STEREOLOGICAL QUANTIFYING OOCYTES AND ITS APPLICATION TO THE ARGENTINE HAKE *Merluccius hubbsi* (Merlucciidae, Marini, 1933)

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ABSTRACT

We analyze the applicability of free-assumption stereological methods to estimate total number of oocytes, related to gonadal development. These results were similar to values for immature oocytes found in others *Merluccius* species and confirm the reliability of the application of these techniques.

Keywords: Stereology, fishes, reproduction, oocytes

RESUMEN

Cuantificación de ovocitos por estereologia y su aplicación a la merluza argentina *Merluccius hubbsi.* Se analizó la aplicabilidad de los métodos estereológicos para estimar el número total de ovocitos, relacionada con el desarrollo gonadal. Los resultados encontrados fueron similares a los valores de ovocitos inmaduros encontrados en otras especies de *Merluccius* y confirman la fiabilidad de las técnicas utilizadas.

Palabras clave: estereologia, peces, reproducción, ovocitos.

INTRODUCTION

Reproductive biology of fishes provides basic information to understand population dynamics, especially in those species that support important fisheries, such as the Argentinean hake *Merluccius hubbsi*. This species is distributed in the southwest Atlantic Ocean, between latitudes 25º South (Cabo Frio, Brazil) and 48º South (Patagonian Shelf, Argentina) related to subantartic waters of the Malvinas Current (Angelescu & Prensky, 1987) and is the main fishing resource in Uruguay and Argentina fisheries (Bezzi *et al*., 1995; DINARA, 2010).

The study of the reproductive effort in fishes is a critical variable in stocks that show large fluctuations in abundance as a result of the dependence between spawning stock and recruitment (Murua & Saborido-Rey, 2003). Stereology is a technique that has been applied to measure fecundity as a biologic parameter for assessing fish populations. Several studies have shown the usefulness of stereological techniques to better describe the reproductive status of molluscs e.g. Alvarez-Fariña & Gual-Arnau (2006) and fishes (Christiansen *et al*., 1986; Emerson *et al*., 1990; Macchii & Christiansen, 1992; Medina *et al*., 2002; Kjesbu *et al*., 2010a; Witthames *et al*., 2009). Most of those studies use a model-based approach to count oocytes such as the method of Weibel & Gómez (1962) and the Emerson formula (Emerson *et al*., 1990). Recent advancements to estimate the number of oocytes (Aragón *et al*., 2010; Korta *et al*., 2010a; Kjesbu *et al*., 2010b) have incorporated stereological techniques such as the dissector method.

The aim of this work was to combine two free-assumption stereological methods to estimate ovarian volume (Cavalieri method) and number of oocytes (dissector method) to verify the applicability of these techniques in studies on gonadal development, maturity and fecundity in Argentinean hake as example to explore the possibilities on a population-based study using stereology.

MATERIAL AND METHODS

The material used was obtained from the right ovary of each three adult Argentinean hakes (Table 1) sampled in August 2009 in a commercial fleet vessel at the Argentine-Uruguayan Common Fishing Zone (all animals were recently dead when coming aboard). Taking into account that the estimated size at first maturity for females varies between 33 and 36 cm in this species (Simonazzi & Otero, 1986), we considered that the length range used was adequate.

Whole ovaries were fixed onboard in buffered Formalin 10% and later on dehydrated and embedded in paraffin in the laboratory. Embedded tissues were cut in serial transverse blocks of pieces of gonad following a systematic sampling method, i.e. the first cut was made at a random distance from the beginning of the organ, and the consecutive blocks were performed at fixed five mm intervals. From each consecutive pieces histological layers of five μ thickness were obtained and then stained with Hematoxylin and Eosine. Images of these histological sections were obtained using a stereo microscope mounted up with a digital camera Nikon Eclipse E200 from the ECIMAT Station of Marine Sciences (Vigo, Spain). The diameter of each oocyte was measured with the image analysis software Sgmascan Pro 5. The classification of oocytes into categories followed the criteria proposed by Honji *et al*., (2006) for Argentinean hake *Merluccius hubbsi* as well as by Murua & Motos (2006) for the European hake *Merluccius merluccius*. The stereological method of Cavalieri (Gundersen, 1988; Howard & Reed, 1998) was applied to estimate the total volume (V) of the ovary (Fig. 1). The histological procedures used in this method result in gonad shrinkage and therefore the volumetric values afforded herein are smaller than those of fresh gonads. However, this fact does not affect calculation of oocyte number since oocyte density is proportionally affected.

