Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 - 6131
Vol. 26(6): 1083- 1094 (2022)
ejabf.journals.ekb.eg

Histochemistry of Oogenesis in the Flathead Grey Mullet *Mugil cephalus* (Mugilidae) in Natural Habitat

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ARTICLE INFO

Article History:
Received: Nov. 18, 2022
Accepted: Dec. 15, 2022
Online: Dec. 30, 2022

Keywords:
Histochemistry,
Ovary,
Development,
Oogenesis,
Vitellogenesis,
Mugil cephalus

ABSTRACT

Understanding the structure of the reproductive system of fishes for commercial production allows the development of productive strategies. In the present study, the histochemistry of the oogenesis of *Mugil cephalus* in the natural habitat was investigated. The oocyte development was followed up via both histological and histochemical techniques during the ovarian cycle of *M. cephalus* in Bardawil Lagoon. During ovarian development, only one set of oocytes was annually found. Five stages of oocyte development were identified in *M. cephalus*; namely, primary, vesicles, primary yolk, secondary yolk, and tertiary yolk oocytes. Yolk deposition occurred during four (vitellogenic) stages, including vesicles, primary yolk, secondary yolk and tertiary yolk oocytes. The histological and histochemical findings revealed that three types of yolk inclusions are formed during vitellogenesis. These included lipid yolk droplets, yolk globules and cortical granules. Lipid yolk droplets were deposited first, followed by yolk globules and cortical granules. The former inclusions had lipoid nature; the cortical granules consisted of polysaccharides, while yolk globules contained both proteins and polysaccharides. Such analysis should provide a basis for the study of the endocrine control of vitellogenesis in oocytes and give important information for hatchery management for inducing the spawning of *M. cephalus* in aquaculture systems.

INTRODUCTION

Histological analysis of the reproductive system of fishes provides more comprehensive knowledge on the gonad functions and highly precise characterization of the oogenesis phases and the reproductive cycle of species (**Ortiz-Delgado *et al.*, 2008; Mandal, *et al*., 2020; Senarat *et al.*, 2021)**. Oogenesis is a complicated process, which includes the transition of oogonia into oocytes and their growth to fully developed eggs, ready for propagation (**Grier *et al*., 2009; Viana *et al*., 2018**).

The histological investigation of fish oocyte development during the reproductive cycle of fish species has been studied by several authors (**Ortiz-Delgado *et al.*, 2008; Charitonidou *et al*., 2022; Shilta *et al*., 2022; Porcu *et al*., 2022**). In

addition, the histochemical analysis of oogenesis has received attention regarding teleosts (**Mayer *et al.*, 1988; Sarasquete *et al.*, 2002a, b; Corriero *et al.*, 2004; Motta *et al.*, 2005; Calabro *et al.*, 2008**; **Reading *et al.*, 2018**).

The grey mullet, *M. cephalus* is a euryhaline teleost that lives in environments of different salinities (coastal waters "seawater", estuaries "brackish water", and lagoons "hypersaline"). The usual commercial fishing in natural waters can not satisfy the present demand for the juveniles of *M. cephalus*. The resources of mature female breeders are vitally important, especially when joined to the production of juveniles at large scale in the hatcheries. However, the collection of mature females of mullet is not possible from the natural habitat since the prespawning females would have then migrated to the sea for spawning in the usual manner (**Mousa, 1994**). In such cases, continuous maintenance of broodstock in captivity and the use of special techniques to control and regulate gonadal development are therefore highly significant.

Morphological investigations were performed on the ovaries of mullet species using morphological, histological, and ultrastructural descriptions for *Liza carinata* (**Abou-Seedo & Al-Khatib, 1995**), *M. cephalus* (**Kim et *al.*, 2004**; **Das *et al.*, 2013**), *Mullus barbatus* (**Balci & Aktop, 2019**), *Liza parsia* (**Bose, 2019**) and *Liza ramada* (**Abdal Monem *et al.*, 2021**).

Thus, the present work was planned to investigate the histochemistry of oogenesis in *M. cephalus*.

