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Regular Article

Comparative Ultrastructures of the Fertilized Egg Envelopes in *Nothobranchius foerschi* and *Nothobranchius rachovii*, Nothobranchiidae, Teleostei

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In the case of genus *Nothobranchius*, Nothobranchiidae, the morphology of fertilized eggs and ultrastructures of fertilized egg envelopes have been reported in only two fishes. Therefore it is hard for sure to these morphological characteristics show genus specificity because of lower research samples. So, we studied the morphology of fertilized egg, and compared the ultrastructures of outer surface, micropyle, and section of fertilized egg envelopes under the light and electron microscopes from the other two species, *Nothobranchius foerschi* and *Nothobranchius rachovii*, Nothobranchiidae to find out whether these structures have the species specificity or not. Both fertilized eggs were spherical, demersal and adhesive, and have a large oil droplet. The adhesive whip-like structures were distributed on the outer surface of egg envelope, and a micropyle located on the animal pole. The fertilized egg envelope consisted of two distinct layers: an outer electron-dense layer with adhesive structures and an inner lamellae layer in both species. The external shapes of fertilized egg and ultrastuctures of outer surface, micropyle, and section of fertilized egg envelope have same structure including results before. Our data indicate that these morphological characteristics of fertilized egg and fertilized egg envelope show genus *Nothobranchius* specificity.

Key Words: Fertilized egg envelope, Fertilized egg, *Nothobranchius foerschi*, *Nothobranchius rachovii*, Ultrastructure

INTRODUCTION

The foerschi killifish (*Nothobranchius foerschi* Wildekamp & Berkenkamp, 1979) found in temporary pools of upper Ruvu, Mpiji and lower Rufiji drainage, Tanzania (Hanssens & Snoeks, 2006), and Bluefin nothos (*Nothobranchius rachovii* Ahl, 1926) inhabit lower Zambezi and lower Pungwe River systems in Mozambique, also likely to occur in floodplains of other rivers between Zambezi and Pungwe Rivers (Shidlovskiy et al., 2010). These two species are an annual fish. Their natural habitats are small pools or water depressions where the water dries up annually. Before the water dries up, these fish are able to lay their eggs in mud for the next generation to be born and survive. *N. rachovii* mature in about twelve weeks live for up to a year or year and a half, and die at the end of the breeding season (Shidlovskiy et al., 2010). In such a reason, fertilized egg and egg envelopes of these species have to have a special structure to sustain the dry season.

Genus *Nothobranchius* has been studied on ambient temperature reduction (Lu & Hsu, 2015), circadian activity rhythms during the last days (Lucas-Sánchez et al., 2015), and community assembly (Reichard et al., 2017). But there were

studies on the morphology of fertilized egg or ultrastructures of fertilized egg envelope in only two species, *N. guentheri* and *N. patrizii*. In their study on the fertilized eggs and fertilized egg envelopes of *N. guentheri* and *N. patrizii*, fertilized eggs have characteristics of adhesive, spherical, yellowish and demersal, and had a large oil droplet. The whip-like structures were distributed on the outer surface of fertilized egg envelope in both species. The fertilized egg envelope consisted of two distinct layers, an outer electron-dense layer with whip-like structures and an inner lamellae layer in both species (Kwon et al., 2015). These morphological characteristics showed common traits in genus *Nothobranchius* even species were different each other. But, it is hard for sure to these morphological characteristics show genus specificity because of lower research samples. So, we studied the morphology of fertilized egg, and compared the ultrastructures of outer surface, micropyle, and section of fertilized egg envelopes under the light and electron microscopes from the other two species, *N. foerschi* and *N. rachovii*, Nothobranchiidae to find out whether these structures have the genus specificity or not.

MATERIALS AND METHODS

Collection of Fertilized Eggs

The fertilized eggs of *N. foerschi* and *N. rachovii* were purchased from Mr. Urai Wongwian (Thailand) in February, 2017. Dechlorinated water was added in peat moss including fertilized eggs, the fertilized eggs were corrected from the peat moss under stereo microscope (SMZ-168; Motic, Taiwan). Fertilized eggs were measured for size (n=20) under digital microscope (AD-7013MZT; Dino-Lite, Anmo, Taiwan) and used in this study as experimental samples.

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 ethanol, cleared in propylene oxide, and embedded in an Epon mixture. Ultrathin sections of embedded fertilized egg envelope were taken with an ultramicrotome (Ultracut E; Reichert-Jung, Austria) at a thickness of about 60 nm. The ultrathin sections were mounted onto copper grids, double stained with uranyl acetate followed by lead citrate, and observed with a TEM (JEM 1200EX-II; JEOL, Japan).

