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Three-dimensional reconstruction of postovulatory follicles from histological sections

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ABSTRACT

The postovulatory follicle (POF) resorption process is classified in daily phases by examination of their 2-D histomorphological structure and characteristics to indicate the elapsed time since spawning. These characteristics were used to investigate the associated reduction in volume by three-dimensional (3-D) reconstruction of POFs in ovary samples of European hake (*Merluccius merluccius*) and Atlantic cod (*Gadus morhua*). Ovaries were serially sectioned and POFs were analysed to collect the sequence of profiles to generate a 3-D model. The volume of POFs determined from 3-D reconstruction was also compared to results following Cavalieri and Geometrical Best-Fitting methods. The data of POF volumes obtained were shown to be typical characteristic of four and three different POF histomorphological stages defined for hake and cod, respectively. In both species, POFs shrank sharply in the first hours, and then decreased more gradually until their complete resorption. There was no statistical difference in volume measurement of POFs determined by the three methods listed above, and therefore, the volume of POFs may, be used to validate the reliability of 2-D histomorphological staging to age POFs.

1. Introduction

The reproductive strategies of fishes drive, to some extent, the focus of studies into reproductive biology (Murua and Saborido-Rey, 2003). Many species are thought to enhance their annual fecundity by production of ripe eggs from small precursor gametes that are not clearly differentiated at the start of spawning (Hunter et al., 1992). In such cases the annual reproductive potential must be calculated by the product of spawning frequency, batch fecundity and duration of their spawning season (Murua et al., 2006). Therefore, the spawning fraction, defined as the proportion of females spawning per day, is an essential parameter in order to estimate the number of batches, and hence, the realised fecundity. Commonly, spawning fraction is assessed from the prevalence of females at a specific spawning stage identified by the presence of various classes of postovulatory follicles (POFs) (Hunter et al., 1985; Murua et al., 1998, 2006; Macchi et al., 2004). Moreover, the spawning fraction in the population can be used, in conjunction with other adult parameters, in the daily egg production method (DEPM) (Parker, 1980) to estimate a fisheries-independent value of spawning stock biomass, a basic parameter in fish population dynamic studies. Therefore, an accurate classification of the time course of POF degeneration is a critical step for the estimation of spawning fraction and, hence, the application of the DEPM (Hunter and Macewicz, 1985).

Prior to ovulation the follicle comprises two layers of cells, the outer thecal and inner granulosa, with prominent nuclei and regular columnar shape surrounding the developing egg (Guraya, 1986). POFs are produced in cohorts following each ovulation giving rise to characteristic POF stages depending on the inter batch interval (Witthames et al., 2010). Based on observations of wild Northern anchovy (*Engraulis mordax*) that spawns synchronously around midnight (Hunter and Macewicz, 1980) it was possible to link POF histomorphological structure to the time post spawning. However, many species including European hake (*Merluccius merluccius*) do not synchronise their spawning over a 24 cycle (Murua and Motos, 2006) so POF age is assumed without a prior validation of the duration of the degeneration and resorption process. Due to the difficulties described above, the estimation of the spawning fraction often remains imprecisely estimated. In fact, it is considered the most difficult and unknown parameter for the application of the DEPM (Hunter and Lo, 1997; Stratoudakis et al., 2006; Murua et al., 2006).

To date classification of POF stages to estimate spawning fraction is carried out based on histological characteristics seen in 2-D

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sections that may poorly capture the structure of the underlying 3-D structure of the POFs. All of the characteristics used to classify POF morphology, cell shape and organization, and evaluation of the vacuole abundance are essentially qualitative properties, and therefore, subject to sources of bias/subjectivity. Histological treatment may distort the tissue which can modify the perception of the structure especially when combined with a unique interpretation by the observer when reading the histological slides. Moreover, the plane of the section can affect the histomorphological characteristics of the POFs. In short, there is a loss of dimensional information within the 2-D histomorphological characterization. Although the 3-D shape of 2-D silhouettes from histology seems intuitive, it has been argued that 2-D profiles fail to provide any sort of shape discriminator for geometric solids which generated them (Mayhew, 1991). Therefore, accurate information from 3-D reconstruction may add objectivity to the definition of POF classes. 3-D methodology has proved to be very useful to study the adaptative morphology and physiological responses to environmental fluctuations in invertebrates (Quincoces, 1995; Marigómez et al., 1995a, 1995b).

Thus, the main objective of this paper is to investigate the use of 3-D reconstruction to validate standard histomorphological classification of the different types of POFs, using Atlantic cod (*Gadus morhua* L.) and European hake (*M. merluccius* L.) as case studies. The 3-D reconstructions from serial histological sectioning of each of the follicular stages will therefore provide an objective metric to verify the correctness of the 2-D histomorphological characteristics used to age POFs. Moreover, the precision of the 3-D reconstruction technique will be tested by comparison with other quantitative methods; Cavalieri method and Geometrical Best-Fitting.