The following formula was used to estimate the ovarian volume V,

$$V = T \times \left(A/p\right) \times \sum p$$

where

V: volume (mm^3)
T: distance between consecutive sections (mm)
A / p: area associated to the point of grid (mm^2)
p: number of points overlying the ovarian sections to the organ studied

The dissector method (Sterio, 1984) was used to estimate the number of oocytes per unit volume (numerical density, Nv) (Fig. 2).

For counting oocytes per volume unit (mm^3) the following formula was applied,

$$Nv = \frac{1}{A/f \times h} \times \frac{\sum Q^-}{\sum P}$$

where

A / f: Frame area; is the area used for counting, **h:** height of the dissector (distance between the sections of the dissector pair), $\mathbf{Q^-}$**:** is the total number of oocytes counted in each dissector pair, **P:** is the number of frames (dissectors) used.

To obtain an estimate of the total number of oocytes (N) in a given category, the value of its density (Nv) was multiplied by the estimated volume (V) of each single fish ovary. Finally, the diameters of the profiles of oocytes were measured with image analysis to obtain the per individual hake distribution.

Table 1. Size (total lenght), weight, gonad weight, gonad volume, total number and density of oocytes at different stages of initial development in each ovary analyzed per specimen. Coefficients of error (c.e) for the volume following Cavalieri (c.e. cav) and for total number of oocytes (c.e. N) following dissector were calculated by the formulas of Cruz-Orive & Weibel (1981) and Marcos et al. (2006), respectively.

						Oocytes 100-200 µ of diameter. Stage II		Oocytes 150-300 µ of diameter. Stage III		Total oocytes	
Specimen	Size (cm)	Weight (g)	Gonad weight (g)	Single gonad volume (mm^3)	c.e. cav	Oocyte density (Nº cells/mm^3)	Number oocytes	Oocyte density (Nº cells/mm^3)	Number oocytes	Number oocytes	c.e. N
1	53	814.2	32.3	3496	0.073	122	4.265 x 10^5	33	1.154 x 10^5	5.419 x 10^5	0.076
2	73	1846.3	52.5	4874	0.077	286	1.394 x 10^6	7	3.412 x 10^4	1.428 x 10^6	0.055
3	83	2325.2	81.2	5122	0.076	189	9.681 x 10^5	17	8.708 x 10^4	1.055 x 10^6	0.066

