The oocyte development of Kutum, *Rutilus frisii kutum*, K. with special emphasis on the zona radiata structure

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Abstract

Kutum (*Rutilus frisii kutum*, Kamensky 1901) is an economically important Cyprinid species endemic to the Caspian Sea. This species is anadromous, meaning it enters rivers for spawning. In this study, the oocyte development in the developing oocyte and fertilized egg of kutum was investigated with emphasis on the zona radiata (ZR) structure by light and scanning electron microscopy. Histological features of developmental stages of oocytes were described in detail using light microscopy. The results showed that ZR was not observed in the previtellogenic phase. The ZR was observed as a simple structure between the follicular layer and oocyte membrane during the cortical alveolus stage. The study of the ZR ultrastructure showed some pore canals and microvilli at the vitellogenic stage expanded structurally as the vitellogenesis process proceeded. The ZR maximum thickness (14.9 $\pm$ 1.6 μm) and maximum length of extended microvilli (10.8 $\pm$ 1.4 μm) was achieved at the end of the vitellogenic stage. During ovarian maturation, reduction in ZR thickness and microvilli length was found to be gradual. The transformation of ZR structure, i.e., clogging of the pore canals and retraction of the microvilli occurred functionally during fertilization and later on the ZR renamed as chorion (diameter 9.6 $\pm$ 0.4 μm). The chorion on the surface of fertilized eggs possessed microvilli (8.0 $\pm$ 1.1 μm in length) and some slender processes. In general, considering the changes of ZR thickness and microvilli length, it seems that they play an important role in easier transfer of yolk materials into the oocyte. In addition, after fertilization, the chorion (transformed ZR) helps adhesion of eggs to the bottom with regard to the environment of spawning.

Keywords: cyprinid, kutum, oocyte, zona radiata.

Introduction

The Caspian Sea is the largest inland water body with no connection to others seas and oceans. About 17 families, 52 genera and 126 fish species and subspecies have been recorded in the sea and deltas of the Caspian basin in the study area. Most of them belong to the families of Carps (Cyprinidae; 33%), Gobies (Gobidae; 28%), and Shads (Clupeidae; 14%), which make up more than 75% of the species and subspecies (Afraei-Bandpeia *et al*., 2008). Caspian kutum (Cypriniformes; Cyprinidae; *Rutilus frisii kutum* Kamenskii, 1901) populations are generally distributed along the coastal regions of the south Caspian Sea.

Kutum is an economically important endemic cyprinid species that displays anadromy, annually migrating from the Caspian Sea to freshwater inlets (Anzali Lagoon and some rivers of the south Caspian Sea) in March for spawning. The fish has a group-synchronous ovary and spawns on aquatic plants or on pebbly bottoms of rivers in late April at a water temperature of 9-12°C (Paykan-Heyrati *et al*., 2007). It is a very valuable commercial fish in the southern part of the Caspian Sea and has a great demand due to its good taste and culinary customs of the local people. The catch ratio of kutum in the Iranian coasts was about 16,117.5 tons in 2006 and 2007 (www.schilat.com). Today it has been proven that the recognition of oocyte development can be a valuable mean of better understanding of reproductive biology and possibly restocking valuable kutum species (Afraei-Bandpeia *et al*., 2008).

It is generally recognized that oocyte growth occurs in three distinct phases. The primary growth phase involves primary oocyte growth concomitant with nuclear changes. The vitellogenic phase, which is gonadotropin-dependent, involves the deposition of yolk (vitellogenesis), and the third growth phase involves maturation characterized typically by resumption of meiosis, hydration, and germinal vesicle breakdown (GVBD; Tyler and Sumpter, 1996).

A typical event during the vitellogenic phase is the formation of an extracellular matrix between oocyte membrane and granulosa layer. The nomenclature used to describe this envelope is rather confusing while comparing not only different phyla, but also different teleost species (Yamagami *et al*., 1992). The terms vitelline envelope, chorion, zona pellucida and zona radiata have been used to indicate the whole egg envelope or a part of it (Oppen-Berntsen *et al*., 1990). In this paper, the term zona radiata (ZR) and chorion were used for this envelope before and after fertilization, respectively.

The ZR covering the oocyte is generally a complex extracellular matrix with pore canals filled by oocyte microvilli and follicular cell processes (Guraya, 1996; Baldacci *et al*., 2001; Rizzo *et al*., 2002).

The ZR undergoes structural and mechanical changes during oocyte development, and after fertilization it seems to play several physiological roles, ranging from prevention of polyspermy to protection of developing embryos (Hart, 1990). In general, the structure, thickness, and macromolecular composition of the ZR, as well as presence or absence of appendages on the egg cover, reflect the adaptations to different ecological conditions in which fish eggs develop (Stehr and Hawkes, 1979). The demersal eggs that are often exposed to abrasion have a thick ZR comprising two or more layers, whereas the ZR of pelagic eggs is much thinner (Guraya, 1996).

A micropyle at the animal pole of fish eggs allows the spermatozoan direct access to the plasma membrane of the egg without acrosomal reaction (Guraya, 1996). During oocyte development, the micropyle that comprises a vestibule and a micropylar canal is occluded by a micropylar cell. This association constitutes the micropylar apparatus, which usually shows a similar structure within a family or genus (Riehl and Kokoscha, 1993).

The purpose of this study was to obtain background information about oocyte development and morphology of ZR in the developing oocyte and fertilized eggs in the anadromous fish, *Rutilus frisii kutum* by using light and scanning electron microscopy. The effects of fertilization on ZR structure were also investigated.

Materials and Methods

Animals

All samples were collected approximately monthly from October 2007 to May 2008, which corresponds to the period of gonadal recrudescence and spawning. Biometric data, water temperature and the number of samples are given in Table 1 during the related study periods.

Table 1. Mean value of measured factors in kutum within the sampling period.

	Number of samples	Total length(cm)	Weight of body(g)	Temperature (ºC)	Phase	Stages of ovarian growth	Diameter (µm)	GSI (%)	ZR thickness(µm)	Microvilli length(µm)
October	8	41.1 ± 4.4	807.5 ± 91.9	21.4	Previtellogenesis	Primary oocyte	150.9 ± 56.0	2 ± 0.6	-------	-------
November	8	41.9 ± 3.4	766.2 ± 156.6	18.3	Previtellogenesis	Perinucleolus	262.3 ± 18.0	2 ± 0.9	-------	--------
December	8	44 ± 3.2	1093.6 ± 314.3	14.2	Vitellogenesis	Cortical Alveoli	475.5 ± 38.3	7 ± 2.8	5.5 ± 0.69	-------
January	9	42.2 ± 2.0	872.2 ± 139.4	12.1	Vitellogenesis	Vitellogenesis	775.5 ± 38.3	10 ± 0.3	11.8 ± 0.9	5.1 ± 0.3
February	8	46.0 ± 2.9	971.4 ± 187.0	10	Vitellogenesis	Vitellogenesis	1046.7 ± 44.2	14 ± 0.6	14.8 ± 2.1	8.9 ± 1.1
March	8	43.3 ± 2.6	737.1 ± 127.1	10.2	Vitellogenesis	Vitellogenesis	1314.3 ± 87.3	18 ± 0.9	14.9 ± 1.6	10.8 ± 1.4
April 3	8	44.2 ± 3.1	843.7 ± 196.5	11.4	Maturation	Maturation	1321.7 ± 34.3	18 ± 1.9	13.6 ± 1.2	9.3 ± 2.3
April 15	10	41.9 ± 4.9	685.5 ± 316.3	11.3	Maturation	Maturation	1435.9 ± 40.3	20 ± 0.9	12.3 ± 1.4	7.5 ± 0.8
May 1	8	44.0 ± 4.5	791.2 ± 271.2	13.5	-	Fertilized egg	1551.0 ± 65.6	-------	9.2 ± 0.4	8.0 ± 1.1

Specimens of adult kutum were captured using beach seine from the Anzali shore of the South Caspian Sea (37º 27´N, 49º 33´ E; Guilan province, Iran; Fig. 1). The freshwater specimens were captured from the Sefidrood River inlets to the Caspian Sea during their spawning migration in April and May 2008 (water temperature of 11.4–13.5°C). In addition, fertilized eggs were provided by Shahid Ansari Cyprinid Fish Complex, Rasht, Iran, where artificial breeding of kutum is practiced.

Histological preparation

The fish were killed by decapitation and the ovaries were dissected and weighed. Gonadosomatic index (GSI) was calculated as a percentage of the ovarian weight relative to the body weight. To prepare histological sections, small pieces of left ovary were fixed in Bouin's solution (8–12 h), embedded in paraffin after dehydration and infiltration and sectioned at 5–6 µm. Sections were stained with standard hematoxylin-eosin procedure (H&E). Mounted slides were examined using binocular light microscopy.

Scanning electron microscopy and morphometry

Histological sections were prepared for scanning electron microscopy (SEM) by a new method called histoscan for better examination of the ZR and egg chorion.

The objects to be scanned by SEM are usually treated first by glutaraldehyde and later by osmium

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tetroxide fixative using phosphate buffer. It is a time consuming procedure to prepare the objects of unknown details. We offer a new reliable method by which a thoroughly studied histological preparation could give hints to expertise sections of formerly light microscopic

investigated tissues. Including the cost and time saving advantages, this method also allows internal details of any cellular entity (not only the surface) to be revealed. Such ability for a scanner to study an organ, tissue or cell such as egg has not been reported or published.
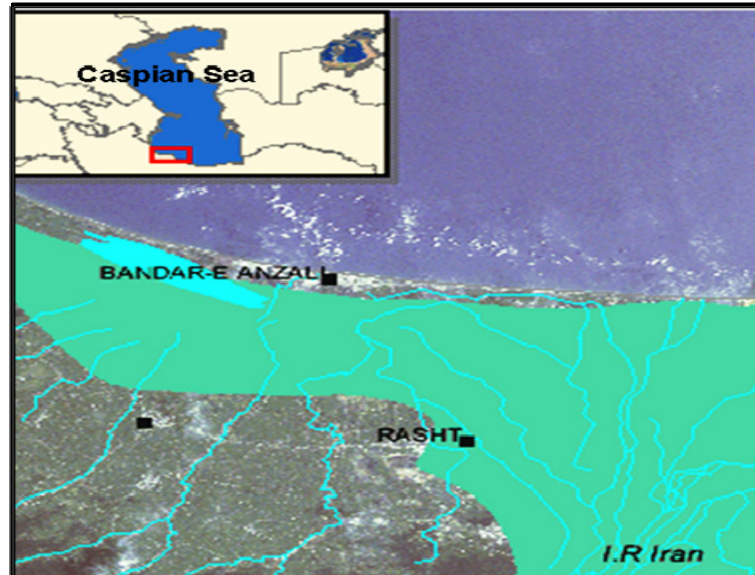

Figure 1. Sampling area of this study (Southern Caspian sea and its rivers from: http://ipieca.unep-wcmc.org/imaps/ipieca/caspian/ viewer.htm).

HistoScan method

Paraffin sections of unknown tissues were mounted on a cover glass of 1×1 cm which was earlier coated by albumin tissue gum. They were allowed to dry and then were over washed many times first by xylene and later by absolute alcohol until sections were free of first paraffin and then xylene. Cover glass containing tissue sections were mounted on stubs and coated with gold and then studied by SEM (LEO 1430VP).

Statistical analysis

The oocyte and egg diameter and thickness of the ZR were measured with an ocular micrometer in randomly selected regions of at least 25 to 40 oocytes and mean values were calculated. Digital SEM images were used for morphometric analyses of ZR during oocyte development. The software LEO Serv-32 installed on SEM was used to measure microvilli length and pores of the chorion.

Results

Based on the histological observation of the ZR structure, stages of oocyte development were classified as previtellogenic, vitellogenic, and maturational phases.

Ultrastructural comparison between the ZR of

these oocytes and that of recently fertilized eggs are described as follows:

Previtellogenic phase

This phase can be divided into two stages, namely the primary oocyte and the perinucleolus. In the primary oocyte stage, small oocytes (150.9 ± 56.0 μm in diameter) containing intense basophilic ooplasm showed a high nucleus-cytoplasm ratio. A few large peripheral nucleoli were also observed (Fig. 2a).

Progress in growth of oocytes and in the perinucleolus stage was characterized by several smaller nucleoli neatly arranged on the inner side of nuclear envelop (Fig. 2b). Size increment of these cells (262.3 ± 18.0 μm) was accompanied by the cells becoming spherical and showing gradual tendency from Hematoxylin to Eosin. Follicular layer existed but ZR was not observed in this phase.