2. Material and methods

Ovarian tissue from spawning hake and cod females were used in this study to conduct the 3-D reconstructions (Table 1). In the case of hake, ovaries were collected by trawling at an average depth of 200 m from the wild population during a survey in the main spawning season (December–April) from a location of high egg production in the Bay of Biscay (North coast of Spain). The water temperature at the site of capture was 13 °C and the ovaries were fixed whole immediately after collecting the fish from the trawl. Cod ovary samples were taken from captive fish at the IMR Matre Research Station (Norway) during the peak of cod spawning (February) in 2003 using a biopsy method (Bromley et al., 2000). A PIT tag (Destron Fearing, USA) was inserted on the back, just below the skin, after sedating the fish by immersion in 60 ppm of benzocaine so each fish could be identified during later biopsy sampling. Around 10 cod producing ripe ovulated eggs were selected from a mixed sex group of fish spawning in 4 m^2 tanks at 9 ± 0.2 °C in order to observe spawning dynamics. Further biopsy samples of 1 cm^3 were taken from sedated females every 6 h during the first 48 h from the start of the experiment and 24 h thereafter from each individual. The time series of biopsy samples were processed to determine resorption rate of POFs and to ascertain whether the follicles disappeared before or after the next batch was produced. In the case of cod, the elapsed time since ovulation was approximately known based on the assumption that the presence of copious egg production indicated recent ovulation. Samples from both species were stored in 3.6% formaldehyde buffered with 0.1 M sodium phosphate to pH 7.0.

Table 1
Number of 3-D reconstructions performed in both species.

Structure	Hake	Cod
Hydrated oocyte	2	2
Very Early POF	2	4
Early POF	6	7
Medium Term POF	3	0
Late PF	2	8
Total	15	21

Table 2
The histological characteristics used to categorize each POF stage of hake and cod (adapted from Murua et al., 2006).

POF resorption stage	General histological characteristics
Very Early POF	The structure of the ovarian follicle is well maintained. There is no sign of deterioration, the granulose and theca layers are distinguishable.
Early POF	Follicular cells are still ordered in layers. First signs of degeneration: granulosa cells are more rectangular, vacuolation and nuclear pycnosis appear.
Medium Term POF	Follicle is being resorbed, granulose and theca layers cannot be differentiated. The follicle structure is disorganized and vacuoles are abundant.
Late POF	The follicle has shrunk substantially and become a small structure, which will eventually disappear.

POFs were staged according to histological classification defined by Murua et al. (1998) and Murua and Motos (2006): Brand-new POF, New POF, Intermediate POF and Old POF that indicate on hake day-zero, day-one, day-two and >2 days since spawning according to those authors (Table 2).

For cod, the histological classification is two-staged, Early POF and Late POF (Murua and Saborido-Rey, 2003). However, it was decided to use the same four stages POF classification of hake for cod in order to see similarities/differences through a comparative analysis. Spawning ovaries in each of these types of POF stages where selected from histology. For the 3-D reconstruction, the POFs with open lumen class that appeared near the cortex of the ovary were chosen because they were better preserved. In addition to this, a few numbers of hydrated oocytes were selected for 3-D reconstruction in order to know the size of the follicular structure prior to ovulation.

2.1. Histological preparation and serial image registration

A 0.5 cm thick cross section was cut from each hake ovary and dehydrated in ascending concentrations of alcohol and infiltrated in methyl-hydroxymethacrylate resin (Technovit 7100) prior to polymerisation into blocks. In the case of cod, a portion from each biopsy was taken with a Pasteur pipette and followed the same dehydration procedure. For hake and cod, each block was serially sectioned at 5 μm and alternate sections were retained on slides for 3-D reconstructions (Fig. 1). All retained sections were numbered to reflect their order in the original block to avoid deformation of the follicle shape during reconstruction. Eighty sections were retained from each block and stained with Haematoxylin & Eosin. In each of the serial sections, the profile corresponding to the same POF was identified and captured as an image at 20× or 40× of magnifications with a camera (Olympus-Camedia 4040-Z) mounted on a Zeiss microscope. The calibration of each magnification was produced by digitising a known distance on a 1 mm calibration slide. This process produced a set of serial images of the 2-D profiles (a POF stack) that covered the full depth of each identified follicle along the direction of sectioning (*Z* axis).

2.2. Quantitative analysis

2.2.1. 3-D reconstruction

The external boundary of each POF profile in the stack was digitised in *X* and *Y* by tracing around the POF's periphery using a mouse to produce a stack of outline traces representing a distance *Z* equivalent to the depth of the POF in the block. The stack of