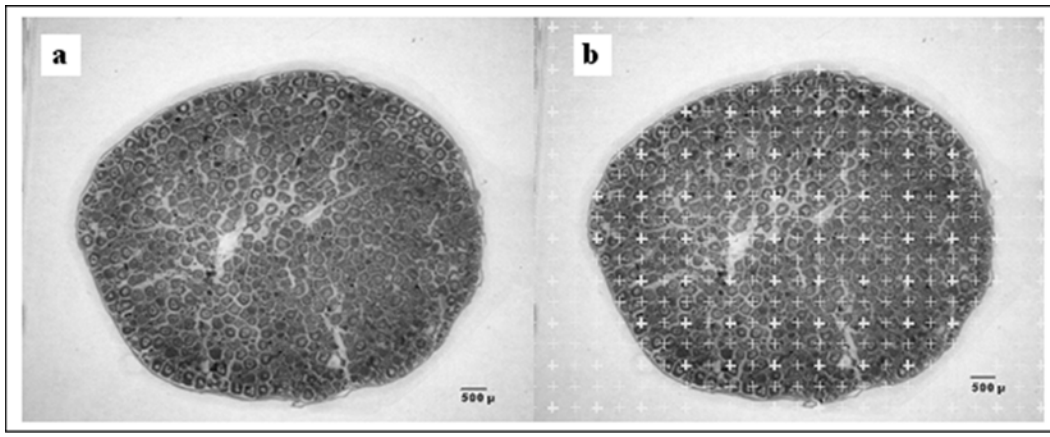

Fig. 1. Cavalieri method was applied in a 10 equidistant and parallel sections (T = 5 mm) from the same number of ovarian blocks that were embedded in paraffin; (a) Section of ovary from hake (*Merluccius hubbsi*); (b) Equidistant point grid onto image of the gonad that is used to estimate the area of a section; each cross or point represents an associated area: A/p = 0.476 mm^2.

RESULTS AND DISCUSSION

The following categories of oocytes were determined and measured:

a) cortical alveoli at stage II is characterized by primary growth oocytes showing cortical alveoli in the periphery of the cytoplasm, whose diameter distribution ranged from 100 to 200 microns, b) oocytes classified as stage III comprise those cells at early vitellogenesis, which contain inclusions of lipid and yolk granules in the cytoplasm and whose diameter distribution ranged from 150 to 300 microns. As inferred from the stereological method, the number of cortical-alveoli oocytes in the sampled gonads ranged between 426.000 and 1.393.000 while the number of oocytes in early active vitellogenesis ranged between 34.000 and 115.000 (Table 1). About 70% of the oocytes measured had 100-200 µm in diameter, indicating that the individuals analyzed were in a gonadal developing phase or early stages of oogenesis and not exceeding stage III (first stage of exogenous vitellogenesis, Vit. I) described in the classification of Murua & Motos (2006) (Table 1) (Fig. 3C). Macchi (2011) has calculated close to 500.000 hydrated oocytes and Rodriguez & Macchi (2010) have reported 120.000 to 830.000 oocytes for batch fecundity spawners of *M. hubbsi* (Table 2). Morover, Korta *et al*., (2010b) in European hake *M. merluccius* have estimated a pool of 500.000 previtellogenic oocytes (Table 2), 50.000 lipid inclusion oocytes and 23.000 cortical alveoli stages, these latter stages being later on recruited into the reproductive active phase. None post-ovulatory follicles or atresia were found in this stage of oocytes with cortical alveoli what suggests that the individuals of Argentinean hake analyzed were in the active phase of their ovarian development (Murua *et al.,* 2003). Korta *et al*. (2010b) in studies for European hake reported cohorts of nearly 70.000 oocytes starting in active vitellogenesis from a stock of 500.000 previtellogenics gametes. The results of our study show similar values for immature oocytes. Even though the present study was unable to