Vitellogenic phase

This phase involves two stages: the cortical alveolus and the vitellogenic stages. The cortical alveoli appeared initially at the peripheral zone of the ooplasm and gradually progressed towards the center of the oocyte. The simple structure of ZR was observed under light microscopy for the first time in this stage (Fig. 2c). The oocyte diameter reached 475.5 ± 38.3 μm. At the commencement of vitellogenic stage, yolk globules

characterized the oocytes (diameter 1046.7 ± 44.2 µm). With progress of vitellogenesis, both the size (1314.3 ± 87.3µm) and accumulation of yolk bodies increased remarkably (Fig. 2d). Germinal vesicle migration to the animal pole was started at the late stage (Fig. 2d).
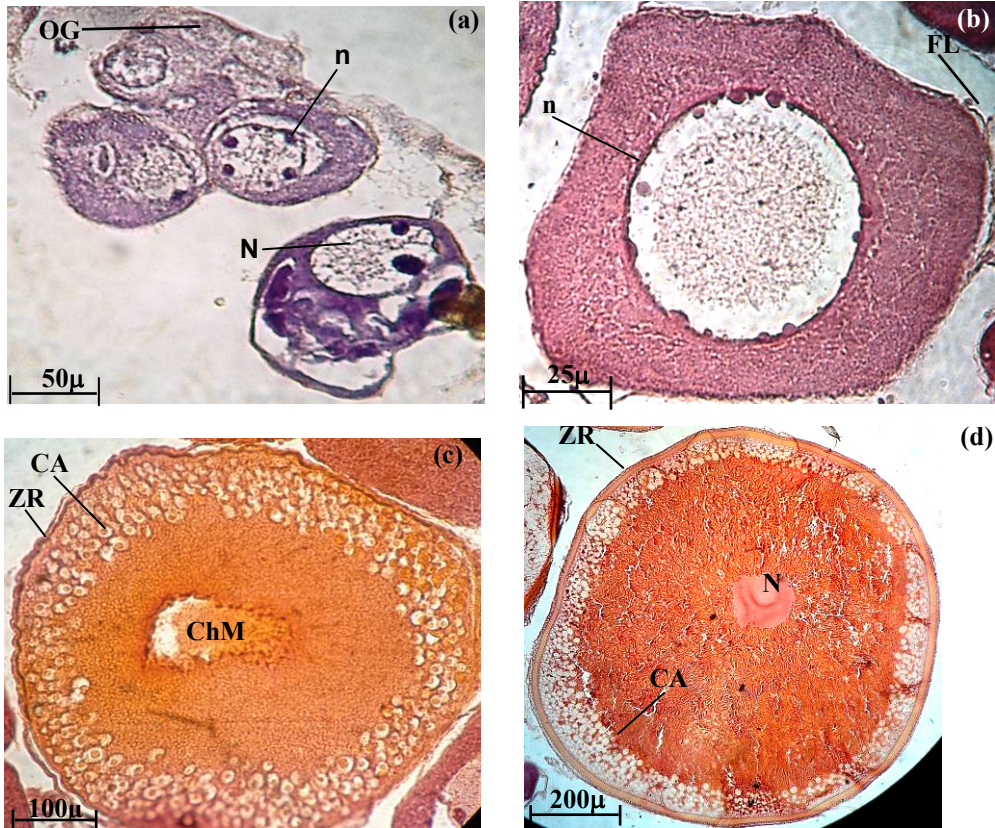

Figure 2. Photomicrographs of the oocyte at different developmental stages. (a) Primary oocyte. (b) Oocyte at the perinucleolus stage. The nucleoli (*n*) located on the inner side of nuclear envelop. (c) Oocyte at the cortical alveolus (CA) stage. The first appearance of the ZR occurred at this stage. (d) Oocyte at the advanced vitellogenic stage. The bulk of yolk bodies and migration of nucleus (*N*) toward the animal pole were obvious. *OG,* oogonia; *ChM,* chromatin materials; *FL,* follicular layer.

The ultrastructural photomicrographs at early vitellogenesis showed microvilli (length 5.1 ± 0.3 µm) that were observed from the ZR (diameter 11.8 ± 0.9 µm) toward the follicular layer. The most prominent feature of the ZR ultrastructure was the presence of numerous pore canals (Fig. 3a) which created the striated appearance of the ZR under the light microscopy (Fig. 3b). With further development of the oocyte, both the ZR thickness and microvilli length increased (14.8 ± 2.1 and 8.9 ± 1.1 µm respectively). This trend continued until the end of vitellogenesis where most of the ZR thickness (14.9 ± 1.6 µm) was acquired. In addition, microvilli in the form of finger projections with the longest length (10.7 ± 1.4 µm) were determined (Fig. 3c).

