that can be determined by means of an analysis of the morphology of the ovary. Using the size distribution of the ovocytes, the species can be classified in the Wallace& Selman (1981) types:

1. **Ovaries with synchronic development:** All ovocytes develop and ovulate at the same time. Only one wave of development can be seen in those ovaries.
2. **Ovaries with synchronic groups.** Two groups of ovocytes of synchronic development can be distinguished. The first, or more developed, use to be more homogeneous than the second.
3. **Asynchronic ovaries.** Ovocytes are found, at the same time, in all the intermediate stages of the development. The distribution of the ovocytes's size frequency is continuous, except for mature ovaries, where the ovocytes are separated from vitelogenic ones.

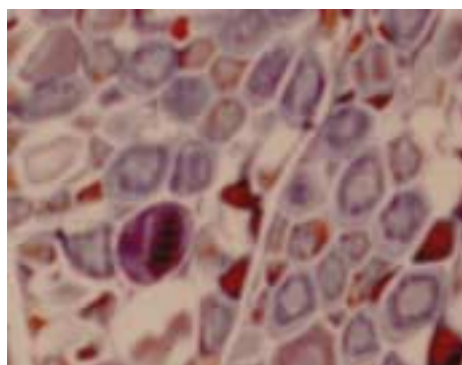
II.1. Oocyte Class:

A good developmental study is the one by Yamamoto (1956), that includes these classes:

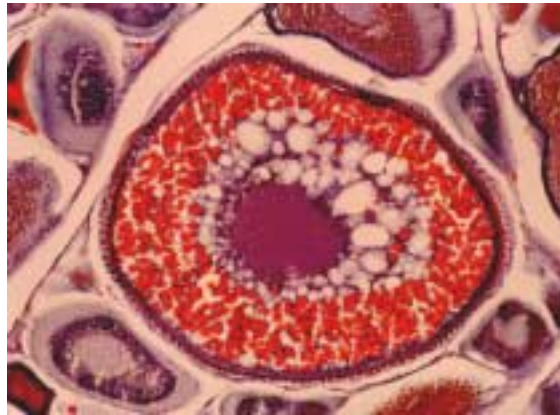
1. Status of nucleolar chromatine
2. Perinucleolar status.
3. Status respect the formation of cortical alveoli.
4. Vitelogenic status.
5. Mature or preovulatory status. Some species comprise the ovocytes that develop an hidratation before the (usually marine pelagic species)

In this work we are going to use a more simple cassification with only 4 stages (Hunter and Macewicz, 1985):

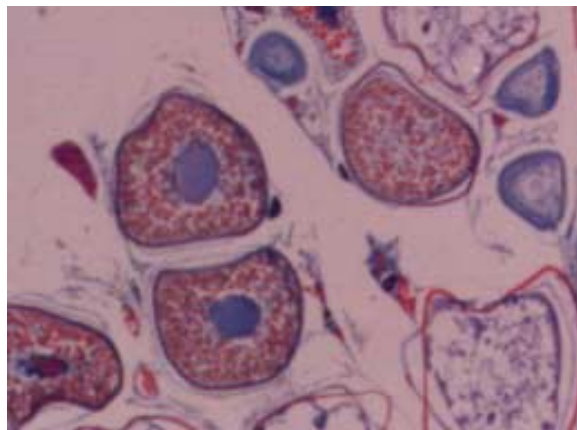
1. **Unyolked Oocytes.** This class includes all oocytes without yolk. Usually thes oocytes are spherical, have a large nucleus with a narrow homogenous and very densely staining cytoplasm. The larger oocytes in this class have an oval nucleus with several nucleoli and are surrounded by a granular perinuclear zone.



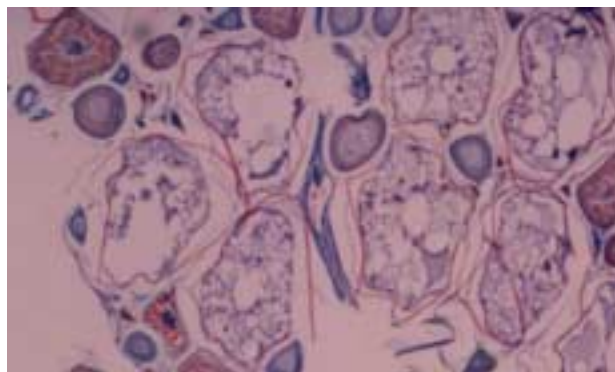
2. **Partially Yolked Oocytes.** These oocytes are in the early stages of yolk deposition (vitellogenesis). The class includes oocytes in the initial stage of yolk deposition up to (and including) those in which yolk granules or spherules extend three-fourths of the distance from the periphery to the perinuclear zone.



