

Structure of Oocyte Surface in Two Korean Minnow Species, *Rhynchocypris kumgangensis* and *R. oxycephalus* (Pisces: Cyprinidae)

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Oocyte surface in two Korean minnows, *Rhynchocypris oxycephalus* and *R. kumgangensis* was examined by light and electron microscope. In two species, the development of the oocyte was similar, but the follicular layer surrounding full-grown oocyte showed an evident difference. In *R. oxycephalus*, the follicular layer at the yolk vesicle stage became bilaminar with the retention of its outer squamous cell layer and the acquisition of an inner cuboidal or round cell layer just over the zona radiata. As the oocyte grows, the cuboidal cells of the inner follicular layer began to be replaced by columnar cells. At the yolk granule stage, the columnar cells secreted mucin to their cytoplasm (adhesive materials) and then surround the entire oocyte, as bundles of fence-shaped structures. Whereas, although the follicular layer of *R. kumgangensis* had an outer squamous layer and an inner cuboidal or round cell layer at the yolk vesicles as in *R. oxycephalus*, no inner cells were more changed with the retention of its cuboidal or round cells. Finally, in *R. kumgangensis*, the adhesive materials did not occur. In Korean two minnows, the structural difference in the oocyte surface seems to be related to their habitats and spawning characteristics as well as taxonomic characters.

Key words : Oocyte structure, *Rhynchocypris*, adhesive material, zona radiata

Introduction

Genus *Rhynchocypris* belonging to family Cyprinidae (subfamily Leuciscinae) is small freshwater fishes and comprises 5 species in Korea (Kim and Park, 2002; Kim *et al.*, 2005). Among the *Rhynchocypris*, *R. oxycephalus* and *R. kumgangensis* coexisted here. Studies on these species were mostly focused on the early life history, ecology and physiology (Song and Choi, 1997; Han *et al.*, 1999; Kang and Min, 1999; Song, 2000; Baek *et al.*, 2002; Park *et al.*, 2004; Choi *et al.*, 2005). However, no egg surface structure has

been known until now in *Rhynchocypris*.

Modifications in the egg surface of various teleosts are closely related to eggs properties: the attachment of the eggs to the substratum in stream bed, and sticks or debris; the retention of water or pressure; the protection of embryo; the chorionic respiratory system; the process of water hardening (Blaxter, 1969; Wourms, 1976; Laale, 1980; Groot and Alderdice, 1985; Erickson and Pikitch, 1993; Riehl and Greven, 1993; Thiaw and Mattei, 1996; Riehl and Patzner, 1998). Also, it has been noted that the morphological differences of the adhesive material and the associated inner layers could be a useful aid in the identification of eggs from different taxa of fish (Johnson and Werner, 1986) and as well as assist in deter-

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mining the phylogenetic relationships between species (Laale, 1980; Groot and Alderdice, 1985; Riehl and Greven, 1993; Park *et al.*, 2001; Park and Kim, 2001, 2003).

Therefore, we are going to describe the structure of the egg surface and discuss the relationship between their habitats and spawning characteristics in the coexisted two species, *R. oxycephalus* and *R. kumgangensis*.

Materials and Methods

10 Females of two species, *Rhynchocypris oxycephalus* and *R. kumgangensis*, were collected from Muju-gucheon dong valley, Muju-gun, Chollabuk-do, Korea, during the spawning season, May to June, 2006. Muju-Gucheon dong valley where is the uppermost stream of the Keum River is keeping cold condition in water, compared to other rivers or streams.

After anaesthetizing with MS222 gravid specimens, their full-grown ovaries were excised and

fixed in 10% neutral buffered formaldehyde. We dehydrated these sections through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford). We deparaffinized 5 μm sections and stained them with Harris hematoxylin, Ehrlich hematoxylin, counter-stained with eosin, and Masson trichrome stain (Gurr, 1956) for general histology. Mucin of gland was demonstrated by alcian blue solution (AB) at pH 1.0 and 2.5 (Steedman, 1950; Lev and Spicer, 1964), and periodic acid-Schiff (PAS) method (Lillie and Greco, 1947).

For scanning electron microscopy (SEM), the extracted ovaries were prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. Postfixation was performed in 1% osmium tetroxide in the same buffer. The samples were dehydrated in a graded alcohol series and critical point dried in CO_2 . The dried samples were coated with gold-palladium. Normal eggs and eggs with the follicle-removed were observed with a JEOL JSM-T330A scanning electron microscope. For photographs and evaluations of the egg sur-

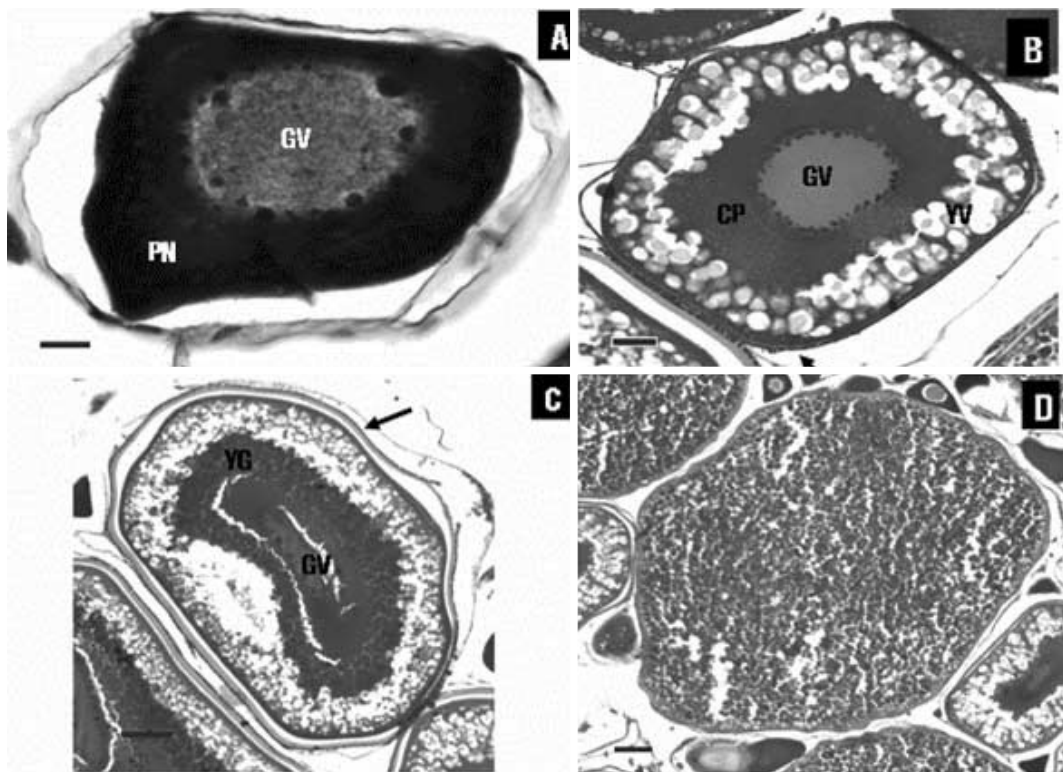


Fig. 1. Developmental stage of the oocyte of *Rhynchocypris kumgangensis* with Ehrlich haematoxylin and eosin. A, Perinucleolus stage (Bar=10 μm); B, Yolk vesicle stage (Bar=20 μm); C, Middle yolk granule stage (Bar=20 μm); D, Mature stage (Bar=20 μm). *Abbreviations*: Arrows, follicular layer; CP, cytoplasm; GV, germinal vesicle; PN, perinucleolus; YG, yolk granules; YV, yolk vesicle.

face, it was used Carl Zeiss vision (LE REL. 4.4, Germany).