For scanning electron microscope 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 (EM CPD300; Leica, Austria). The samples were coated with Pt by ion coater (EM ACE600; Leica). Subsequently, the fertilized eggs were observed under the field emission scanning electron microscope (Merin; Carl Zeiss, Germany).

RESULTS

Morphology of Fertilized Eggs

The fertilized eggs of *N. foerschi* and *N. rachovii* were demersal, spherical, and adhesive, and have a large oil droplet. There were no morphological differences between two species under the light microscope (Fig. 1). The size of the fertilized eggs of *N. foerschi* was 0.98±0.02 mm (n=20). That of *N. rachovii* was 0.97±0.03 mm (n=20). But there was no statistical significance in the two species. The diameter of oil droplet in fertilized eggs of *N. foerschi* was about 0.35±0.01 mm (n=20) and that of *N. rachovii* was 0.36±0.02 mm (n=20). The perivitelline space was almost not developed in both species as the fertilized egg envelope adhere to vitelline membrane.

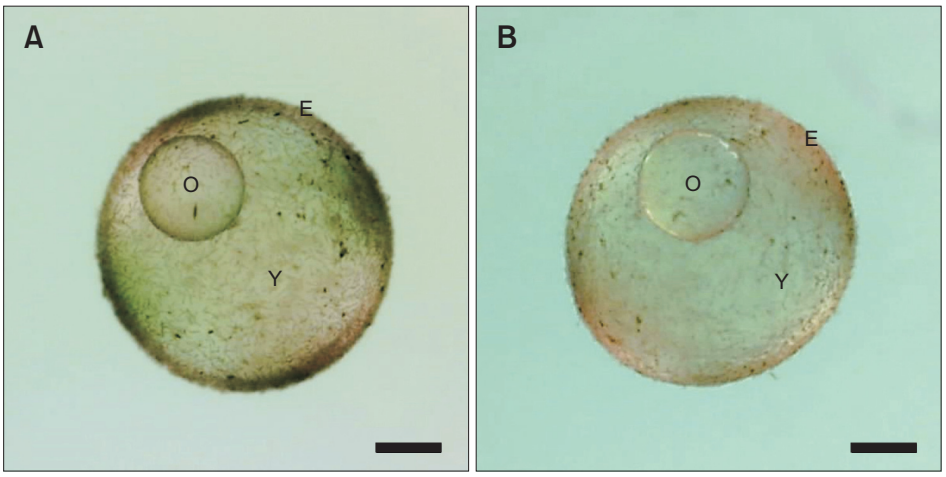

Fig. 1. Fertilized eggs of (A) *Nothobranchius foerschi* and (B) *Nothobranchius rachovii*. E, egg envelope; Y, yolk; O, oil droplet. There were no morphological differences in the two species under the light microscope (scale bar=200 μm).

Outer Surfaces of the Fertilized Egg Envelopes

In both species, micropyle was located in the animal pole of fertilized egg. It looks like a plate coral mouth. The adhesive whip-like structures were distributed on the outer surface of fertilized egg envelope and total length of adhesive structure was about 40~50 μm and adhesive structures present in 3~4 per 400 μm^2 (Fig. 2). Each adhesive structure was covered with fibrous structures (Fig. 3). There were no morphological differences between of *N. foerschi* and *N. rachovii*.

Fertilized Egg Envelope Sections

In *N. foerschi*, the thickness of the fertilized egg envelope was about 24~26 μm, and the fertilized egg envelope consisted of two layers, an outer electron-dense layer with adhesive structures and an inner lamellae layer and the inner part of adhesive structures was filled up materials with median electron density (Fig. 4A). In the *N. rachovii*, the thickness of the fertilized egg envelope, the number of fertilized egg envelope and section of adhesive structures were very similar to that of *N. foerschi* (Fig. 4B). There were no morphological differences such an external shape of fertilized egg in both species.

DISCUSSION

The fertilized egg envelope has functions as gas exchange, selective transport, protections of embryo from physical impact, chemical impact and infection during the embryogenesis and prevention of polyspermy (Cameron & Hunter, 1984; Donovan & Hart, 1986; Harvey et al., 1983).

In general, the morphology of fertilized eggs in teleost is known to differ according to the family. The fertilized eggs of fish belong to Cyprinidae, Characidae and Belontiidae are spherical (Joo & Kim, 2013; Kim et al., 1996, 1999). But fertilized egg of Cichlidae is oval (Kim et al., 2009), and fertilized eggs of fish belong to Eleotrididae and Pomacentridae are long ellipsoidal (Kim et al., 1998b, 2002).

