

Comparative Ultrastructures of the Fertilized Egg Envelopes in *Danio rerio* and *Danio rerio* var. *frankei*, Cyprinidae, Teleostei

Kyung Bok Joo, Dong Heui Kim^{1,*}

Department of Ophthalmic Optics, Chodang University, Muan 534-701, Korea

¹Department of Environmental Medical Biology, Wonju College of Medicine, Yonsei University, Wonju 220-701, Korea

The leopard danio, *Danio rerio* var. *frankei* is a spotted color morph of the zebrafish, *Danio rerio* caused by a pigment mutation. The structural differences of fertilized egg and egg envelope are poorly documented. To clarify this, we compared the fertilized egg morphology and ultrastructures of surface structures, the micropyle and the cross section of fertilized egg envelopes of zebrafish and leopard danio, variation species of zebrafish using a light and electron microscopes. Although the fertilized egg sizes were different, the external shapes of the fertilized eggs of two species couldn't be differentiated under the light microscope. The characteristics of fertilized eggs, such as a spherical shape, a non-adhesive quality and a large perivitelline space, were shown to be related to spawning habit. In ultrastructure of fertilized egg envelope, there is no morphological difference of micropyle between two species. By contrast, the ultrastructure and the numbers of knob-like structures and semihemisphere-like structures per unit area on the outer surface, and the number of lamellae of inner layer on the fertilized egg envelope section displayed definite species specificity. Collectively, our data indicate that the ultrastructure of fertilized egg envelope in the zebrafish could be differentiated by species variation.

*Correspondence to:

Kim DH,
Tel: +82-33-741-0332
Fax: +82-33-732-4446
E-mail: fish7963@yonsei.ac.kr

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INTRODUCTION

Fertilized fish eggs are covered by an acellular egg envelope which protects the developing embryo from physical and chemical damage from the external environment and exchanges gas through diffusion (Laale, 1980; Grierson & Neville, 1981; Harvey et al., 1983; Cameron & Hunter, 1984). In addition, a micropyle in the area of the animal pole plays a role as a passageway for sperm without an acrosome and is known to work as a physical barrier to limit polyspermy (Ohta & Nashirozawa, 1996; Yoon et al., 1996).

In the fish egg, a perivitelline space is formed after fertilization following a cortical reaction on the vitelline membrane (Ohta, 1985). The formation of this perivitelline space is related to the inhibition of polyspermy and to the blastodisc formation. The liquids between the vitelline membrane and

the egg envelope protect the egg against physical impacts with the external environment (Donovan & Hart, 1986). The fertilized eggs were divided into adhesive and non-adhesive type according to species and separated them by specific gravity into the buoyant type that floats on the water, and the demersal type that sinks. In some species, adhesive eggs have accessory structures which attach the fertilized egg to the spawning bed until the egg hatches. The structures may of various shapes and function, depending on the species (Thiaw & Mattei, 1991; Kim et al., 1998a). Even eggs of the same species have been reported to have different shapes depending on their geographical distribution (Brummett & Dumont, 1981). The internal layer of the egg envelope decomposes when hatching enzymes are secreted by the embryo just prior to hatching. The thin egg envelope is then torn by the movement of the embryo, allowing hatching to occur

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(Yasumasu et al., 1989).

In fish, the morphology of the fertilized egg envelope varies according to the physical and chemical characteristics of the water environment (Lønning, 1972). Structures vary according to environmental factors such as light intensity, water pressure and wave action (Stehr & Hawkes, 1979) and according to type of spawning ground, such as rock, stone, sand or mud (Ivankov & Kurdyayeva, 1973). In addition, the structure and protein composition of the egg envelope differ according to species as well as by family (Deung et al., 1999; Kim et al., 2001, 2002). The egg envelopes of three species of Belontiidae—*Trichogaster trichopterus*, *Trichogaster leeri* and *Trichogaster trichopterus trichopterus*—have been reported to have the same morphology (Kim et al., 1999). The fertilized egg of zebrafish, *Danio rerio* is of the non-adhesive type, and the ultrastructure of the egg envelope has been reported (Hart & Donovan, 1983). The leopard danio, *D. rerio* var. *frankei* is a spotted color morph of the zebrafish caused by a pigment mutation (Meyer et al., 1993). Leopard danio had a mutation in the zebrafish connexin 41.8 gene, indicating that the mutation of connexin 41.8 gene might induce a variety of leopard spot patterns and other phenotypic defects including the fertilized egg envelope (Watanabe et al., 2006).