MATERIALS AND METHODS

Fish collection and preservation

Mature females (at least two-years-old) of *M. cephalus* were monthly collected alive throughout a whole year from Bardawil Lagoon (natural marine habitat). At the prespawning with the onset of spawning (November and December), females were obtained bi-monthly to ensure that all stages of ovarian development are collected. After gross examination of the paired ovary and subsequent maturity staging, the ovaries were weighed and fixed in either 10% neutral buffered formalin or Bouin's fluid for 72h and stored at 4°C.

Histological and histochemical methods

The fixed ovary was thereafter dehydrated through an ascending series of increasing concentrations of ethanol, cleared and embedded in paraplast (m.p. 56-58 °C). Consecutive transverse sections were made at 5µm thickness. Sections of ovaries were stained with either Harris's alum haematoxylin or Heidenhain's iron haematoxylin according to **Conn (1953)**, and aqueous solution of eosin was used as a counterstain for general histology. Five stages of oocytes were recognized according to **Mousa (1994)**; stage I (primary oocytes), stage II (vesicles oocyte), stage III (primary yolk oocyte), stage IV (secondary yolk oocyte) and stage V (tertiary yolk oocyte).

The histochemistry of oogenesis was addressed through the following procedures:

- The Sudan black B technique **(Chiffelle & Putt, 1951)** was applied for detecting lipid inclusions in cryostat cut sections.

- The mercury bromophenol blue method "Hgbb" (**Bonhag, 1955**) was applied for illustrating the total proteins.
- The periodic acid Schiff (PAS) reaction (**McManus, 1948**) was applied to demonstrate the general carbohydrates.

Oocyte measurement

Oocyte size was obtained by taking the mean of the maximum and minimum diameters of the oocytes sectioned through the nucleus.

RESULTS

In *M. cephalus*, the oocytes appeared to exhibit bead-like arrangement (Fig. 1a) instead of the random scattering which is familiar in ovaries of other animals. During ovarian development, only one clutch of oocytes was found to mature annually. The oogonia pass through a number of successive stages to produce the final growth stage of oocytes. These stages are involved in general certain complicated changes in the cytoplasm and nuclei of those cells.

Histology of oocyte development

The terminology used for staging the individual oocytes is based on their histological appearance **(Yamamoto *et al*., 1965; Mousa, 1994)**. Oogenesis comprises generally five stages, viz. primary oocytes (chromatin- and peri-nucleolus oocytes), vesicles, primary yolk, secondary yolk and tertiary yolk oocytes.

I. Primary oocytes stage

The primary oocytes are small in size, with a diameter ranging from 20-120µm in spherical or oval shapes. Following oogenesis, chromatin nucleolus stage is the newly formed primary oocytes measuring 20- 40µm and containing a single large prominent nucleolus (Fig. 1a). It was observed that, at the beginning of the peri-nucleolus stage, the large nucleolus of the chromatin nucleolus stage gave rise to more than one small nucleoli that were still relatively scattered in the nucleus. With the progression of the primary oocyte development, the nucleoli became numerous and small in size, being located close to the nuclear membrane (Fig. 1a).

II. Vesicles stage

Observations recorded that, the measure of the oocytes in this stage ranged from 110- 160µm. The nuclear membrane became wavy, exhibiting pocket-like depressions in which the nucleoli were located (Fig. 1a, b). A number of small vesicles appeared in the cytoplasm, and initially in the peri-nuclear area. These vesicles gradually increased in size and number, and by the end of this stage, they formed a densely packed perinuclear zone (Fig. 1b).

III. Primary yolk stage

The diameters of the individual oocytes in this stage measure was 150- 370µm. The nuclei appeared in some cases nearly spherical or oval in shape. The minute yolk globules and the circum-nuclear ring of colorless lipid droplets characterized this stage (Fig. 1c). It was clear that yolk globules appeared first in the outer most part of the ooplasm, and increased in both number and size accompanying the oocyte growth Then, globules became more abundant, providing continuous thick layer. The oocyte

at this stage is covered by three layers; the outer layer called theca, the middle layer is the granulosa and the inner one is the zona radiata, which is thicker than the other two layers (Fig. 1c).