3. **Yolked oocytes.** This class includes all oocytes that contain yolk spherules or globules throughout the region between the periphery of the oocyte and the perinuclear zone.

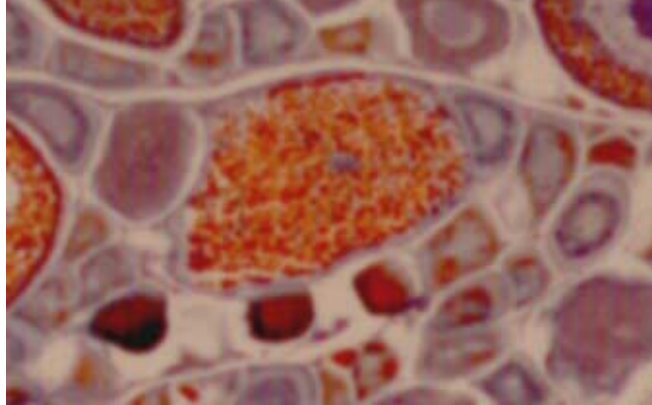


4. **Hydrated oocytes.** In this class the nucleus migrates to the animal pole, the yolk globules first fuse to form yolk plates, finishing when the hydrated oocytes are ovulated.



II.2. Atresic stages.

1. **Alpha (α) Stage Atresia.** During this stage the oocyte is reabsorbed, leaving only the follicular layers. The early phase of α -atresia is characterized by the disintegration of the nucleus, a phenomenon that is evident by an irregular shape, and the disintegration of some of the yolk globules.



2. **Beta (β) Stage Atresia.** At the beginning of the process the β -atretic follicle is a compact structure composed of numerous disorganized granulosa cells surrounded by a thin theca and a layer of blood vessel layer.

Three different patterns of atresia may occur at the conclusion of β stage:

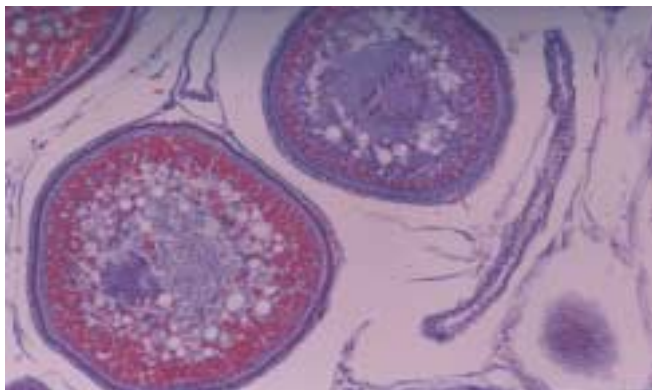
a) The follicle may follow the classic pattern and pass through subsequent gamma and delta stages (characterized by an increased pigmentation and an much smaller size).

b) The follicle may be completely resorbed during the β -stage leaving no histological evidences.

c) The follicle may pass directly from the β -stage directly to the delta stage.

II.3. Postovulatory follicles.

The postovulatory follicles are the follicular waste after ovulation. They are usually composed of a granulosa and a theca and present a great cavity in the center that is usually obliterated or collapsed. Its presence indicates a recent spawning because they are fastly reabsorbed. The postovulatory follicles that are older than 48 hours can be easily mistaken with advanced stages of β -atresia in vitellogenic ovocytes, so they do not constitute a good criterium to determine a recent spawning.



Appendix 2: Practical forms

Identification Code:

Oocytes class:

Unyolked oocytes:

Patially yolked oocytes:

Yolked oocytes:

Hidrated oocytes:

Postovulatory follicles:

Atresia Stages

α -Atresia:

β -Atresia:

Another atresia stages:

Activity:

Maturity Stage:

Observations:

Identification Code:

Oocytes class:

Unyolked oocytes:

Patially yolked oocytes:

Yolked oocytes:

Hidrated oocytes

Postovulatory follicles:

Atresia Stages

α -Atresia:

β -Atresia:

Another Atresia stages:

Activity:

Maturity Stage:

Observations: