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Developmental Changes of the Oocyte and Its Enveloping Layers, in *Micropercops swinhonis* (Pisces: Perciformes)

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In the goby *Micropercops swinhonis*, the development of its egg's enveloping layers could be divided into 4 stages. In the earliest developmental period, stage I, there is a simple oocyte surrounded by a layer of squamous follicular cells. Stage II corresponds to the yolk vesicle stage of vitellogenesis. Here the initial follicular layer has become bilaminar with the retention of its outer squamous cell layer and the acquisition of an inner cuboidal cell layer just over the zona radiata. The number and size of the cuboidal cells increases throughout this stage. Stage III corresponds to the yolk granule stage of true vitellogenesis. Here the cuboidal cells begin to be replaced by columnar cells. As the oocyte grows, the columnar cells increase in size. The columnar cells produce cytoplasmic neutral mucins and by the end of this stage their cytoplasm has been filled with this mucin. In stage IV a single layer of squamous cells still remained as the outer follicular layer of the oocyte. The secretory activity of the inner follicular layers' columnar cells has ceased and they had lost their cell wall integrity and ended as a series of bullet-shaped, neutral mucin deposits.

Once laid, teleost fish eggs may be considered as either buoyant or demersal eggs. Many of the latter are characterised by having an outer sticky adhesive coating (Lagler et al., 1977; Mito, 1979). The ultrastructure and many patterns of the adhesive materials (adhesive coat, adhesive membrane, secondary egg envelope) have been described in many taxa (Kanoh, 1952; Honma and Tamura, 1962; Yorke and McMillan, 1979; Hart et al., 1984; Erickson and Pikitch, 1993; Riehl and Greven, 1993; Kim and Park, 1995, 1996; Park and Kim, 1996, 1997; Thiaw and Mattei, 1996). It has been noted that the morphological differences of the adhesive material and the associated inner layers are a useful aid in the identification of eggs from different taxa of fish (Johnson and Werner, 1986) and assist in determining the phylogenetic relationships between species (Laale, 1980; Groot and Alderdice, 1985; Kjesbu and Kryvi, 1989; Riehl and Greven, 1993) as well. It has been established that the primary function of the adhesive material is for the attachment of the eggs to the substratum (Blaxter, 1969; Laale, 1980; Riehl and Greven, 1990, 1993). Worum and Sheldon (1976) claimed that these layers may also function as a chronic respiratory system in the *cyprinodontid Cynopoecillus melanotaenia.*

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The goby *Micropercops swinhonis* is a small freshwater fish of the family Odontobutidae. This family consists of 4 genera and 6 species all having a relatively narrow distribution between China and Korea. In Korea, *M. swinhonis* has been reported to be restricted to the small region of Chollabuk-do (Kim et al., 1986; Kim and Kang, 1993; Kim, 1997). Kim and Kim (1996) detailed their breeding habitats, as well as commented on their spawning behaviour and early embryonic development. Details of the structure of the outer-most egg layer including the adhesive material may give an indication of their taxonomic position. In addition to this, the information would assist in the evaluation of environmental factors that determine their habitat and spawning characteristics.

Therefore, we describe the development of the outer egg layer as well as the architectural structure of the adhesive material. We also comment on the relationship between their habitat and adhesive structures in this species.

Material and Methods

Females of *M. swinhonis* were collected from Chong-Ho reservoir as well as from small streams in Puan-gun, Chollabuk-do in Korea throughout 1996 including the spawning season. Ovaries holding immature and mature eggs were dissected from the abdomen.

Tissues for light microscopy were fixed in 10%

neutral buffered formalin. These were dehydrated through a standard ethanol series to 100%, cleared in chloroform and then embedded in wax (Paraplast, Oxford). Blocks were sectioned at 5 μm. Sections were deparaffinized and stained using standard techniques. Stains used to describe and characterize the adhesive layers were Harris haematoxylin and eosin (H&E), alcian blue-periodic acid Schiff's (AB-PAS, acid and neutral mucins), peripheral nerve stain (Spoelstra, unpublished), and Von Kossa's method (divalent cations notably calcium). Egg samples for lipid examination were frozen by direct immersion in liquid nitrogen and cut on the freezing-bar of an Ames Cryostat. Sections were 8-10 μm thick and stained with Oil-Red O. A calibrated eyepiece micrometer was used to measure all layer thicknesses. The nomenclature used follows Nagahama (1983).

Results

The development of the layers enveloping the oocyte in *M. swinhonis* occurs in 4 stages according to the stage of oocyte development as defined cytologically by its size, the appearance of its nucleus and nucleolus, as well as the type and localization of its cytoplasmic inclusions.

Stage I

Initially this stage was characterised by a conspicuous nucleolus associated with chromatin threads (chromatin-nucleolus stage) within the nucleus of the oocyte. No follicular layer existed. As the oocyte grew, its germinal vesicle (defined earlier as the nucleus) increased in size, and its nucleoli increased in number up to four, each about 1 μm in diameter. Later in this stage the nucleoli continued to proliferate, but decreased in size (0.3 μm), and became located at the periphery of the enlarging germinal vesicle (perinucleolus stage) (Fig. 1A). By this time the oocyte was surrounded by a thin, single layer of squamous epithelial cells (outer follicular layer) (Fig. 2A). Simultaneously the ooplasm became more basophilic.

Stage II

Yolk vesicles first appeared in the ooplasm during the second stage of the oocyte's rapid growth and development. Throughout this stage the germinal vesicle continued to enlarge, becoming highly irregular in shape. Here there was a dramatic increase in oocyte size (Fig. 1A). Within the acidophilic ooplasm were membrane-limited vesicles of varying size which were negative to eosin as well as to AB-PAS.

Early in this stage, the yolk vesicles lay in the periphery of the ooplasm (early yolk vesicle stage) (Fig. 1A). As the oocyte grew, the yolk vesicles increased in size and number until by the end of the stage they occupied much of the ooplasm (late yolk vesicle stage). Later, the yolk vesicles moved to the periphery of the oocyte, where they are commonly referred to as the cortical alveoli.

Another prominent feature observed during the early yolk vesicle stage was the formation of a single, thick, well-differentiated eosinophilic zone, the zona radiata, between the outer follicular layer and the oocyte (Fig. 2B). At this stage the zona radiata was between 0.2-0.5 μm in thickness. By the late yolk vesicle stage, AB-PAS demonstrated that the zona radiata had two distinct layers; an outer, thin zone (0.5 μm) staining strongly and an inner thicker and paler zone which stained weakly. The zone had thickened to a maximum of about 1.5 μm.

At the beginning of stage II, the follicular layer became bilaminar by the addition of a single, cuboidal cell layer immediately below the outer squamous layer and above the zona radiata. These cuboidal cells all had a pale cytoplasm (Fig. 2B). They had a central nucleus with granules which stained darkly with H&E, and were negative to AB-PAS. The cuboidal cells were approximately 1-2 μm in the early yolk vesicle stage and 3.5 μm by the late yolk vesicle stage. The thickness of the combined outer and inner follicular layers was 4 μm by the end of this stage. Later in this stage, large oil droplets had accumulated in the central region of the ooplasm near the germinal vesicle. Small calcium deposits lay in the ooplasm near the peripherally lying yolk vesicles. The calcium deposits were not birefringent (Fig. 1F).

Stage III

In this stage, the oocyte's germinal vesicle became less crenate and developed a smooth outline, as it began to move away from the central ooplasm. This stage was characterised by true vitellogenesis where eosinophilic yolk granules surrounded by a limiting membrane were interspersed throughout most of the ooplasm (Fig. 1A-C). In the primary yolk granule stage, the yolk granules (Fig. 1A) were very small, then as time progressed the granules began to fuse with each (secondary yolk granule stage) (Fig. 1A-C). As the oocyte continued to grow, the yolk granule fusion continued until several large yolk masses occupied most of the ooplasm (tertiary yolk granule stage) (Fig. 1D).

Over this period, the zona radiata retained its dense, thin outer layer and less dense, thicker inner layer (Figs. 1B and D). The radial striation were more evident. The thickness of the zona radiata increased from about 2.5 μm thick in the primary yolk granule stage to a maximum of 4 μm in the secondary and tertiary yolk granule stages (Figs. 2C and D).

The oocyte follicular layer retained its outer squamous cell layer and inner cuboidal cell layer (Figs. 1B, C, and 2C). The combined follicular layers were up to 5 μm in height by the end of this stage. During the primary

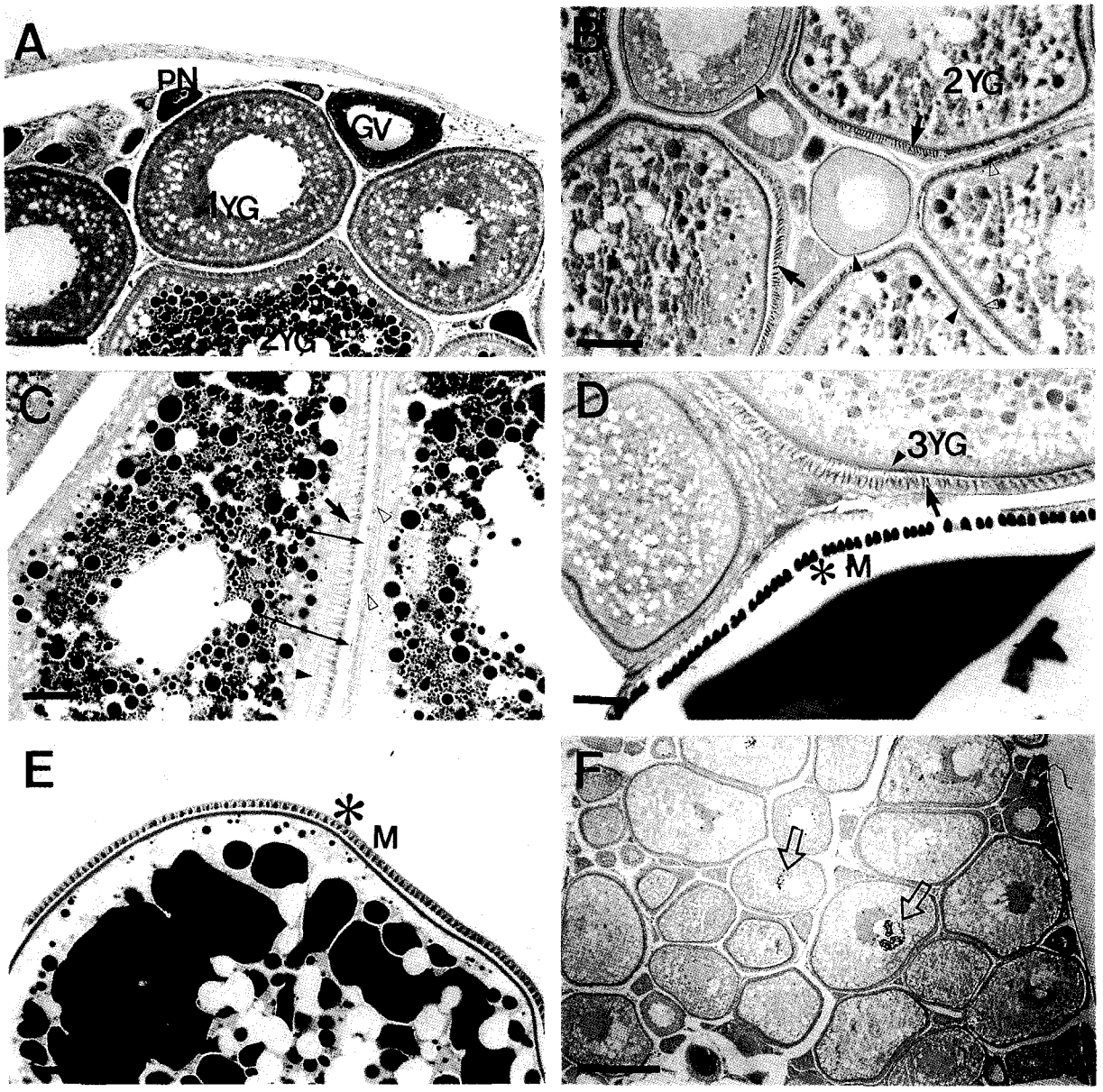

Fig. 1. Light microscopic histophotographs of the development of the oocyte and its enveloping layer of *Micropercops swinhonis*. A, Developmental oocyte (H&E stain). B, Development of zona radiata and alternative cell shapes of inner follicular layer (enveloping layer). In the secondary yolk granule stage, there were cuboidal cells and columnar cells staining positively with the combined AB-PAS. C, In the secondary yolk granule stage, the oocyte was surrounded by outer squamous follicular cell and inner cuboidal/columnar follicular layer (H & E). D-E, In tertiary yolk granule stage, the inner follicular layer consisted mostly of the columnar cell. In the mature stage, the oocyte consisted of outer squamous follicular layer and bullet-shaped structure (adhesive material) of the inner follicular layer (combined AB-PAS). F, Deposits of calcium laid in the ooplasm near the yolk vesicles or yolk granules (Von Kossa's stain). GV, germinal vesicle; M, mature oocyte; PN, perinucleolus stage; 1YG, primary yolk granule stage; 2YG, secondary yolk granule stage; 3YG, tertiary yolk granule stage; solid arrowheads, zona radiata; long arrows, flattened squamous follicular cells; short arrows, columnar follicular cells; white arrowheads, cuboidal follicular cells, white arrows, calcium deposits; *, bullet-shaped structures. Scale Bars=100 μm.

yolk granule stage, a small number of cuboidal cells became columnar. These columnar cells had a pale cytoplasm and an apically placed nucleus with nucleoli that stained darkly with H&E and negatively to AB-PAS.

In the secondary yolk granule stage, a cytoplasmic accumulation of AB-PAS positive material was first detected within the columnar cells (Fig. 1B). As the oocyte grew, the columnar cells increased greatly in size and number. The cuboidal cells were about 5-6 μm in size and the columnar cells were about 10 μm in height and between 3-5 μm in width (Fig. 1B and C). The thickness of the combined follicular layers had increased up to 11 μm. Some of the columnar cells had AB-PAS positive mucins suggesting neutral mucins in their ooplasm. No AB-PAS positive material was present in

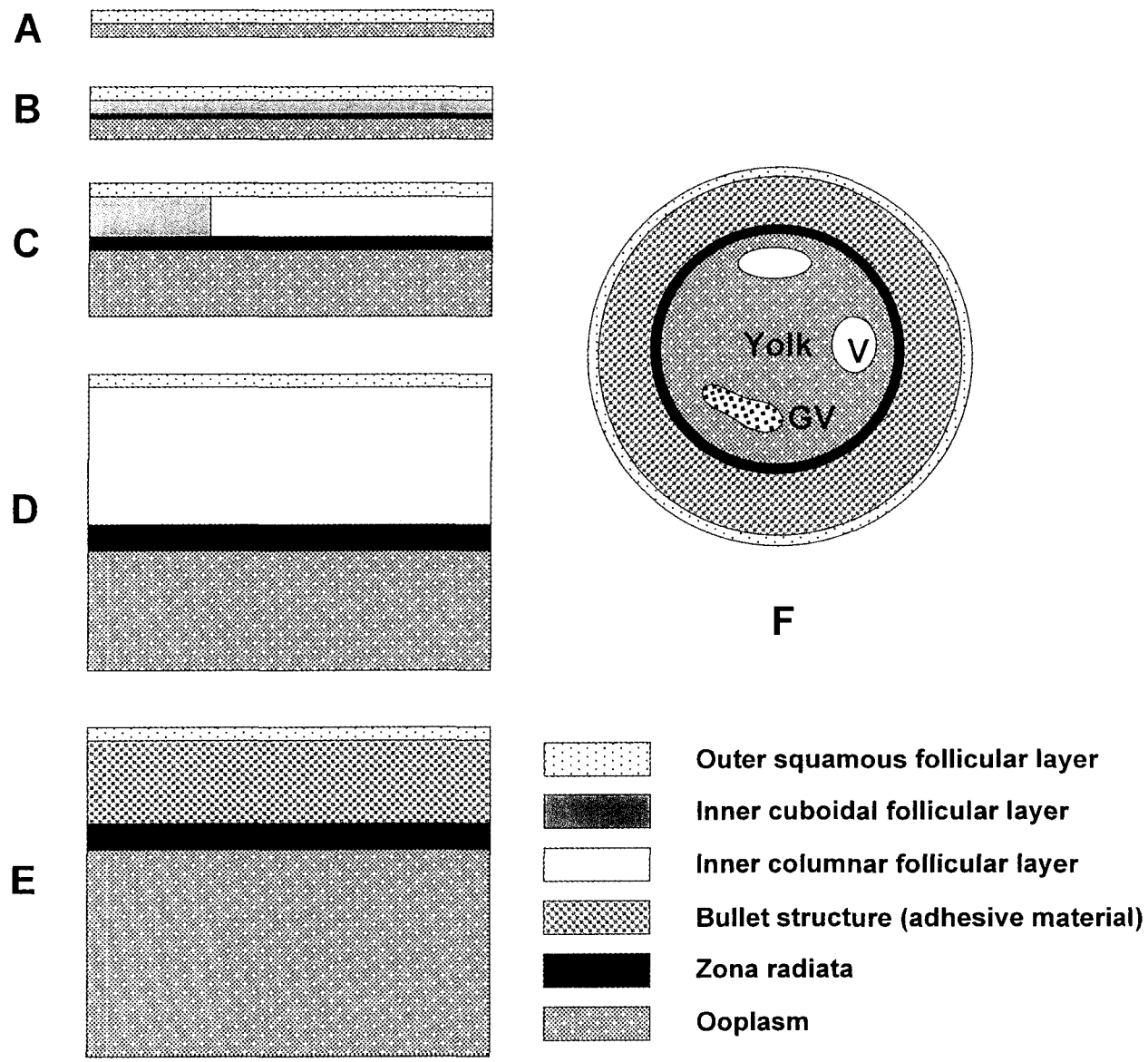

Fig. 2. Developmental schematic diagrams of the enveloping layer and oocyte of *Micropercops swinhonis*. A, Perinucleolus stage. B, Early yolk vesicle stage. C, Primary yolk granule stage. D, Tertiary yolk granule stage. E, Mature stage. F, The follicular constituents in the mature (pre-ovulation) stage of the oocyte. GV, germinal vesicle; V, yolk vesicle.

cuboidal cells.

By the tertiary yolk granule stage, most cuboidal cells had been replaced by columnar cells which had their cytoplasm virtually filled with mucins (Figs. 1D and 2D). The columnar cells had lengthened to about 20 μm. The combined follicular layer had thickened to approximately 22 μm.

During this stage large deposits of calcium lay in the ooplasm near the yolk granules (Fig. 1F).

Stage IV

The final stage (pre-ovulation stage) was characterised by the fusion of numerous masses of yolk granules to form a single mass of yolk (Figs. 1E, 2E, and F). Their germinal vesicle was difficult to detect. The zona radiata had altered from being bilaminar to becoming an acellular amorphous layer. The follicular layer still had two layers (Fig. 2E and F). The cytoplasmic mucins of the inner follicular layer columnar cells had coalesced