Results

1. General morphology and development

The oocyte development of *Rhynchocypris oxycephalus* and *R. kumgangensis* was very similar, except for some modifications of inner follicular layer during vitellogenesis in *R. kumgangensis*. Their oocytes became developed from peri-nucleolus stage which the oocyte has nucleoli located at the periphery of the germinal vesicle (large nucleus) and was surrounded by a thin, single layer of squamous epithelial cells (Fig. 1A and Fig. 2A). As the oocyte grow, the oocyte increased

in size and its cytoplasm was occupied with yolk materials (vitellogenesis stage) (Fig. 1A to 1D). At the beginning of the vitellogenesis (early yolk vesicle stage), the follicular layer became two layers (Fig. 1B and Fig. 2B and 2E). By this time, a zona radiata was formed between the follicular layer and ooplasm. As the oocyte grow, the yolk vesicles increased in size and number, and moved to the periphery of the oocyte (late yolk vesicle stage) (Fig. 1C). At this stage, AB-PAS demonstrated that the zona radiata had two distinct layers; an outer thin zone staining strongly and an inner thicker and paler zone which stained weakly (Fig. 3D). As the vitellogenesis proceeds, the cytoplasm became occupied with many dense yolk granules which are a limiting membrane (early yolk granule stage). During later yolk gran-

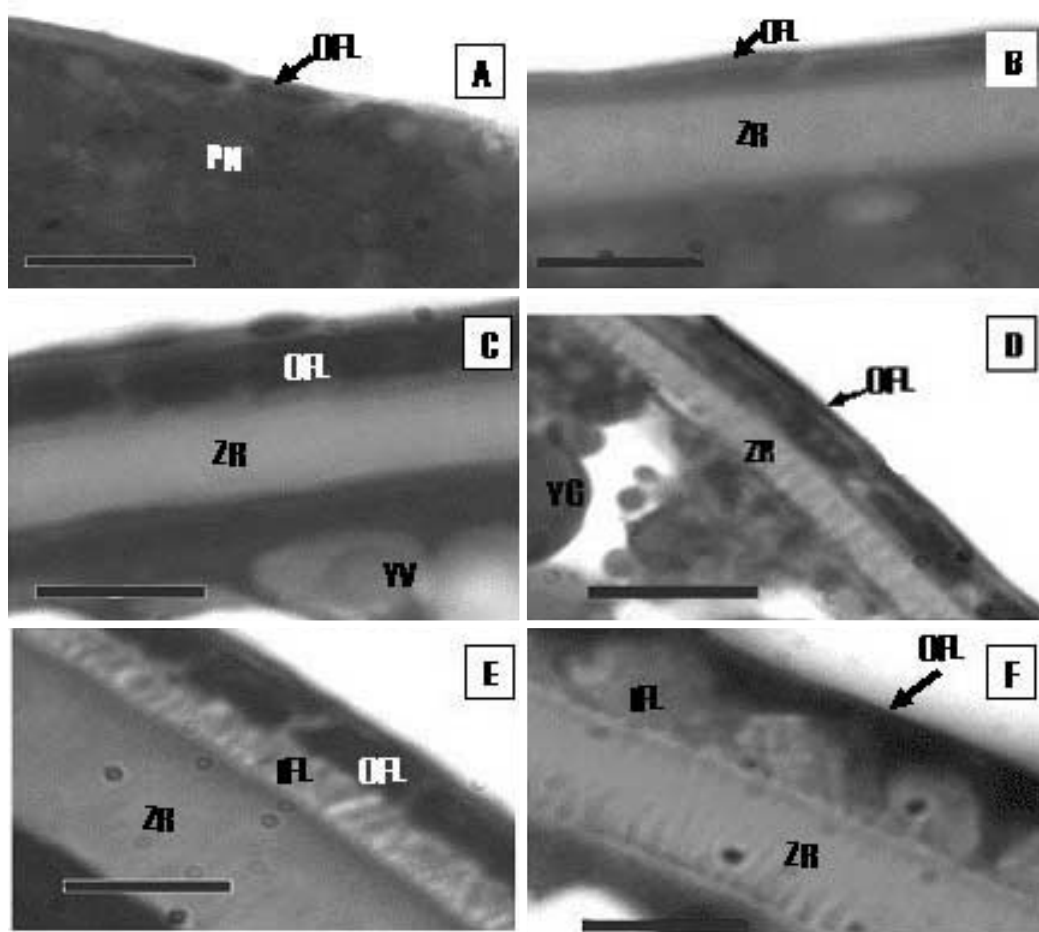


Fig. 2. Developmental stage of the oocyte surface of *Rhynchocypris* with Ehrlich haematoxylin and eosin (Bar=20 μ m). A, Peri-nucleolus stage of *Rhynchocypris kumgangensis*; B, Yolk vesicle stage of *R. kumgangensis*; C, Yolk granule stage of *R. kumgangensis*; D, Mature stage of *R. kumgangensis*; E, Yolk vesicle stage of *R. oxycephalus*; F, Yolk granule stage of *R. oxycephalus*. Abbreviations: IFL, inner follicular layer; OFL, outer follicular layer; PN, peri-nucleolus; YG, yolk granules; YV, yolk vesicle; ZR, zona radiata.

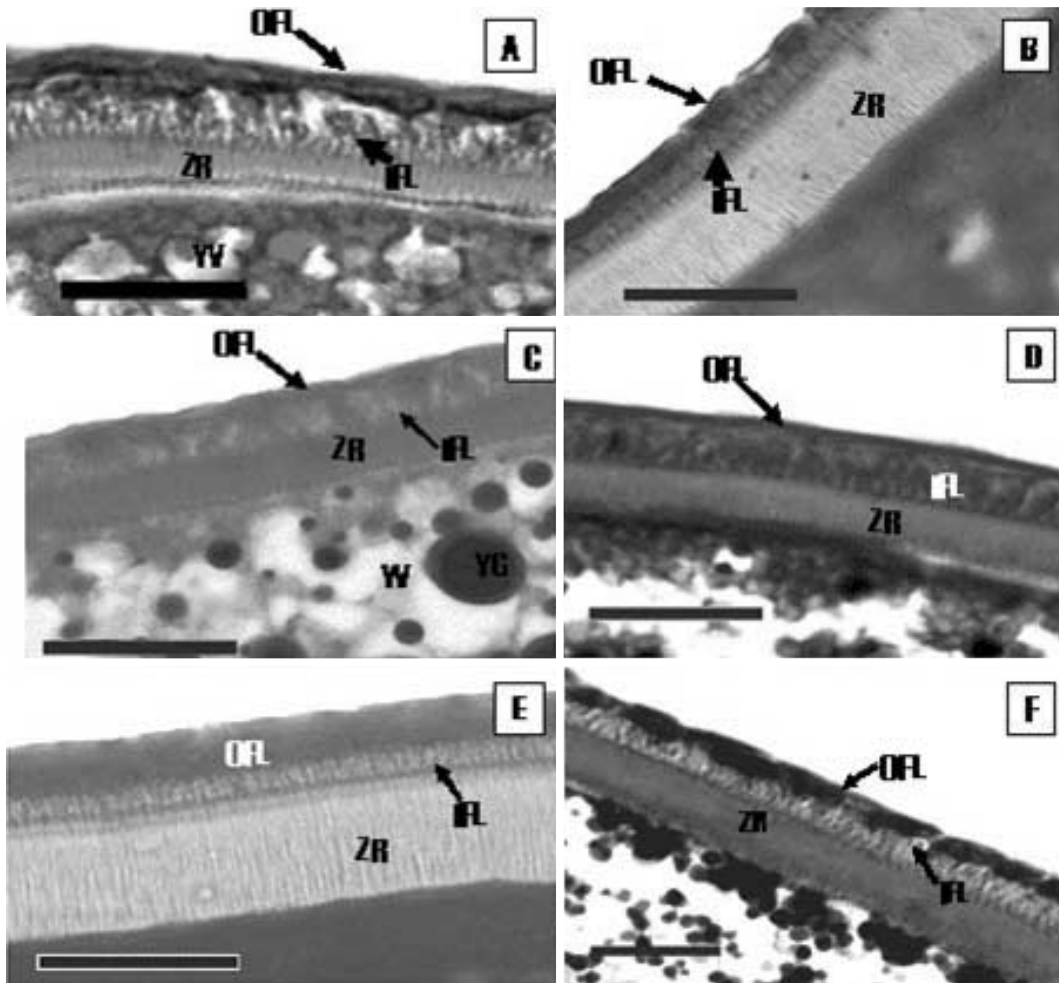


Fig. 3. Special staining reactions on the oocyte surface of *Rhynchocypris oxycephalus* at the yolk granule stage (Bar=20 μ m). A, AB (pH 1.0) reaction; B, AB (pH 2.5) reaction; C, PAS reaction; D, AB-PAS reaction; E, Toluidin blue reaction; F, Masson trichrome reaction. *Abbreviations:* IFL, inner follicular layer; OFL, outer follicular layer; YV, yolk vesicle; YG, yolk granules; ZR, zona radiata.

ule stage, the yolk granules became fused each other to form several yolk masses (Fig. 1D).

2. Structure of the egg surface

1) Observation by light microscope

R. kumgangensis

By the early yolk vesicle stage, the follicular layer had an outer squamous layer and an inner cuboidal or round cell layer at the yolk vesicles. But no inner follicular cells were more changed with the retention of its cuboidal or round cells (Fig. 2A to 2D). As the vitellogenesis proceeded, its ooplasm was displaced to many dense yolk granules. By the late yolk granule stage, the oocyte was characterized by the fusion of numer-

ous masses of yolk granules to form a single mass of yolk. At this stage, the zona radiata was 3.0~7.0 μ m thin.

R. oxycephalus

At early yolk vesicle stage, the inner follicular layer which consisted of a single cuboidal cell layer (inner follicular layer) immediately below the outer squamous layer (outer follicular layer) showed dramatic changes (Fig. 2E to 2F). At early yolk granule stage the follicular bilayer, an outer squamous cell layer and an inner cuboidal cell layer, increased in height. Some of inner cuboidal cells became columnar cell (Fig. 2E). As the oocyte grow, the follicular cells and the zona radiata increased greatly in size, thickness, height, or number. By this time, the oocyte became