In this study, the fertilized eggs of *N. foerschi* and *N. rachovii* were demersal, spherical, and adhesive, and have a large oil droplet such as that of *N. guentheri* and *N. patrizii* belong to same family (Kwon et al., 2015). Also, the diameters of egg and oil droplet size, color, poor development of perivitelline space and adhesive property were same to compare to that of *N. guentheri* and *N. patrizii*. Therefore these morphological characteristics of fertilized eggs showed genus specificity. The fertilized eggs of fish belong to Belontiidae have a large oil droplet, and this oil droplet plays a role to making buoyancy and nutrients source (Kim et al., 1999). The oil droplet in fertilized egg of genus *Nothobranchius* seems to be utilized as food source than buoyancy because of the fertilized egg is non-floating type.

In teleost, sperm has no acrosome. So sperm needs a sperm

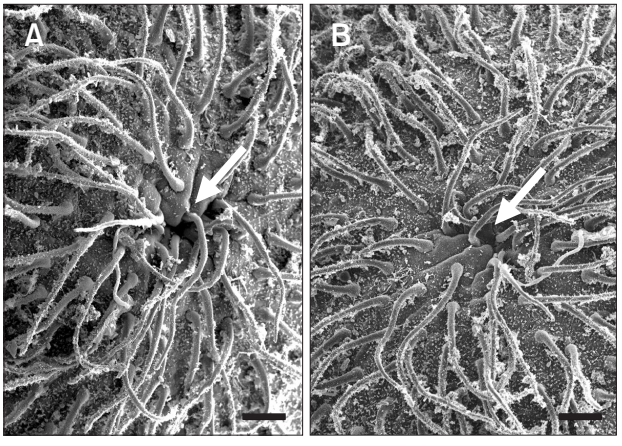

Fig. 2. Scanning electron micrographs of outer surface on the egg envelopes of (A) *Nothobranchius foerschi* and (B) *Nothobranchius rachovii* (scale bar=10 μm). The adhesive whip-like structures were distributed on the outer surface of egg envelope in all both species, and a micropyle (arrows) located on the animal pole.

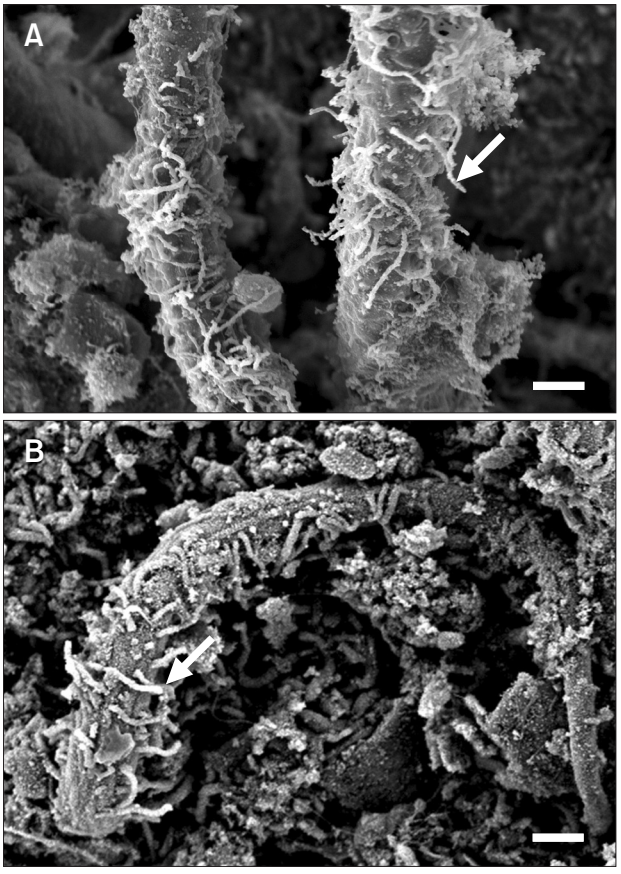

Fig. 3. Magnified adhesive structures of (A) *Nothobranchius foerschi* and (B) *Nothobranchius rachovii* (scale bar=1 μm). Fibrous structures (arrows) were distributed on the adhesive whip-like structure, and there were no morphological differences in two species.

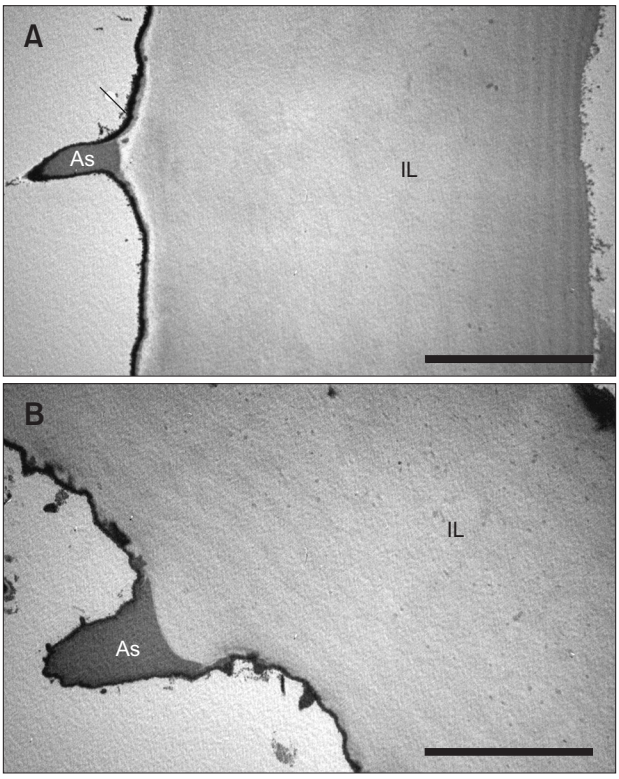

Fig. 4. Transmission electron micrographs of the fertilized egg envelopes in (A) *Nothobranchius foerschi* and (B) *Nothobranchius rachovii* (scale bar=10 μm). Arrows, outer electron dense layer; AS, adhesive structure; IL, inner layer.

entry site, micropyle on the egg envelope for fertilization. In general, the micropyle is funnel shape, but micropyles of the fertilized eggs from *N. foerschi* and *N. rachovii* are form of plate coral mouth. The morphology of this micropyle seems to be a common trait of genus *Nothobranchius*.

In our study, the adhesive whip-like structures were distributed on the outer surface of egg envelope, and size of adhesive structure was about 40~50 μm. Adhesive ship-like structures present in 3~4 per 400 μm^2 under scanning electron microscope. Each adhesive whip-like structure was covered with fibrous structures. There were no morphological difference between of *N. foerschi* and *N. rachovii*. In study on *N. guentheri* and *N. patrizii* belong to same family, ultrastructures of adhesive structures in both species are same, but these species have species specificity because of the size of whip-like structure in *N. patrizii* was smaller than that of *N. guentheri* (Kwon et al., 2015). In comparison of ultrastructure of egg envelope in both Danio rerio and Danio rerio frankei, morphology and the number per unit area of appendicular structures were showed species specificity (Joo & Kim, 2013). Even if same family, the fine structure of the fertilized egg envelope is known to showed species specificity in Salmonidae (Schmehl & Graham, 1987), Characidae (Kim et al., 1996), Cichlidae (Deung et al., 1997) and Cyprinidae (Kim et al., 1998a). However it showed family specificity in Belontiidae (Kim et al., 1999) because of the fine structures of fertilized egg envelopes are same in all three species. In this study, the sections of fertilized egg envelope of *N. foerschi* and *N. rachovii* consisted of two layers, outer electron dense layer with whip-like structure and inner lamellae layer. According to the study on the other species in same family, that of *N. guentheri* and *N. patrizii* the egg envelope consisted of two distinct layers, an outer electron-dense layer containing adhesive structures and an inner layer of horizontal electron-dense lamellae alternating with interlamellae of lower electron density. The adhesive structures filled up with electron dense (Kwon et al., 2015). These ultrastructural characteristics including thin outer layer and thick inner layer, electron dense adhesive structures, and distorted micropyle may be referable to special life cycle to sustain the dry environment. Also, we suggest that these ultrastructures of fertilized egg envelope section are same in 4 species belong to genus *Nothobranchius*. Collectively, the external shapes of fertilized egg and ultrastuctures of outer surface, micropyle, and section of fertilized egg envelope have same structure including results before. Our data indicate that these morphological characteristics of fertilized egg and fertilized egg envelope show genus *Nothobranchius* specificity.

CONCLUSIONS

We compared the fertilized egg morphology and ultrastructures of fertilized egg envelopes of *N. foerschi* and *N. rachovii* under light and electron microscopes. The external shapes of fertilized egg and ultrastuctures of outer surface, micropyle, and section of fertilized egg envelope have same structures including results before. In conclusion, these morphological characteristics of fertilized egg and fertilized egg envelope showed genus *Nothobranchius* specificity.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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