into basally located bullet-shaped structures (Fig. 1E). These structures were strongly positive to AB-PAS. Subsequentially, the columnar cells lost their cellular integrity and remained as bullet-shaped structures adherent to the zona radiata. The bullet-shaped structures appeared to be longitudinally differentiated as indicated by alternate light and dark staining properties along that axis. The thickness of the combined follicular layer had decreased to 12 μm.

Discussion

The oocyte follicular layer of the goby *Micropercops swinhonis* was bilaminar i.e. an outer single squamous cell layer and an inner cuboidal/columnar cell layer. The outer follicular layer described in this study has been reported as the outer thecal layer with its underlying basal lamina (Iwamatsu, 1980; Nagahama, 1983; Kobayashi and Yamamoto, 1985; Park and Kim, 1996, 1997). In other fish the inner follicular layer, initially of cuboidal cells and then ultimately replaced by columnar cells, has been referred to as the granulosa cell layer (Hurley and Fisher, 1966; Worum and Sheldon, 1976; Riehl and Greven, 1993; Abraham et al., 1993; Koya et al., 1995; Thiaw and Mattei, 1996). It has been suggested that in fish the outer squamous cell layer gives rise to the inner cuboidal/columnar layer (Riehl and Greven, 1993; Selman et al., 1993; Koya et al., 1995).