Maturational phase

This phase, which includes two stages (early and final maturation), began with anadromic migration in April when the fish entered rivers. The oocytes matured, follicles ruptured, and the fish spawned in the late April. Highest mean values for GSI (20 ± 0.9%) and oocyte diameter (1321.7 ± 34.3 µm) were observed in this phase.

At the early maturational stage, coalescence of yolk globules, completion of the germinal vesicle migration to the animal pole and appearance of micropylar vestibule were observed (Fig. 3d) but GVBD and formation of micropylar canal occurred only at the final maturation (Fig. 3e).

Ultrastructural study showed that ZR thickness with microvilli length decreased during this phase (Table 1). This trend continued up to the final maturational stage when ZR thickness and microvilli length reached their minimum values (12.3 ± 1.4 and 7.5 ± 0.8 µm, respectively; Fig. 3f).

Fertilized egg

The fertilized eggs possessed a diameter of 1551.0 ± 65.6 μm. Formation of spherical bodies and perivitelline space occurred in the spawned oocyte exactly before fertilization (Fig. 4a). These changes, including disappearance of the pore canals, were followed by the transformation of the ZR into the chorion layer after fertilization (Fig. 4b). In addition, appendages on the egg surface were formed after fertilization (Fig. 4b, c, d). The length of appendages, distance between them and diameter of chorionic pores (Fig. 4d) present on the egg surface were 8.0 ± 1.1 μm, 1.8 ± 0.1 μm and 157.5 ± 20.1 nm, respectively.
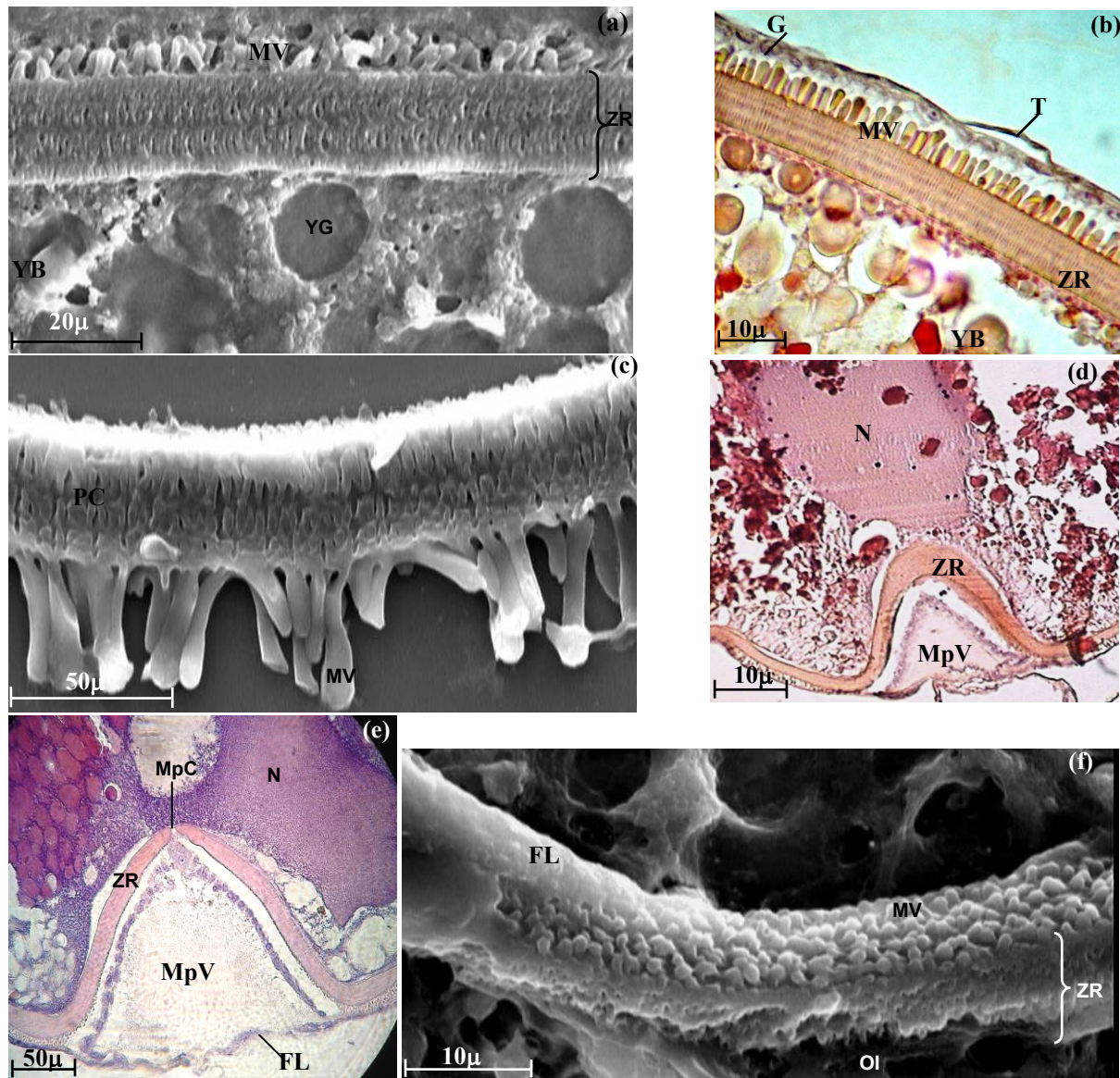

