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▶ To cite this version:

Manon Lesage, Mak Sully, Jérôme Bugeon, Manon Thomas, Stéphanie Gay, et al.. Quantitative analysis of asynchronous oogenesis dynamics in fish: Application of a modern 3D analysis approach to an old problem. 12. International Symposium on Reproductive Physiology of Fish (ISRPF), May 2023, Heraklion, Greece. hal-04187726

HAL Id: hal-04187726
https://hal.inrae.fr/hal-04187726

Submitted on 25 Aug 2023

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Quantitative analysis of asynchronous oogenesis dynamics in fish:
Application of a modern 3D analysis approach to an old problem

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INTRODUCTION

In multiple spawning fish (such as medaka and zebrafish), oogenesis involves anatomical structures in permanent turnover, the ovarian follicles, which support the development and growth of oocytes until spawning. Although many regulatory actors have already been identified, we still have an incomplete view of the dynamics of the follicular growth during life and reproductive cycles. This gap is mainly due to the lack of quantitative data describing the follicular population and its evolution. Traditionally, studies use manual methods of counting from dissociated follicles, or semi-automatic methods from two-dimensional (2D) ovarian sections. Such approaches are extremely time-consuming and greatly limit the accuracy of the data. More recently, the development of modern 3D imaging methods and automatic image data analysis tools has raised new opportunities to overcome this limitation and reduce methodological bias. The objective of the present work was to exploit these novel approaches to visualize and precisely enumerate the whole population of ovarian follicles of Medaka females of different ages, in normal and disrupted conditions.

METHODS

Ovaries were collected from Medaka females of different stages (larvae, juvenile and adults). Juvenile and adult samples were processed for staining of follicular contours (using the MG nuclear dye) and subjected to clearing treatment to allow confocal 3D imaging. To this aim, we established a clearing protocol (C-ECi) that combines both the CUBIC hyperhydration and the organic solvent ECi methods. For larvae samples, an iDISCO-inspired permeabilization protocol followed by pH3 immunostaining and ECi clearing were used for full 3D imaging. In order to accurately quantify and measure intra-ovarian follicles, we exploited recent Deep Learning algorithms (Noise2Void, Cellpose) that we integrated into an end-to-end processing pipeline allowing reliable measurement of diameters of almost all follicles, regardless of the female stage.

RESULTS & DISCUSSION

The follicular diameter distributions revealed different patterns of follicular density over the fish life. At the larval and juvenile stages, the density profile exhibits a single peak corresponding to the early follicle reserve. This profile then converges, in adults, to a more complex profile including 2 peaks of early follicles, intermediate follicles in a stationary phase and large follicles in a fast daily maturation cycle. The detection of two early follicle peaks strongly suggests the existence of two distinct recruitments in adults (and two oocyte reserves). The first recruitment (between stages II and III) supplies pre-vitellogenesis and the second one (between stages IV and V) marks the transition from pre-vitellogenesis to vitellogenesis. Analysis of follicular density in ovaries from females lacking miR-202 (KO *mir-202*-/-), a key regulator of fish fecundity, revealed a decrease in the number of follicles at stages I and IV and an increase at stages II/III, thus suggesting lower recruitment rates in absence of miR-202. Overall, the quantitative and temporal analysis of the ovary in 3D enabled to accurately describe the dynamics of the asynchronous oogenesis in Medaka and revealed the existence of two follicular reserves at the adult stage. This approach also allowed to highlight the role of *mir-202* in the regulation of these reserves. In the future, such exhaustive data will be of great value to build mathematical models to better understand the dynamics of Medaka oogenesis in relation to fecundity and its regulation by internal or secreted factors.

This work was supported by the Agence Nationale de la Recherche (DYNAMO project, #ANR-18-CE20-0004) and INRAE DIGIT-BIO métaprogamme (IMMO project).