In fish, once the outer follicular layer has been discarded when the ova have been expelled into the external water mass for fertilization, the inner follicular layer becomes a critical factor in the success not of the subsequent development of the fish larvae. Many architectural patterns of the mature inner, follicular layer (adhesive membrane, adhesive material, egg envelope) have been reported ranging from "wart-like appendages" in some Pleuronichthidae (Mito, 1963), a "hexagonal pattern" in *Cynolebias melanotaenia* and *C. ladigesi* (Worum and Sheldon, 1976), polygonal ornaments in the genus *Epiplatys* (Thiaw and Mattei, 1996), "lamella structures" of Pleuronectinae (Hirai, 1993), "equidistant concentric ridges" of Perciforms (Briz et al., 1995), through to an unadorned jelly coat in *Silurus glani* L (Abraham et al., 1993) as well as in *Petromyzon marinus* (Yorke and McMillan, 1979). In the family Cobitidae the adhesive material surrounding the oocyte has been classified by external architecture into 7 types based on external appearances i.e. granule, villus, saw tooth-like, vine, undulating (hillock), fence-like, and finally a number of unadorned forms (Kim and Park, 1995, 1996; Park and Kim, 1996, 1997). A similar variety of structures have been documented in teleosts. Occasionally their morphology has been used for taxonomic purpose (Laale, 1980; Groot and Alderdice, 1985; Hirai, 1993; Britz et al., 1995; Kim and Park, 1995, 1996; Thiaw and Mattei, 1996; Park and Kim, 1996, 1997). In our study, the mature inner follicular layer (the adhesive material) in *M. swinhonis* has the appearance of bullet-shaped, membrane-bound structures covering the entire surface. This appearance is quite different to that reported previously for any teleost species. As such it may be a useful character in the determination of the phylogenetic relationships of this *M. swinhonis*.

Specific adhesive materials have been reported to be associated with particular substrates as well as with spawning conditions in many fish species (Blaxter, 1969; Laale, 1980; Riehl and Greven, 1990, 1993). In the American smelt *Osmerus mordax*, the egg has a short adhesive stalk which in the spawning season is attached to the stony bottoms of streams (Lagler et al., 1977). In the brook silverside *Labidesthes sicculus*, the egg has a single elongate filament that serves first for temporary flotation, and then for final attachment (Lagler et al., 1977). Koya et al. (1995) reported that the "down-like" layer formed on the outermost zone of the vitelline envelope of *Hexagrammos octogrammus*, functions as an adhesive membrane. Thiaw and Mattei (1996) reported that the polygonal ornaments of the egg surface in the genus *Epiplatys* may retain water and may alter the egg's buoyancy. When the ova of the sheatfish (*Silurus glanis* L.) are laid, their mucus coat swells, becomes buoyant and adhesive thus facilitating the egg's adherence to the nest wall and to each other (Abraham et al., 1993). There have been few forms of adhesive material related with their inhabiting bottoms in benthic species: Cobitidae; granular form in sands, villus form in pebbles, vine form in deep sands, unadorned, saw tooth like, form in muds, and fence like form in weeds (Kim and Park 1995, 1996; Park and Kim 1996, 1997).

In our study the adhesive materials covering the oocyte were neutral mucins primarily of mucoproteins and mucopolysaccharides. It has been reported in numerous fish species that the adhesive material consisted of several materials as mucus, mucin, and mucilage, or gelatin, and the material had adhesive properties which enables the eggs to become attached to vegetation, submerged objects, and to one another (Yorke and McMillan, 1979; Laale, 1980; Abraham et al., 1993; Thiaw and Mattei, 1996).

The zona radiata of *M. Swinhonis* demonstrates a number of forms during its development. Early in stage II, it consists of a single cellular layer, by the end of stage II has two cellular layers and by the end of the tertiary yolk granule period of stage III it has reverted to a single layer which in this case is acellular. It has been reported in the zebra fish *Brachydanio rerio* (Selman et al., 1993), that the changing morphology of the zona radiata is related to the development of the oocyte. The radial striations of the zona radiata correspond to the interdigitations of microvilli and processes from both the oocyte and the follicular cells (Hurley and Fishes, 1966; Groot and Alderdice, 1985; Park and Kim, 1996, 1997). Detailed electron microscopic

studies will be necessary to understand the dramatic changes observed in the zona radiata. Such studies may shed some light on the functional significance of this dynamic structure.

The primary habitat of *M. swinhonis* appears to be weedy patches in slowly flowing bodies of water where it has been reported that their fertilized eggs as egg masses are attached to the plant stem or the surface of pebbles (Kim et al., 1986; Kim and Kang, 1993; Kim, 1997). We believe that in *M. swinhonis*, the egg envelopes that hold the egg masses together, as well as the retention of the specific structures within the habitat to which these egg masses are adherent to, are critical to the successful, continued breeding of *M. swinhonis* in the wild.

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