Their egg structures are poorly documented. To clarify this, we compared the fertilized egg morphology and ultrastructures of surface structures, the micropyle, and the cross section of fertilized egg envelopes of zebrafish and leopard danio, variation species by means of a light and electron microscopes to determine whether these fertilized eggs and egg envelopes have the same morphology or not.

MATERIALS AND METHODS

Collection of Fertilized Eggs

The fish specimens used in this study were purchased from Sanho Aquarium (Wonju, Korea). The tap water used for rearing was treated with Fritz-guard (Fritz Co., Ltd., Mesquite, TX, USA) to remove chlorine, and its temperature and pH were maintained at $25\pm 1^\circ\text{C}$ and 6.5 ± 0.5 , respectively. Biological filtration was performed using a sponge filter (Brilliant Sponge Filter; Tetra Co., Ltd., Melle, Germany), and excrement settled to the bottom of the water tank was eliminated by exchanging one-quarter of the water each day. An artificial light was illuminated for ten hours per day to simulate a daytime environment, and frozen bloodworms (Blood Worms; Hikari Sales USA Inc., Hayward, CA, USA) and Tetra Min (Tetra Co., Ltd.) were provided as food two times per day, at 9 a.m. and 5 p.m. One day prior to the collection of fertilized eggs, 10 males and 5 females of each species in a 2 to 1 ratio were put into a glass water tank (45×30×30 cm) with a net in the evening. The fertilized egg was corrected at the bottom of glass water tank after spawning

the next morning. Fertilized eggs which confirmed the formation of perivitelline space with a light microscope were measured for size (n=100) and used as samples for analysis.

Electron Microscopy

For transmission electron microscope (TEM) observation, fertilized eggs were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C . After prefixation, the specimens were washed twice in the same buffer solution and then postfixed in 1% osmium tetroxide solution in 0.1 M phosphate buffer solution (pH 7.4) for 2 h at room temperature. Specimens were dehydrated in increasing concentrations of ethanol, cleared in propylene oxide, and embedded in an Epon mixture. Ultrathin sections of Epon-embedded fertilized egg envelope were taken with an Ultracut E (Reichert-Jung, Vienna, Austria) ultramicrotome at a thickness of about 60~80 nm. Tissue sections were mounted onto collodion-coated copper grids, double stained with uranyl acetate followed by lead citrate, and observed with TEM (JEM 1200EX-II; JEOL, Tokyo, Japan) at 80 kv. For scanning electron microscope (SEM) observation, prefixation, postfixation and dehydration were conducted by following the same procedure as that for TEM. The samples were replaced with isoamyl acetate and critical point dried. The samples were coated with 20 nm gold-palladium using a ion sputter (JFC-1100; JEOL). Subsequently, the outer surface and micropyle of the fertilized egg envelope were observed with SEM (JSM 6300; JEOL).

Statistical Analysis

The statistical differences of the size of fertilized egg, the numbers of knob-like structures and semihemisphere-like structures found on the outer surfaces of the fertilized egg envelopes between zebrafish and leopard danio were analyzed with Student's t-test. A p-value<0.01 was considered to be statistically significant. All data were shown as mean±SEM.

RESULTS

External Shape of Fertilized Eggs

Fertilized eggs of both species were transparent, demersal and non-adhesive, and had large perivitelline spaces. There were no morphological differences between two species under the light microscope (Fig. 1). The sizes of the fertilized eggs were also comparable across species. The sizes of the fertilized eggs of zebrafish (*D. rerio*) were 1.19 ± 0.35 mm (n=100). Those of leopard danio (*D. rerio* var. *frankei*) were 1.14 ± 0.35 mm (n=100). There was a statistically significant difference of the size of fertilized egg between the two species (p<0.001).

Outer Surfaces of the Fertilized Egg Envelopes

In zebrafish, the outer surface was covered with fiber-

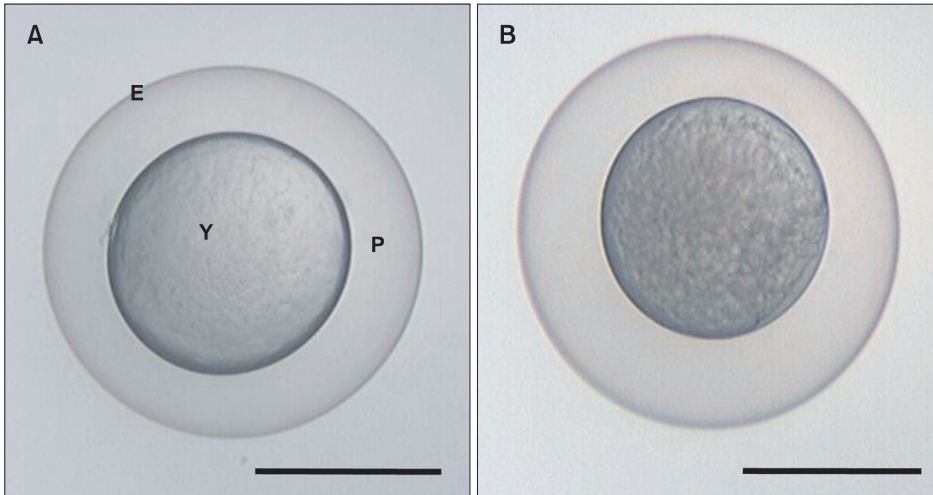


Fig. 1. (A) The fertilized egg of zebrafish. E, egg envelope; Y, yolk; P, perivitelline space. (B) The fertilized egg of leopard danio. The external shapes of the fertilized eggs of the two species of *Danio*, Cyprinidae could not be differentiated with the naked eye, nor were there differences under the light microscope (scale bar=500 µm).

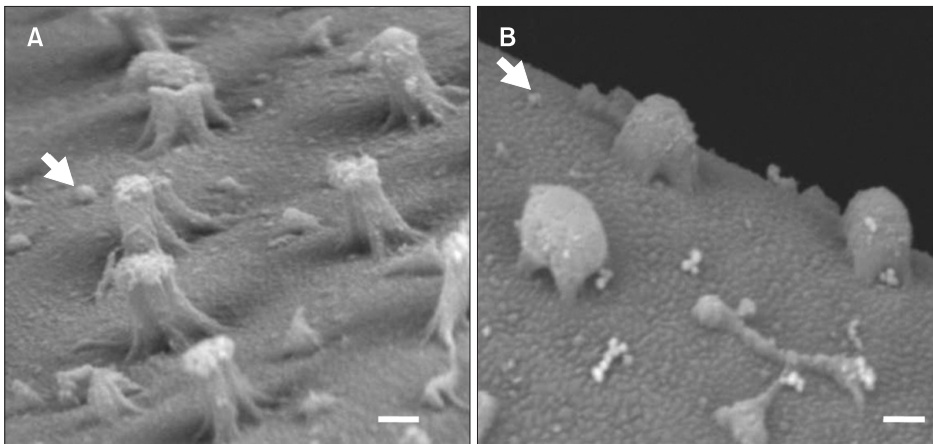


Fig. 2. (A) Outer surface of the egg envelope of zebrafish. The outer surface was covered with fiber-supported knob-like structures (scale bar=2 µm). (B) Outer surface of the egg envelope of leopard danio. The outer surface was covered with semihemisphere-like structures (scale bar=2 µm). Arrows indicate.

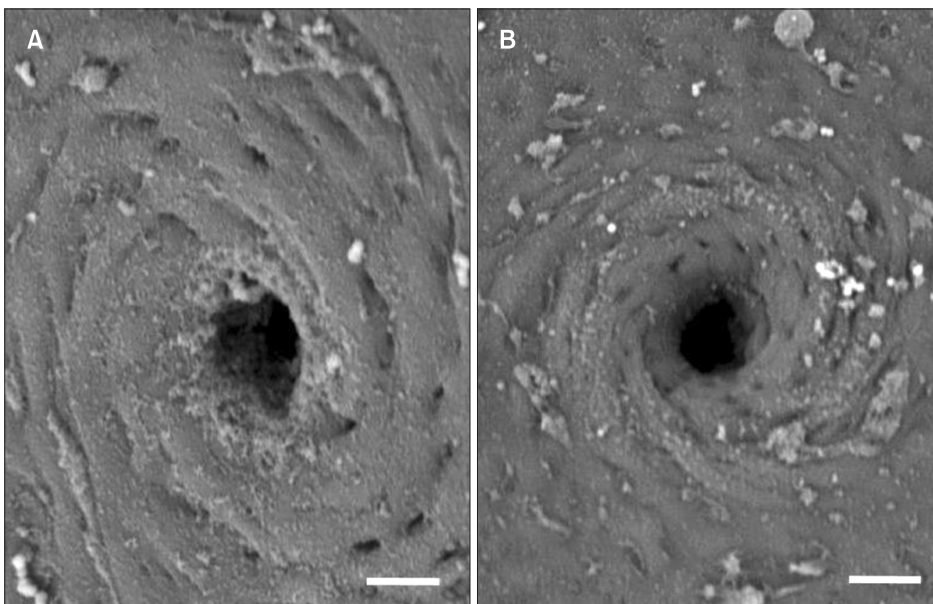


Fig. 3. Scanning electron micrograph of micropyle in zebrafish (A) and leopard danio (B). There is no ultrastructural difference between two species. Scale bar=2 µm.