IV. Secondary yolk stage

The oocytes in this stage measure 370- 500µm. The nuclear membrane started to be rather indistinct. The ooplasm of secondary yolk stage was heavily impregnated with yolk globules. The lipid droplets, which had been concentrated around the nucleus were notably distributed in the ooplasm (Fig.1d).

V. Tertiary yolk stage

The diameters of the tertiary yolk oocytes range from 500- 660µm. The nuclei at this stage are commonly known as "germinal vesicles", having irregular outlines (Fig. 1d). The yolk globules appeared accumulated throughout the entire ooplasm, and the lipid droplets coalesced and became less in number (Fig. 1e).

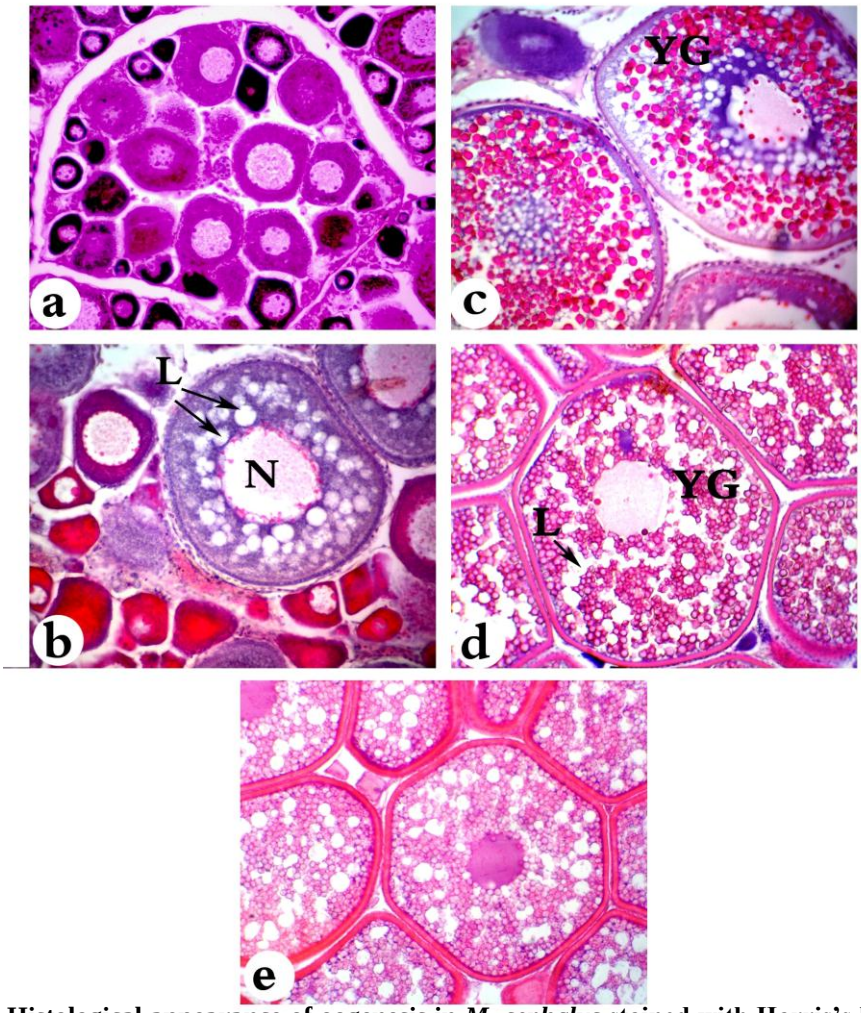

Fig. 1. Histological appearance of oogenesis in *M. cephalus* stained with Harris's haematoxylin and eosin. (a) Early vitellogenic ovary showing primary oocytes, X100; (b) Vesicles oocyte exhibiting lipid droplets (L) in the ooplasm around the nucleus (N) (X200); (c) Mid-vitellogenic ovary, showing primary yolk oocyte. Note that yolk globules (YG) in the peripheral ooplsm among lipid droplets which first appear in the perinuclear area (X100); (d) Late-vitellogenic ovary showing numerous protein yolk globules (YG) and lipid droplets (L) in the ooplasm of secondary yolk oocyte (X100), and (e) Prespawning ovary showing tertiary yolk oocyte impregnated with yolk globules (X100).