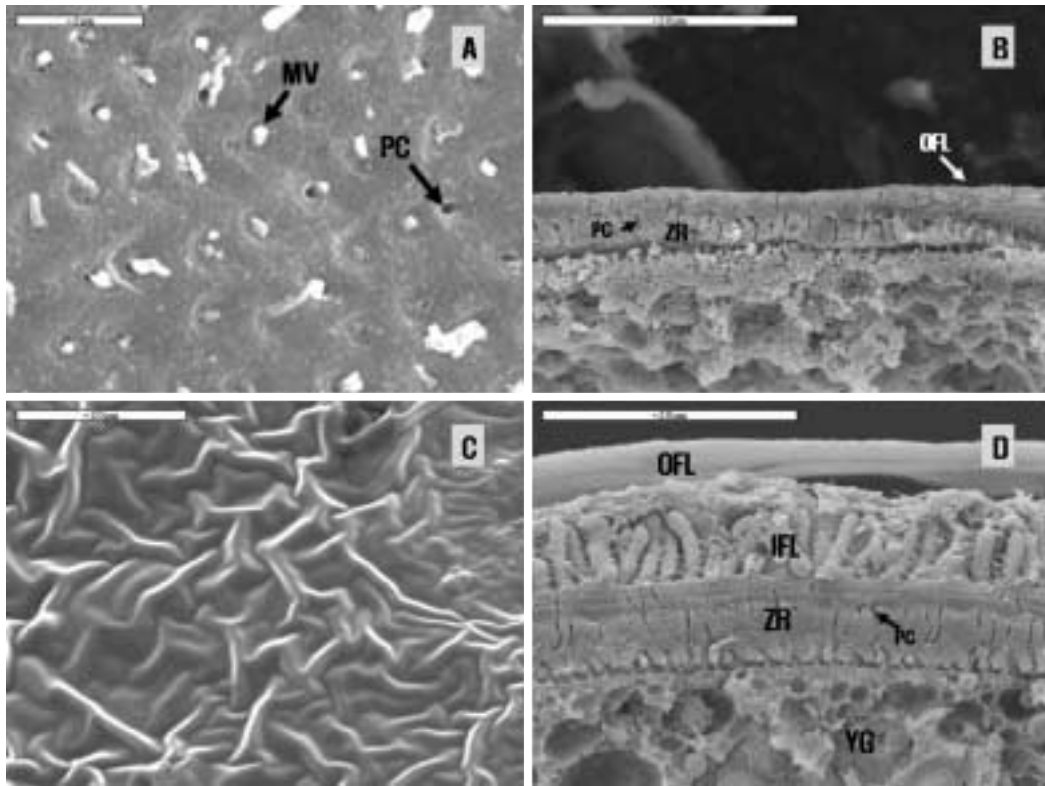


Fig. 4. Photographs of scanning electron micrograph on the oocyte surface of *Rhynchocypris* (Bar=20 μm). A, Surface structure of oocyte of *Rhynchocypris kumgangensis*; B, Fine structure of oocyte of *R. kumgangensis*; C, Surface structure of oocyte of *R. oxycephalus*; D, Fine structure of oocyte of *R. oxycephalus*. Abbreviations: IFL, inner follicular layer; OFL, outer follicular layer; MV, microvilli; PC, pore canal; YG, yolk granules; ZR, zona radiata.

larger and thicker, and also its inner follicular layer became thicker than the opposition part toward ooplasm (Fig. 2F). At later yolk granule stage, most cuboidal cells of the inner follicular layer was replaced by columnar cells filled with secretions (Fig. 3A to 3F). The inner follicular layer became thicker. By the end of this stage, subsequently the cytoplasm of the columnar cells lost their cellular integrity with top-situated nucleus and remained as fence-shaped structures, called adhesive material. These secretions were positive to AB (pH 1.0 and 2.5), AB-PAS and Toluidine blue (Fig. 3A to 3F). The thickness of the zona radiata was thicker, 7.0~8.0 μm .

2) Observation by scanning electron microscope

R. kumgangensis

Without adhesive structure, the full-grown oocyte of *R. kumgangensis* had a thin zona radiata, mean 3.0~7.0 μm , and follicular layer, mean 2~5 μm (Fig. 4A and 4B). The zona radiata showing striated appearances in light microscope had

plenty of pore canals and microvilli (Fig. 4A). The microvilli projected from the oocyte toward the follicle cell through the pore canals that traverse the zona radiata (Fig. 4B).

R. oxycephalus

With adhesive structures, the full-grown oocyte had a thicker zona radiata and follicular layer (Fig. 4C and 4D): in the thickness, the zona radiata was mean 7.0~8.0 μm and the follicular layer comprising of inner layer (adhesive material) and outer layer mean 4.0~8.0 μm (Fig. 4D). There were numerous pore canals in the zona radiata as in *R. kumgangensis* (Fig. 4D).

Discussion

Two Korean minnows, *Rhynchocypris oxycephalus* and *R. kumgangensis* are sympatric in some regions as Muju Gucheon-dong, the uppermost stream of Keum River. The two species have similar habitats which inhabit valley or the uppermost

streams of the river, and spawn in sands or on the surface of gravels during mostly April to June. Also, their eggs were known as a demersal and adhesive type (Song and Choi, 1997; Han *et al.*, 1999; Kim and Park, 2002).

However, through observation on the surface structure of the full-grown oocytes in two minnows, we could find out a new fact. The adhesive materials to attach the eggs to the substratum or form of egg clumps was not existed in *R. kumgangensis*, but occurred in *R. oxycephalus*. During vitellogenesis, the inner follicular layer of *R. kumgangensis* kept the retention of its cuboidal or round cells, but that of *R. oxycephalus* underwent a dramatic change to secrete adhesive materials. Due to this developmental difference in two species, the entire egg surface of *R. oxycephalus* became covered with bundles of fence-shaped structure. Based on this histological observation, it could be considered that the egg of *R. Kumgangensis* is demersal but non-adhesive, whereas that of *R. oxycephalus* is demersal and adhesive. As regards ecological aspect, *R. oxycephalus* with adhesive materials may be related to more turbid habitat as cool and fast current freshwater systems, compared to *R. kumgangensis*. Although closely related two species are sympatric, they must have unique environmental factors as egg deposit site, spawning habitat, or behavioral strategy by species. These adhesive structures resembled bullet-shaped structure of *Micropercops swinhonis* in appearance and development (Park *et al.*, 1998 and 2001).