Figure. 3. The ZR structure of kutum oocyte in different developmental stages. (a) SEM of oocyte at the vitellogenic stage. (b) Oocyte at the vitellogenic stage. Striated appearance of ZR, follicular layers and microvilli (*MV*) were observed. (c) SEM of ZR during vitellogenesis in which the thickest ZR and the longest microvilli (*MV*) at the end of vitellogenesis were measured. (d) Oocyte at the early maturational stage in which completion of nucleus (*N*) migration toward the animal pole occurred where micropylar vestibule (*MpV*) existed. (e) Oocyte at the final maturational stage. GVBD and appearance of micropylar canal (*MpC*) occurred at the final maturation. (f) SEM of oocyte at the final maturational stage in which the minimum value of the ZR and microvilli (*MV*) were measured. *FL,* follicular layer; *OI,* oocyte inner; *YB,* yolk body; *PC,* pore canals; *n,* nucleolus; *T,* theca; *G,* granulosa; *YG,* yolk globule.
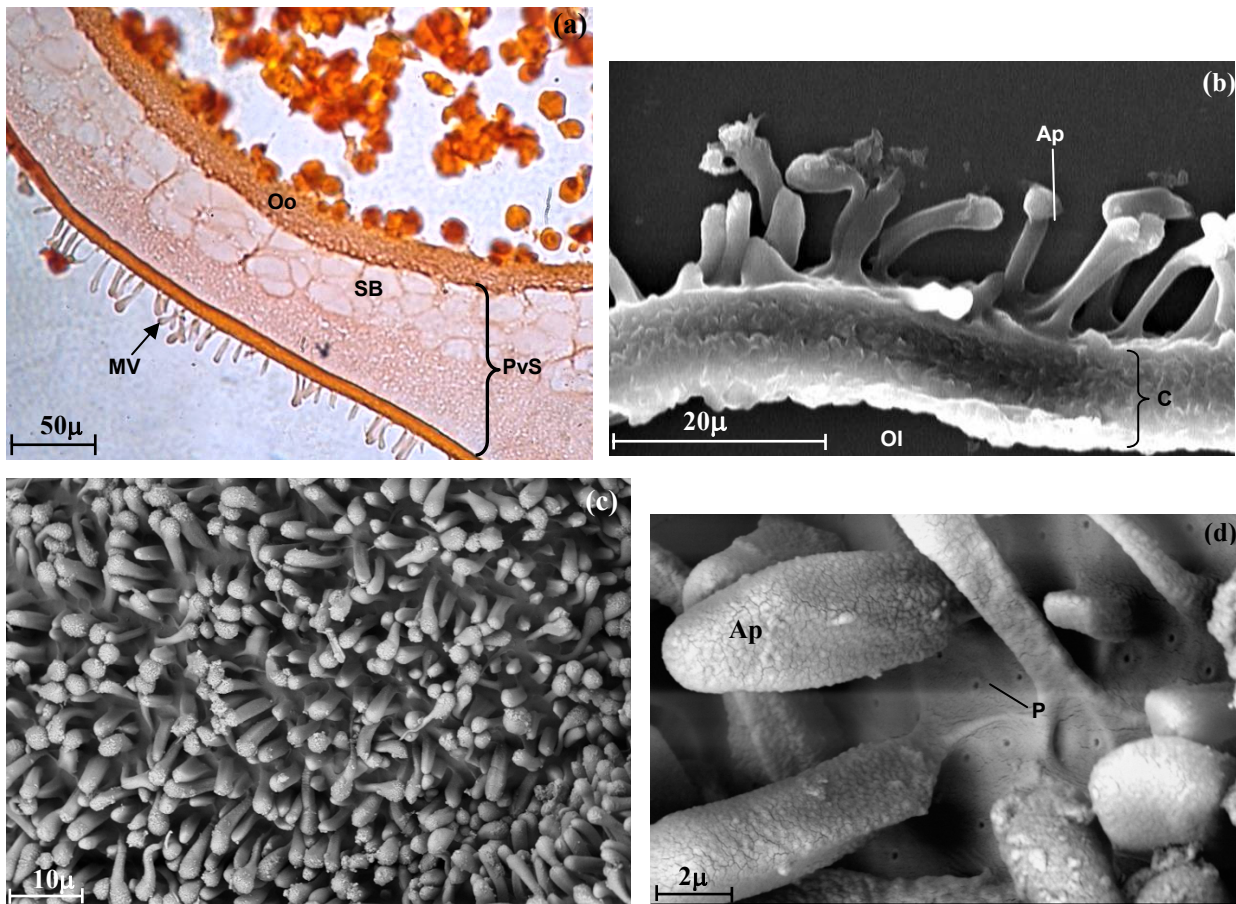
Figure 4. Events occurring in the spawned oocyte before fertilization (a) and structure of chorion after fertilization (b-d). (a) Formation of spherical body *(SB)* and perivitelline space (*PvS*) exactly before fertilization occurred. (b) SEM of egg chorion (*Ch*) after fertilization. The pore canals were not present in the egg chorion. (c) Condensed density of appendages (Ap) on the egg surface (SEM). (d) SEM of egg surface in which the pores (*P*) and appendages (Ap) were evident. *Oo,* oolema; *OI,* oocyte inner.