Histochemistry of oocyte development

The enlargement of oocytes is brought about mainly by the accumulation of yolk material, which is an essential constituent of oocytes in general. Three distinct types of yolk material could be distinguished in *M. cephalus*; namely, lipid yolk droplets, yolk globules and cortical granules.

Lipid yolk droplets

In paraffin sections, the lipid droplets appear as vascular structures (Fig. 1) but this is an artifact feature since the fat solvents used in such procedures dissolve them out. Nonetheless, in Sudan black stained cryostat-cut frozen sections, lipid droplets are readily distinguished by their intense sudanophilia **(Fig. 2)**. The appearance of these droplets in the developing oocytes marks the initiation of the process of yolk formation. Lipid droplets are seen firstly as a ring of small droplets lying around the nuclei of the vesicles oocytes **(Fig. 2a)**. Then they were noticed to undergo gradual increase in both number and size until they occupy almost the whole ooplasm of the primary **(Fig. 2b)**, secondary **(Fig. 2c)** and tertiary **(Fig. 2d)** yolk oocytes.

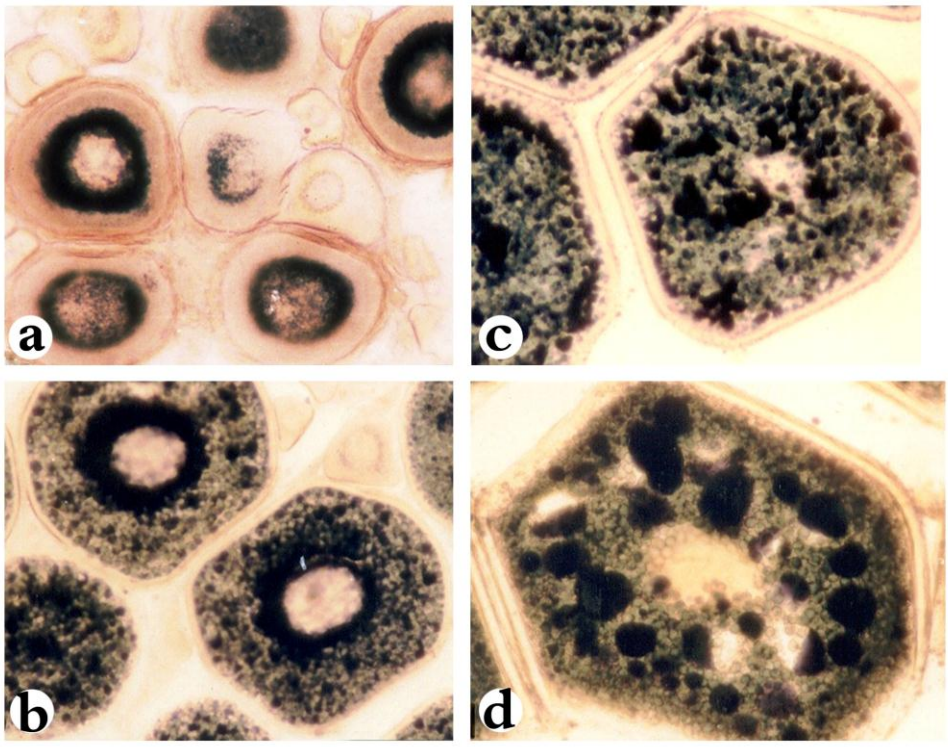

Fig. (2): Histochemistry of oocyte development in *M. cephalus*, sections stained for lipids with Sudan black, X100. (a) The first appearance of lipid droplets is a minute droplets in the perinuclear area of the ooplasm in the vesicles oocyte stage. (b) In the primary yolk oocyte, the lipid droplets not only form a densely packed perinuclear zone but a larger number interspersed between yolk globules. (c) Lipid droplets have enlarged and distributed in all ooplasm of the secondary yolk oocyte. (d) At the end of vitellogenesis, during the tertiary yolk oocyte stage the lipid droplets have further enlarged through coalescence.