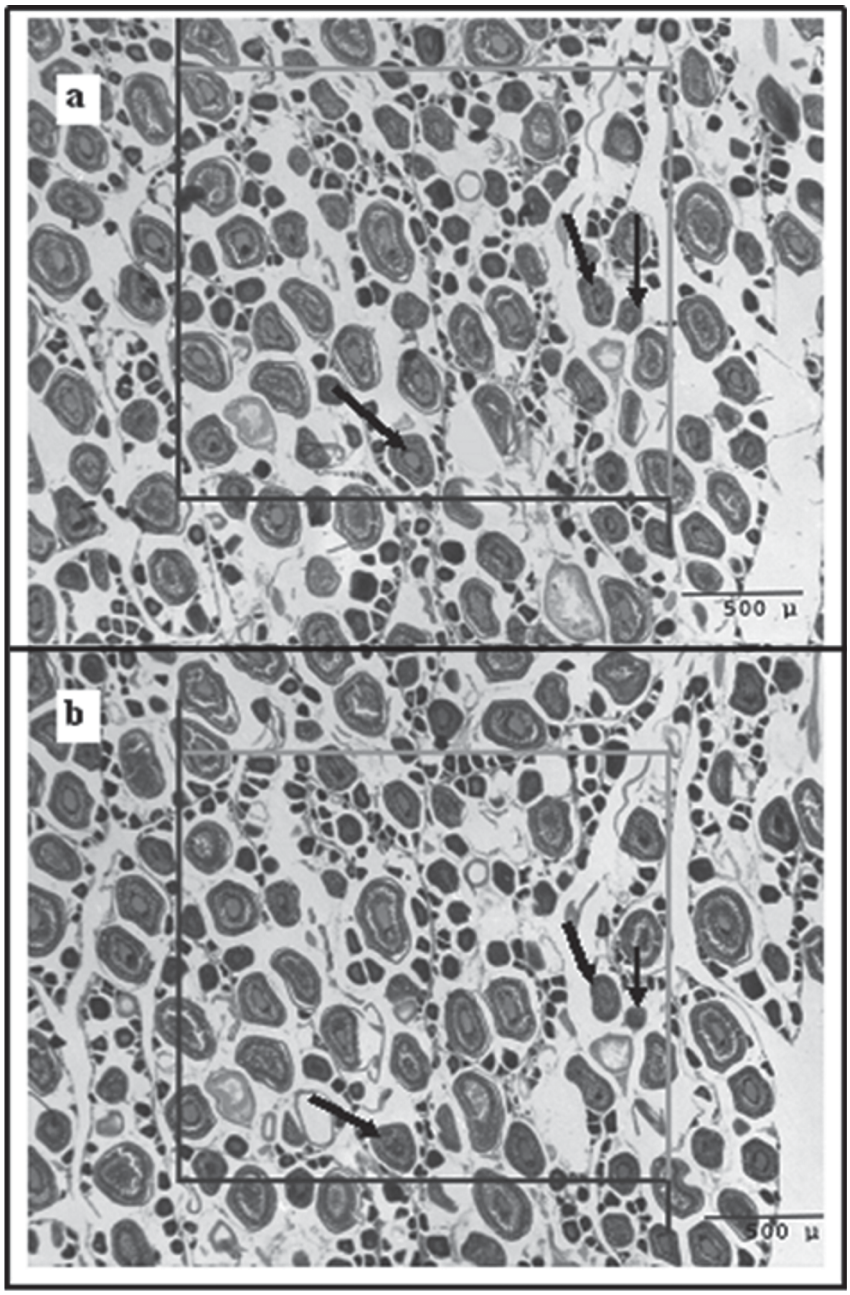

Fig. 2. Disector. Hake gonad sections used to determine the number of oocytes using the method Disector.
a) Section "look-up" b) Reference Section. (see text for details).

Table 2. Number of oocytes at differents developing stages reported by studies. PVO. Previtellogenic oocytes. The initial stages of oocytes are the sum of: lipid inclusions oocytes, Cortical alveoli and early vitellogenic stages (100-300 µ) for individuals in gonadal developing phase. B.F. = Batch Fecundity; R.F. = Relative Fecundity.

Specie	PVO (g^{-1})	Initial vitellogenesis Stages of Oocytes (g^{-1})	B.F. Hydrated oocytes	R.F. Hydrated oocytes (g^{-1})	Total maturity Eggs	Source
Argentine hake (*Merluccius hubbsi*)	Not Count	54.399*			Not found	Present work. In the individuals analyzed
Argentine hake (*Merluccius hubbsi*)			604.644	545		Macchi *et al*., 2004; Rodrigues & Macchi 2010
Chilean Hake (*Merluccius gayi*)			150.000	125-140	3.700. 000	Cerna & Oyarzun 1998
European Hake (*Merluccius merluccius*)	500.000	73.000	200.000	167		Korta et al. 2010 a or b
Cod (*Gadus morhua*)	250.000	150.000		460	3 to 5 million	Kjesbu *et al*.,1998; Kjesbu *et al*., 2011