Discussion

In this study, the morphological changes of the ZR during previtellogenic, vitellogenic and maturational phases of the oocyte development as well as transformation of the ZR into the chorion layer in *Rutilus frisii kutum* were investigated.

Ovarian follicular growth is the result of complex processes of hormonally controlled development and differentiation, which involves cellular and molecular changes of the oocyte, ooplasmic components, egg envelopes and somatic cells (Guraya, 1996). Oocyte development of kutum is almost similar to other fish such as trahiras species (Gomes *et al*., 2007), zebrafish (*Danio rerio*; Maack and Segner, 2003), pearl mullet *(Chalcalburnus tarichi*; Unal *et al*., 2007) and common carp (*Cyprinus carpio*; Gupta, 1975) having the demersal eggs. Unlike kutum, the pelagic eggs of marine fish such as golden grey mullet (*Liza aurata*; Heidari *et al*., 2009) contain oil droplets that are essential for their buoyancy. It seems that the main

difference between benthophil fish (producing demersal eggs) and pelagophil fish (producing pelagic eggs) is related to the presence or absence of oil droplet(s).

Depending on fish species, ZR appears in different developmental stages. Ravaglia and Maggese (2002) studied the ultrastructure of ZR in *Synbranchus marmoratus* and reported it in the late perinucleolar stage. The ZR became thicker as the oocyte reached the vitellogenic stage. The ultrastructural studies of a lentic piscivorous teleost *Serrasalmus spilopleura* showed that ZR was present during late previtellogenic stage (Guimarães and Quagio-Grassiotto, 2001). In *Oligosarcus hepsetus*, the ZR was detected primarily in the yolk vesicle phase, in which it appeared as a thin and inconspicuous layer between the follicular cell layer and the oocyte membrane (Santos *et al*., 2005). ZR of *Oreochromis niloticus* oocyte was observed as a single layer at the cortical alveolus stage, becoming thicker during the vitellogenic stage (Srijunngam *et al*., 2005). In previtellogenic phase of kutum oocyte as in most other teleosts (Begovac and Wallace, 1988), only

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follicular layer could be observed with no trace of ZR. The ZR was first evident when kutum oocyte entered the cortical alveolus stage and it became thicker along with progress of oocyte development and yolk deposition. It is therefore assumed that ZR has no functional features at early oocyte developmental stages.