Yolk globules

Proteinic materials were perceived in the yolk globules (proteid yolk) as well as in the zona radiata as indicated by their strong reactivity with bromophenol blue **(Fig. 3)**. The deposition of yolk globules appeared at first as a few minute spherical globules situated in the outermost part of the ooplasm of vesicles oocytes **(Fig. 3 a&b)**. Thence they were noticed to undergo a progressive increase in both number and size concomitant with the oocyte growth (i.e. in the primary, secondary and tertiary yolk oocytes), and they soon accumulated in aggregates or clusters in the ooplasm of the tertiary yolk oocytes **(Fig. 3 b-d)**.

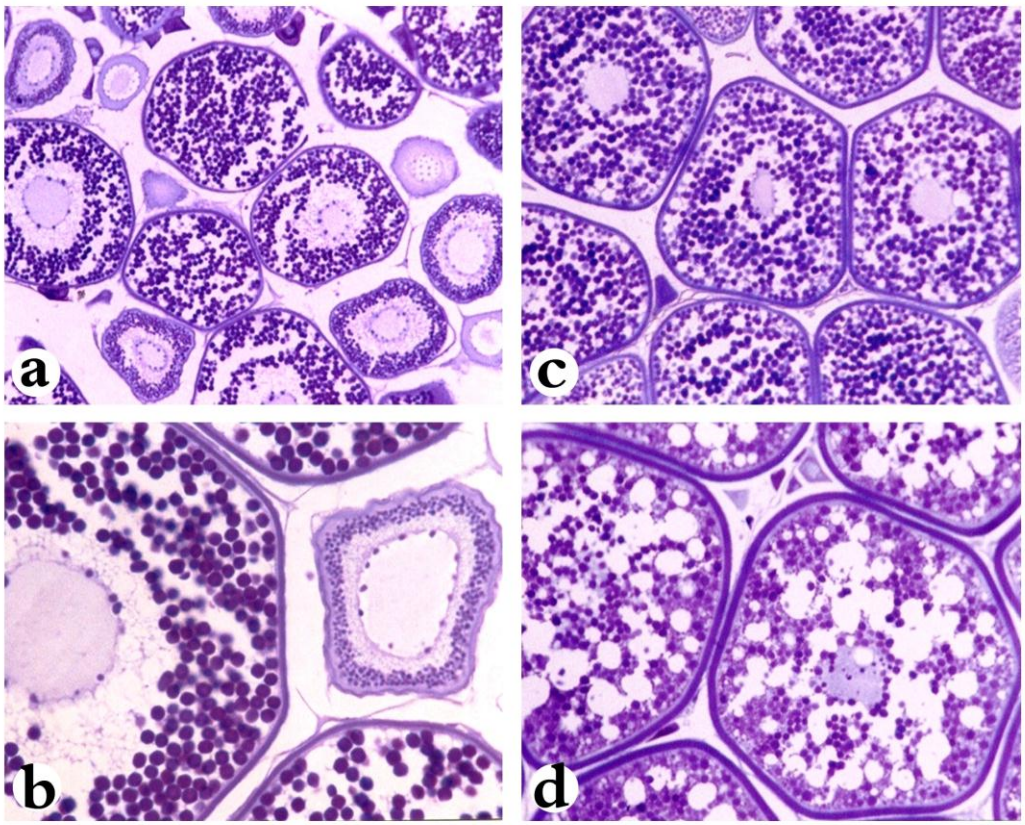

Fig. (3): Fragments of *M. cephalus* ovary stained for proteins with bromophenol blue. The yolk granules stained intensively for protein. Also, the nucleoli and zona radiata stained positively for proteins. **(a)** Vitellogenic ovary at mid-vitellogenic stage, X100. **(b)** The appearance of numerous small protein yolk granules in the outer cortex of the ooplasm in the primary yolk oocyte, X200. **(c)** The protein yolk globules have multiplied and increased in size during the secondary yolk oocyte stage, X100. **(d)** In the tertiary yolk oocyte, the protein yolk globules coalesced and appeared accumulated throughout the entire ooplasm, X100

Cortical granules

Cortical granules appeared at first as small granules scattered along the periphery of the vesicles oocytes; exhibiting a positive PAS-reactivity indicating their contents of carbohydrate materials **(Fig. 4)**. Accompanying the growth of the oocytes, the cortical granules were increased in number constituting a very well expressed layer beneath the vitellogenic (secondary and tertiary yolk) oocyte membrane **(Fig. 4)**.

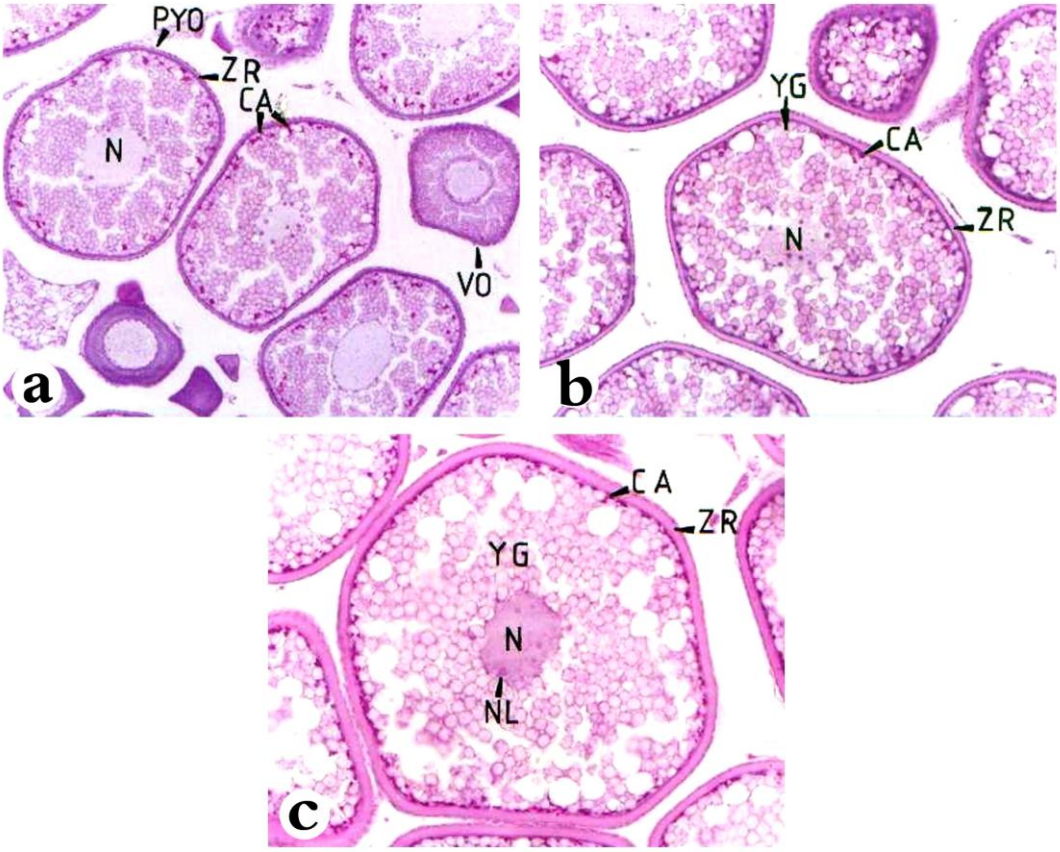

Fig. (4): Histochemistry of carbohydrates during oocyte development in *M. cephalus*. Sections stained for carbohydrates with PAS and counter-stained with Harris's haematoxylin. X100. The cortical granules or alveoli (CA) have a PAS-positive reaction. These small granules first appear in the outer cortex in the vesicles oocyte (VO) and the primary yolk oocyte (PYO) **(a)** stages, at the beginning of vitellogenesis, becoming more prominent by the secondary **(b)** and tertiary **(c)** oocyte stages. Also, zona radiata (ZR) and yolk globules (YG) contained an average amount of carbohydrate material as reflected by their moderate reactivity with PAS; nucleus (N) and nucleolus (NL)