determine the number of mature oocytes, it was feasible to determine the amount of oocytes per developing stage that will successively enter the active vitellogenesis stage (i.e., 1.450.000 oocytes) (Table 1). Considering that oocyte number was estimated on one single ovary per individual fish, and making a rough extrapolation, both ovaries would account for 1 - 3 million oocytes in developing. Therefore, each individual might be producing batches of about 500.000 eggs per individual that can be released into the sea in every spawning event, in agreement to previous observations in this species (Macchi, 2011). Current application of stereological methods for both, cell counting and estimation of ovarian volume are free-assumption models for stereology methods. The applications to the study of gonads per individual of fishes were recommended relevant in those situations focusing to increase the accuracy of the estimation procedure as occurs in irregular shaped cells, such as follicles in vitellogenesis (Weibel & Gomez, 1962; Sterio, 1984; Aragón *et al*., 2010). Moreover the main disadvantage of any stereological method is its laborious and time-consuming investment in routine analyses; its advantages are the precision gain to obtain unbiased individual estimates of number of cells such as oocytes at each development stage. The present study is a test on technical advantages

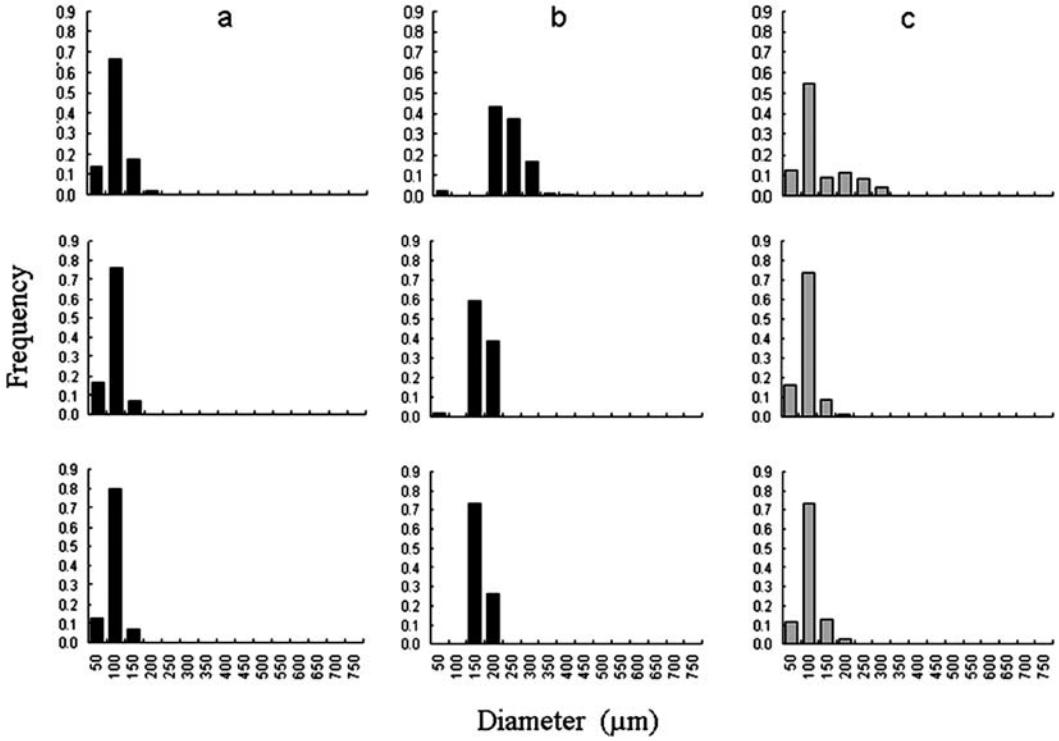

Fig. 3. Relative frequency of oocytes diameter on each individual analyzed hake. (a) Stages I and II oocytes. (b) Stage III oocytes. (c) Total oocytes in ovaries.

and disadvantages of a theoretically unbiased methodology to estimate total number of oocytes in fishes, especially related to gonadal development stages (Brown-Peterson *et al.*, 2011). This would mean that in a population study the number of individuals to be sampled and analyzed with free-assumption stereological methods will be less, but more laborious analysis on each individual.

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