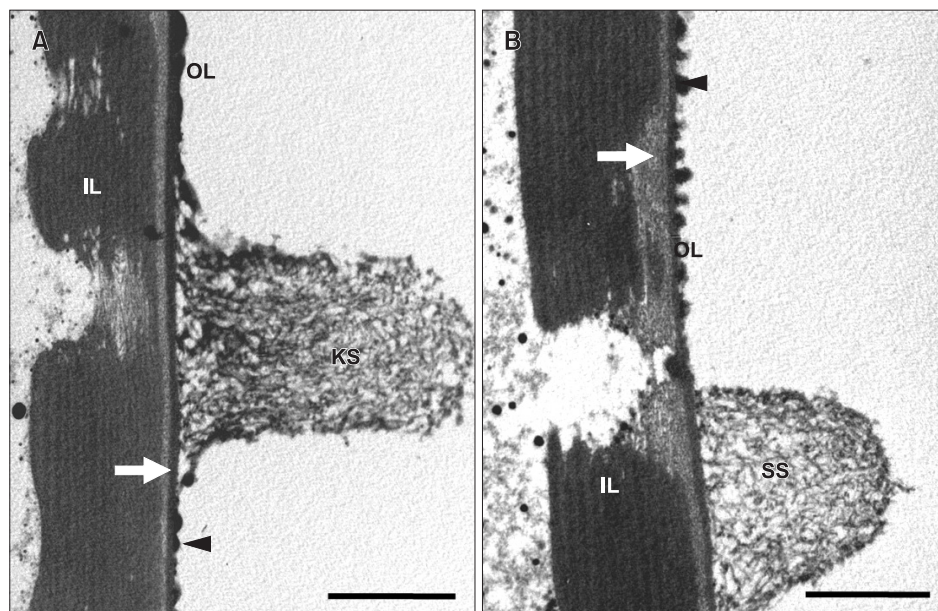


Fig. 4. Transmission electron micrograph of the section of the fertilized egg (scale bar=500 nm). (A) Zebrafish, (B) leopard danio. IL, inner layer; white arrow, middle layer; OL, outer layer; KS, knob-like structure; SS, semihemisphere-like structure; black deltoid, pore canal plug.

supported knob-like structures (Fig. 2A). These structures were observed to exist at a density of $5.1 \pm 1.4 / 100 \mu\text{m}^2$ ($n=30$), and the pore canal plugs between the structures had a density of $10.6 \pm 1.6 / 25 \mu\text{m}^2$ ($n=30$). In leopard danio, semihemisphere-like structures were found on the outer surfaces of the egg envelopes (Fig. 2B). These structures were present in a 2.9 ± 0.9 per $100 \mu\text{m}^2$ ($n=30$) concentration, and the density of pore canal plugs was 7.7 ± 1.2 per $25 \mu\text{m}^2$ ($n=30$). There were statistically significant differences of the number of both structures and pore canal plug between the two species ($p < 0.001$).

Both species had a micropyle in the area of the animal pole, and the envelope around the micropyle showed a right-handed swirl shape (Fig. 3). But, there were no morphological differences between species.

Fertilized Egg Envelope Sections

In zebrafish, the thickness of the fertilized egg envelope was about $0.53 \sim 0.60 \mu\text{m}$ except for the knob-like structures, and the egg envelope consisted of three distinct layers: an outer, electron-dense layer containing pore canal plugs; a middle fibrillar layer; and an inner zone of 16 horizontal electron-dense lamellae alternating with 15 interlamellae of lower electron density. The knob-like structures were loose and fibrous with high electron density (Fig. 4A).

The thickness of the fertilized egg envelope of leopard danio was about $0.58 \sim 0.63 \mu\text{m}$ except for the semihemisphere-like structures. Although the cross section of the egg envelope was very similar to that of zebrafish, its inner zone contained 13~15 electron-dense lamellae alternating with 12~14 interlamellae of low electron density (Fig. 4B). The cross sections of the knob-like structure of zebrafish and the

semihemisphere-like structures of leopard danio had a loose fibrous shape with low density and no interspecies variability.

DISCUSSION

In teleost, the external shapes of most fertilized eggs are oval for Cichlidae (Kim et al., 2009) and long ellipsoidal for (Kim et al., 2002) and Pomacentridae (Kim et al., 1998b). The fertilized eggs of Cichlidae are of the adhesive type and receive nearly no physical impact from the surrounding environment because it spawns by placing its ovipositor on the spawning bed. Therefore, the egg envelopes and the vitelline membranes of these species are nearly fully adhered and do not develop perivitelline spaces (Deung et al., 1997). In our study, although the fertilized egg sizes were different, the external shapes of the fertilized eggs of two species couldn't be differentiated with the light microscope. The characteristics of fertilized eggs, such as a spherical shape, a non-adhesive quality and a large perivitelline space, were shown to be related to spawning habit. Considering their egg scatter, a reduction in the physical impact with the ground after spawning is considered to play a critical role in the protection of the embryo.

The formation of a perivitelline space is known to follow expansion of the egg envelope due to osmotic pressure caused by the release of hydrophilic glycoconjugate molecules from the egg cortical layer into the egg envelope due to an increased permeability (Laale, 1980), and the formation of this space has been shown to be induced by a poke with a glass needle or by chemicals, heat, light, electricity or ultrasound in unfertilized eggs (Yamamoto, 1961), depending largely on the concentration of calcium ions and on the pH (Gilkey, 1983). Also, the formation of perivitelline space is related to

formation of blastodisc and polyspermy prevention (Donovan & Hart, 1986).

In this study, both species had process-like structures, but their shapes were different, with zebrafish having knob-like structures and leopard danio having semihemisphere-like structures. These structures were thought to be very helpful in defending against the physical impact experienced after spawning. For three spine stickleback egg envelopes of the non-adhesive type with regard to the spawning bed, knob-like structures were observed (Deung et al., 1999). *Hemibarbus longirostris* of Cyprinidae also displayed knob-like structures, although their fertilized eggs were of the adhesive type (Kim et al., 2001). Therefore, these knob-like structures or process-like structures were not considered to be a common characteristic of non-adhesive eggs.

Adhesive eggs without adhesive filaments or other accessory structure secrete the mucous mucin, mucin-like materials or gelatin on their egg envelope surfaces. For *Acerina vulgaris*, *Fundulus heteroclitus* and *Carassius auratus*, the adhesive property is known to disappear after fertilization (Laale, 1980). In *Oryzias latipes*, both adhesive and non-adhesive fibers have been observed on the egg envelope surfaces, some with a single fiber and others with a bundle of many fibers (Hart et al., 1984).

In *Lampetra fluviatilis* and *Lampetra planeri*, two-thirds of the fertilized eggs were covered by gelatin and adhesive filaments (Kille, 1960). Tomato clown anemonefish and dark sleepers have a bundle of adhesive filaments in the area of the animal pole in order to attach the fertilized egg to the spawning bed. The surface structure of the egg envelope tended to be morphologically conserved within families (Kim et al., 1999), although some inter-species differences have been observed according to region (Brummett & Dumont, 1981). For fish belonging to Cichlidae, reticular structures are distributed on the egg envelopes, with morphological differences noted according to species (Deung et al., 1997). This study found that the pore canal plugs on the egg envelope surface between structures were variably distributed. These pore canal plugs were considered to be formed by the closing of the remaining pore canals after disappearance of microvilli during formation of the egg envelope. Although the shapes of the two species were identical, the numbers were quite different ($p < 0.001$). We recorded densities of 10.6 ± 1.6 in a $25 \mu\text{m}^2$ for zebrafish and 7.7 ± 1.2 in a $25 \mu\text{m}^2$ for leopard danio.

As the sperm of teleost do not have acrosome, unlike other vertebrates (Gwo & Gwo, 1993; Kim et al., 2009; Lee et al., 2009), sperm cannot penetrate the egg envelope. Instead, fertilization occurs through a micropyle in the area of the animal pole of egg envelope. Because the internal diameter of the micropyle becomes smaller in the inner area of the egg envelope, polyspermy is limited (Kim et al., 1999).

The external ultrastructure of the micropyle varies by species.

Although there are similarities within some families (Deung et al., 1997; Kim et al., 1999), morphological differences are also observed, such as those seen in the fertilized eggs of Cyprinidae (Kim et al., 1998a). *Zacco platypus* has five specific peak-like structures surrounding the micropyle, which is not observed in other species. Therefore, these structures can be used for taxonomical identification (Deung et al., 2000). However, in the tomato clown anemonefish (*Amphiprion frenatus*) and the dark sleeper, a micropyle is not observed because the adhesive accessory structures are located in the area of the animal pole in order to hide the micropyle (Kim et al., 1998b, 2002). Head and tail light fish (*Hemigrammus ocellifer*), black tetra (*Gymnocorymbus ternetzi*) and Buenos Aires tetra (*Hemigrammus caudovittatus*) of the same family were reported to have uplifted structures arranged in radial fashion around a micropyle. The structures not observed in other families were considered to be common morphological characteristics of the Characidae (Kim et al., 1996).

Although *Squalus suckleyi* and *Lampetra japonica* belong to Chondrichthyes, they contain acrosomes without a micropyle on the egg envelope (Stanley, 1971). Some species, such as *Acipenser transmontanus*, have many micropyles and acrosomes (Cherr & Clark, 1984).

The structure of the egg envelope varies according to the species, even within a same family. The egg envelopes of the head and tail light fish and the Buenos Aires tetra, both of which belong to Characidae, consist of three layers. The envelope of the head and tail light fish has an outer layer with a low electron density, a middle layer with a high electron density and an inner zone with a lamellar structure of variable electron density. That of the Buenos Aires tetra consists of an outer layer with a high electron density, a middle layer with a low electron density and an inner zone with a lamellar structure of variable electron density. For the black tetra of Characidae, the egg envelope consists of two layers. The inner zones of the three species have different numbers of layers, with three, four and five layers in the head and tail light fish, the black tetra and the Buenos Aires tetra, respectively (Kim et al., 1996). However, Belontiidae species have the same two-layer structure: an adhesive outer layer and a saw-like inner layer (Kim et al., 1999).

In this study, the cross sections of the fertilized egg envelopes of both species were very similar morphologically. The egg envelopes consisted of three distinct layers: an outer, electron-dense layer containing pore canal plugs, a middle fibrillar layer, and an inner zone of horizontal electron-dense lamellae alternating with interlamellae of lower electron density. The middle fibrillar layer is believed to function as protection for the embryo from external physical impacts, as it helps to maintain the elasticity of the envelope. However, the number of lamellae varied, with measurements of 16 and 13~15 in zebrafish and leopard danio, respectively.

The fertilized egg envelope is known to be thicker in buoyant types than in demersal types, in oviparous than in ovoviviparous species. Although the spawning environment and habits are the same, some species have egg envelopes of different structures (Guraya, 1986). Moreover, the structure of the egg envelope varied even inside of the same family. The egg envelopes of all pink salmon, chinook salmon and chum salmon of Salmonidae consist of inner and outer layers. Only pink salmon have a single adhesive layer, while the other two species have two adhesive layers. The morphologies of the outer surface of the egg envelope and the pore canal plug are known to differ by species (Schmehl & Graham, 1987). A pore canal was reported to undergo morphological changes during development (Lønning & Davenport, 1980). In addition, various structures of the egg envelope were considered very critical for the preservation of the species under changing environmental conditions (Schmehl & Graham, 1987).

While the fertilized egg sizes were different, the external shapes of the fertilized eggs of *D. rerio* and *D. rerio* var. *frankei* could not be differentiated with the naked eye, nor were there differences under the light microscope. The characteristics of fertilized eggs, such as a spherical shape, a non-adhesive quality and a large perivitelline space, were shown to be

related to spawning habit. In the case of micropyle, we couldn't find morphological difference between two species. By contrast, the ultrastructure and the numbers of knob-like structures and semihemisphere-like structures per unit area on the outer surface, and the number of lamellae of inner layer on the fertilized egg envelope section displayed definite species specificity. Collectively, our data indicate that the ultrastructure of fertilized egg envelope in the zebrafish could be differentiated by species variation.

CONCLUSIONS

We compared the fertilized egg morphology and ultrastructures of surface structures, the micropyle and the cross section of fertilized egg envelopes of zebrafish and leopard danio, variation species of zebrafish using a light and electron microscopes. But ultrastructure and numbers of knob-like structures and semihemisphere-like structures per unit area on the outer surface, as well as the number of lamellae on the egg envelope were concluded to show species specificity. Our data indicate that the ultrastructure of fertilized egg envelope in the zebrafish could be differentiated by species variation.

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