DISCUSSION

The histological and histochemical data have demonstrated that three types of yolk inclusions (lipid yolk droplets, yolk globules, cortical granules) were produced during vitellogenesis in *M. cephalus*. These yolk inclusions differed distinctively in their morphology, tinctorial properties as well as their chemical nature. The histochemical procedures, hereby applied have revealed that the above mentioned lipid yolk droplets were of lipoid nature, the cortical granules consisted of polysaccharides whereas yolk globules were constituted of both proteins and polysaccharides. Similar observations were obtained in *Thunnus thynnus* and *Xiphias gladius* (**Sarasquete *et al.*, 2002**; **Ortiz-Delgado *et al.*, 2008**). Many researchers agree that the cortical alveoli (yolk vesicles) consist of polysaccharides (**Guraya, 1965** and **Maksimova, 1991**). But **Anderson (1968)** remarked that the cortical alveoli, of oocytes of *Syngnathus fuscus* and *Fundulus heteroclitus* contained both polysaccharides and proteins. Furthermore, **Khoo (1979)** reported that such vesicles were constituted of polysaccharides and proteins rich in sulphydryl groups. Concerning the chemical nature of the yolk granules, some authors marked that they consist mainly of protein and lipoprotein (**Guraya, 1965** and

Rastogi, 1969). In the oocytes of *Syngnathus fuscus* and *Fundulus heteroclitus*, yolk granules were noticed to contain phospholipids (**Anderson, 1968**), while the yolk granules of *Carassius auratus* consisted of proteins, phospholipids and neutral lipids (**Khoo, 1979**). However, other studies demonstrated that the yolk granules of oocytes were formed of proteins, together with a small amount of glycogen and glycoproteins **(Maksimova, 1991; Sarasquete *et al.*, 2002a** and **Ortiz-Delgado *et al.*, 2008)**.

The occurrence of such three types of yolk inclusions in teleost oocytes supported previous reports **(Kjesbu & Kryvi, 1989; Maksimova, 1991; Sarasquete *et al.*, 2002a; Ortiz-Delgado *et al.*, 2008** and **Reading *et al.*, 2018)**. However, it seems that the occurrence of these types of yolk inclusions is not universal incidence among all teleosts, since in some species only yolk vesicles and yolk granules were observed as postulated by **Yamamoto (1956)** and **Khoo (1979)**. The first type of yolk inclusions accumulate in developing oocytes of *M. cephalus* is lipid yolk, in the form of distinct lipid droplets, the appearance of which can be considered to mark the start of endogenous vitellogenesis (**Schackley & King, 1977; Mayer *et al.*, 1988** and **Reading *et al.*, 2018**) although the endogenous origin of lipid yolk has yet to be fully proved. The accumulation of lipid yolk (endogenous vitellogenesis) prior to that of protein yolk (exogenous vitellogenesis) is common in most studied teleosts, whereas in other studied teleosts the lipid yolk inclusions firstly appear in the perinucleolar cytoplasm (**Schackley & King, 1977; Wiegand, 1982; Reading *et al.*, 2018**). In *Dicentrarchus labrax*; they generally first appear in the outer cortex (**Mayer *et al.*, 1988**).

Protein yolk accumulation occurs during oocyte development in *M. cephalus* (Yolk granule stage) after and concomitant to, lipid yolk accumulation. Protein yolk is sequestered in the form of discrete granules, which first appear in the outer cortex. It is now generally accepted that protein yolk is exogenous in origin, the protein yolk precursor having been identified as the female-specific plasma lipophosphoprotein complex, vitellogenin (**Wallace, 1978**). The prevailing view is that the hepatically produced vitellogenin is specifically sequestered, under gonadotropin control, by the developing oocytes (**Wallace & Selman, 1981; Tyler *et al.*, 1987 & 1991**).

The third, and quantitatively minor, type of inclusion is the cortical alveoli (carbohydrate yolk; **Raven, 1961**) which release their contents into the perivitelline space during the cortical reaction at maturation. For this reason they can not be considered yolk in the strict sense. Whereas, in the majority of teleost species studied, cortical alveoli formation occurs prior to both lipid yolk and protein yolk formation (**Khoo, 1979; Wallace & Selman, 1981** and **de Vlaming, 1983**). In *M. cephalus*, histochemical study has revealed that the cortical granules first appear after the start of both lipid yolk and protein yolk formation. Similar histochemical observation was obtained in *D. labrax* (**Mayer *et al.*, 1988**).