The microvilli structures interposed among the pore canals of the ZR were observed in *Iksookimia longicorpus* (Park and Kim, 2001), *Hoplias lacerdae* (Rizzo *et al*., 2002), *Hoplias malabaricus* and *Hoplerythrinus unitaeniatus* (Gomes *et al*., 2007). In kutum, the microvilli began to form on the oocyte surface at the beginning of vitellogenic stage. The microvilli that passed the pore canals concomitant with increment of ZR thickness were developed in the form of finger like projections. It seems that there is close relationship between the vitellogenesis and the ZR structure, including microvillar projections in kutum oocyte. It is probable that longer microvilli provided extended surface to transport yolk of materials via ZR.

It is presumed that the pore canals and microvilli were not necessary to the oocyte ZR after the completion of vitellogenesis, thus the microvilli were retracted gradually and the pore canals normally closed during maturation too (Dumont and Brummet, 1980). Kayaba *et al*. (2001) reported that in the Japanese eel, *Anguilla japonica,* microvilli increased in number and length during oocyte maturation. It is supposed that in kutum, needlessness of microvilli as well as pore canals after completion of vitellogenesis result in gradually decreasing ZR thickness and shortening microvilli length during maturation until the ZR transformed to the chorion.

Some eggs of cyprinid fish such as *Rutilus rutilus* and *Vimba vimba*, obtain strong attachment extensions on the chorion after they are fertilized (Glechner *et al*., 1993; Riehl *et al*., 1993; Patzner *et al*., 1996) while other fish such as *Cyprinus carpio*, *Hypophthalmichthys molitrix* and the cardinal fish *Apogon imberbis*, apparently have partly smooth eggs (Riehl and Patzner, 1994; Breining and Britz, 2000; Lahnsteiner, 2003; Esmaeili and Johal, 2005). After fertilization, the structure of chorion can express a close relationship with fish environment. Park and Kim (2001) stated that the structure of the chorion in three-spined loaches, *Iksookimia logicorpus, I. hugowolfeldi* and *I. yongdokensis* could specify the habitats of these fish. The first of these species having chorion with villi, inhabited pebble bottoms with rapidly flowing water, the second having chorion with short thin villi lived on pebble or sand bottoms with moderately flowing water and the third having chorion with round granules inhabited mainly sand bottoms with slow-flowing or stagnant water. The demersal eggs comparing with pelagic ones which often subjected to abrasive forces, generally develop thick chorion with complex lamellae (Guraya, 1996; Suzuki *et al*., 2000; Brandao *et al*., 2003). The thicker chorion can provide a mechanic

protection against abrasion of the bottom, as noted in *Salmo gairdneri* (Nagahama, 1983). Regarding the reproductive ecology of kutum, i.e. attachment of kutum eggs to the substrate and spawning in the rivers with moderate flowing water, its chorion was almost thick and possessed appendages for attachment.

The type of micropyle is one of the useful characters for identification of fish eggs. Micropyles in the freshwater fish have been differentiated into four types: Type I micropyles with a deep micropylar pit or funnel-like depression (vestibule) on the surface oocyte and short micropylar canal; Type II micropyles with a shallow saucer like depression (flat pit) and a correspondingly longer canal; Type III micropyles without a pit, only with a canal; Type IV micropyles with two vestibules (outer and inner) at the egg surface and short canal (Riehl, 1993; Riehl and Kokoscha, 1993). Our observations showed that the micropyle of kutum oocyte should be classified under Type I because a deep micropylar pit and short micropylar canal were found (Fig. 3e).

In conclusion, it appears that the ZR plays a role in the vitellogenic process before fertilization, i.e., the microvilli and pore canals on the ZR helps in easier transportation of yolk materials into oocyte. The chorion of fertilized eggs undergoes changes corresponding abrasive forces of environment, develops attaching appendages to the bottom and helps the eggs to resist against the running water.

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