In other teleost egg, these morphological characters of egg surface have been used for taxonomic perspective (Laale, 1980; Groot and Alderdice, 1985; Hirai, 1993; Britz *et al.*, 1995; Thiaw and Mattei, 1996; Kim and Park, 1996; Park and Kim, 2001, 2003; Rizzo *et al.*, 2002). In this study, the structure of the oocyte surface revealed inter-specific difference in two minnows.

By histochemical tests on the adhesive materials, the fence-shaped materials of *R. oxycephalus* was positive to AB, AB-PAS, and PAS reaction, meaning mucoprotein in nature. The nature of adhesive structure in other teleost eggs has been known as neutral mucins of primarily of mucoproteins and mucopolysaccharides, or gelatin (Yorke and McMillan, 1979; Laale, 1980; Abraham *et al.*, 1993; Riehl and Bless, 1995; Thiaw and Mattei, 1996; Park *et al.*, 1996, 2001).

Formation of various adhesive structures in teleost eggs may be produced by the following

materials: the follicular epithelium in the goby, *Pomatoschistus minutus* and some *Silurus* species (Kobayakawa, 1985; Abraham *et al.*, 1993); additional layers produced by the follicular epithelium in some *Clupea* (Gillis *et al.*, 1990); the ovarian wall in the stickleback, *Puntungia tymensis* (Riehl and Greven, 1993); a special follicular epithelium in the perch, *Perca fluviatilis* (Riehl and Greven, 1993); modification of the zona radiata in Cobitidae (Riehl and Patzner, 1998; Park and Kim, 2001, 2003). Based on our results, the adhesive materials in *R. oxycephalus* may be originated from the follicular epithelium as in *Pomatoschistus minutus* and some *Silurus* species (Kobayakawa, 1985; Abraham *et al.*, 1993).

From our histological and ecological approaches on two minnow species, it seems that the structure of the oocyte surface may be closely related to the systematic relationships as well as the evaluation of environmental factors that determine their habitats and spawning characteristics. For improving these results about the genus *Rhynchocypris*, however, broad studies on the oocyte of other minnows will be needed in future.

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금강모치와 버들치 난모세포의 표피 구조

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한국산 버들치속 (*Rhynchocypris*) 어류인 버들치 (*Rhynchocypris oxycephalus*)와 금강모치 (*Rhynchocypris kumgangensis*) 난모세포의 난막구조에 대해 광학현미경과 전자현미경으로 조사하였다. 두 종에 있어서 난형성과정은 비슷했으나 난모세포를 둘러싸는 여포세포층 (follicular layer)에 있어서는 차이를 보였다. 버들치는 난황포 (yolk vesicle)시기에 있어 여포세포층은 안쪽에 입방형 또는 둥근모양의 세포층 (inner follicular layer)이 난막위에 형성되고 그 바깥쪽으로 편평세포층 (outer follicular layer)의 2층으로 이루어져 있었다. 난모세포의 발생이 진행됨에 따라 inner follicular layer의 입방형세포는 원주형세포 (columnar cell)로 바뀌게 된다. 난황구 (yolk granule)시기에 원주형세포는 세포질에 부착물질인 mucin을 분비해서 난세포 전체를 둘러싸게 된다. 반면에 금강모치의 경우 버들치와 마찬가지로 난황포시기에 안층의 입방형 또는 둥근모양의 세포층과 바깥층의 편평세포층을 가지게 되지만 안층의 세포는 더 이상 변화를 보이지 않았으며, 부착물질 또한 형성되지 않았다. 이처럼 한국산 버들치속에 있어 난막의 구조적 차이는 두 종간에 뚜렷한 분류형질로도 이용될 수 있을 뿐 아니라 그들의 서식처 및 산란습성과도 연관이 있는 것으로 생각된다.