For characterizing the oogenesis developmental phases, size, quantity and standing of oocyte components have been employed. The process of oogenesis in *M. cephalus* found to comprise five successive stages, namely: primary oocytes stage (chromatin-nucleolus and peri-nucleolus stages), vesicles stage, primary yolk stage, secondary yolk stage and tertiary yolk stage. Similar developmental phases were also obtained in *Salmo gairdneri irideus* (**Yamamoto *et al.*, 1965**) and *Xiphias gladius* (**Ortiz-Delgado *et al.*, 2008**).

Vitellogenesis (i.e. yolk deposition) in *M. cephalus* covering the four stages: vesicles stage, primary yolk stage, secondary yolk stage and tertiary yolk stage. Similar vitellogenic stages were described in teleostean species, such *Carassius auratus* (**Khoo, 1979**), *Gadus morhua* (**Kjesbu & Kryvi, 1989**). In some other fishes, the vitellogenic stages were described as two phases only; vacuolization and yolk deposition in *Clarias gariepinus* (**Zaki *et al.*, 1986**) and *Chrysichthyes auratus* (**Ashour *et al.*, 1990**), and lipid yolk vesicles and protein yolk vesicles in *Macrodon ancylodon* (**Vizziano & Berois, 1990**). However, **N'Da & Deniel (1993)** classified the vitellogenesis in *Mullus surmuletus* into three stages: primary, secondary and tertiary vitellogenesis.

In conclusion, this study described the pattern of oocyte development and the histochemical composition of yolk inclusions during oogenesis in the grey mullet. Such an analysis should provide a basis for the study of the histochemistry of vitellogenesis in teleosts.

Acknowledgement

We are extremely grateful to Professor Shaaban Mousa (Klinik fur Anaesthesiologie, Charite-Uńiversitatsmedizin Berlin) for critical review of the manuscript.

Conflict of Interest

The author declares there are no conflicts of interest.

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ARABIC SUMMARY

دراسة هستوكيميائية على تطور البويضات فى سمكة البورى

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يعتبر فهم تركيب الجهاز التناسلي للأسماك من أجل الإنتاج التجاري أساسيا لتطوير الإستراتيجيات الإنتاجية. فى هذا البحث تمت دراسة تطور البويضات لسمكة البورى فى بيئتها الطبيعية باستخدام الطرق الهستوكيميائية. تم تتبع تطور البويضات بالتقنيات النسيجية والكيميائية النسيجية خلال دورة المبيض لسمكة البورى في بحيرة البردويل. وجد أن مجموعة واحدة من البويضات هى التى تنضج سنويا أثناء تطور المبيض فى سمكة البورى. أمكن تمييز خمسة مراحل من تطور البويضات وهى: مرحلة البويضات الابتدائية ، مرحلة الفجوات، مرحلة المح الأولى، مرحلة المح الثانية ومرحلة المح الثالثة. يغطى ترسيب المح أربع مراحل من تلك المراحل (المراحل المحية)، مرحلة الفجوات، مرحلة المح الأولى، مرحلة المح الثانية ومرحلة المح الثالثة. أثبتت النتائج الهستولوجية والهستوكيميائية أن ثلاثة أنواع من المكونات يتم تكوينها أثناء ترسيب المح. تضم هذه الأنواع: قطرات المح الدهنية، كريات المح والحبيبات القشرية. تترسب أولا قطرات المح الدهنية ثم كريات المح والحبيبات القشرية. يتكون النوع الأول (قطرات المح الدهنية) من الدهون وتتكون الحبيبات القشرية من السكريات العديدة، بينما تحتوى كريات المح على بروتينات وسكريات عديدة.

هذه الدراسة يمكن أن تمثل الأساس العلمى للتحكم الهرمونى فى تطور ونضج البويضات وتوفير المعلومات المهمة لإدارة المفرخات لتحفيز تفريخ سمكة البورى فى أنظمة الاستزراع المائى.