

Ultrastructure of the chorion and the micropyle of an endemic cyprinid fish, *Cyprinion tenuiradius* Heckel, 1849 (Teleostei: Cyprinidae) from southern Iran

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Abstract

The scanning electron microscope was used to investigate the ultrastructures of the egg membrane surface (unfertilized egg) of an endemic cyprinid fish, *Cyprinion tenuiradius* Heckel, 1849. The eggs of this species were almost circular in shape, had a smooth surface and one type II micropyle consisting of the flat pit and a long canal in the animal polar region. The micropyle region was not flat, micropyle was circular or oval in shape and the micropyle canal was located in its center. Round or oval accessory pores were also observed in the pit around of the micropylar canal. The surface of zona radiata was smooth with a uniform distribution of almost round pores with lips.

Keywords: Morphology, Fertilization, *Cyprinion tenuiradius*, Micropyle, Accessory openings

Introduction

An important reason of teleost evolutionary success is their reproductive system, which has been functionally acclimated in all aquatic environmental conditions (Koc, 2010). Reproduction represents one of the most important aspects of the different species biology and the maintenance of viable populations depending on its success (Suzuki and Agostinho, 1997).

Different fish species live in special ecological conditions, therefore they have unique reproductive strategy, with special anatomical, behavioral, physiological, and energetic adaptations (Moyle and Cech, 2004). The envelope of teleost fish's egg has a key role in this reproductive success. Teleosts develop mature eggs enclosed in a hard and complex eggshell (chorion), which is formed by protein components organized into a complex structure. This structure plays an essential role in control of the relation between the external and the internal egg environments. It allows for respiratory gas diffusion, provides mechanical protection and thermal insulation, and permits sperm entry (Hamodrakas, 1992).

Ultrastructural characteristics of the chorion and the micropyle of teleost eggs differ in different species, and have recently been considered as a criterion for identification of eggs (Ohta et al., 1983; Chen et al., 2007). Shape, number and size of micropyle and also reinforcement type of the micropyle canal; the number and length of the longest and shortest ridge in the micropyle region; the diameter, number, and arrangement of the accessory

openings; the adhesive structures of egg and mode of them to the substrate; ornamentation and the thickness of the membrane have been used for taxonomic purposes (Hirai and Yamamoto, 1986; Riehl, 1993; Julianini et al., 1994; Chen et al., 1999; Li et al., 2000; Bless and Riehl, 2002; Esmaeili and Johal, 2005; Huysentruyt and Adriaens, 2005; Chen et al., 2007; Costa and Leal, 2009).

Some characters such as shape of the external surface of the egg membrane, number and shape of micropyles, the branching pattern of the tubules in the primary membrane, the width of the membrane and the degree of egg adhesiveness are species typical, although they represent a convergent similarity in some species (Vorobyeva and Markov, 1999; Huysentruyt and Adriaens, 2005; Costa and Leal, 2009).

The chorion surface has also been analyzed using scanning electron microscopy (Ohta et al., 1983; Johnson and Werner, 1986; Costa and Leal, 2009; Koç, 2010). Johnson and Werner (1986) described the external morphology of the chorion of five freshwater fishes and concluded that scanning electron microscopy was a powerful tool for identifying fish eggs. On the other hand, the micropyle and its microstructures in unfertilized eggs are important characters in gamete recognition and fish egg identification, therefore its morphology may be species specific (Ginsburg, 1968; Kobayashi and Yamamoto, 1981; Chen et al., 2007). The microstructure of micropyles and surface ornamentations, however, may change during fertilization.

Therefore, the microstructural features of the micropylar canal are not reliable tools for the identification of fertilized eggs but can be used for unfertilized eggs. The thickness of the egg membrane in different kinds of fishes might be also affected by ecological environments and developmental stages (Ivankov and Kurdyayeva, 1973; Riehl, 1978; Chen et al., 2007).

As mentioned above, SEM studies are an important prerequisite to obtain further insight into the fine structures of teleost eggs. The aim of this study is description of morphology and ultrastructures of egg envelope of poorly known endemic species, *Cyprinion tenuiradius* Heckel, 1843 for the first time using scanning electron microscope. Qarah Aghaj botak, *Cyprinion tenuiradius* is the only endemic species of the genus *Cyprinion* (locally named 'botak') in Iran, which has been reported from the Persian Gulf in southern Iran (Esmaeili et al., 2010; Coad, 2011). Description of morphological characteristics of this fish may be useful to have clear picture of taxonomic position of *Cyprinion* taxa in Iran when the comparative data are completed on *C. macrostomum*, *C. kais* and *C. watsoni* which are closely related species to *C. tenuiradius*.

Material and methods

To investigate the morphology and surface ultrastructures of ripe eggs (stage V), we caught female specimens of *C. tenuiradius* from Rudbal River ($28^{\circ}41'36.18''$ N, $52^{\circ}37'11.52''$ E, at an altitude of 1168 m), Firooz Abad, Fars Province, southern Iran. Rudbal River is a branch of Qarah Aghaj

River and drains to Persian Gulf. The gravid females were distinguished from males by application of slight pressure on the abdominal region of the alive fish, leading to the release of ripe oocytes. Following a modified Nikolsky's scheme (Nikolsky 1963) we assigned a maturity stage by recording color, and texture of the gonads in dissected specimens. For SEM study, we removed the gravid eggs from ovaries, fixed and preserved them in 10% formalin. After fixation, we cleaned the gravid eggs with a fine, short-haired brush and fine forceps, washed them with distilled water and then dehydrated them in a graded series of ethanol (30, 50, 70, 90, and 100%). We placed egg samples in a freeze drier system (30 min) and then mounted dried eggs on aluminum stubs. These mounts were then sputter-coated with gold, and were observed and photographed with a Cambridge 180 scanning electron microscope (SEM) at voltage of 20 KV (Esmaeili et al., 2007). The measurements were performed using Image Tool software and the data were analyzed in SPSS 15 software.

Results

The unfertilized eggs of Qarah Aghaj botak at stage V were spherical in shape (Fig. 1a), and ranging between 0.07-1.3 mm in diameter. The ripe stage (stage V) of gonad was recognized macroscopically according to Nikolsky (1963) method. At this stage, ovaries were dark yellowish and occupied most of the body cavity. They had achieved their maximum weight and size. Large, dark yellowish and spherical oocytes, full of yolk were distinguishable.

Using SEM, the micropyle region was not flat (Figs. 1b, c). The micropyle was almost circular or oval in shape and the micropyle canal was located in its center (Figs. 1c-e). The outer opening diameters of the micropylar canal ranged from 2.56 μm to 18.28 μm with a mean of 10.42 ± 11.11 in circular and oval shape micropyles, and the canal tapering toward the inner membrane surface (Figs. 1d, e). The micropyle region was curved, porous without any microvilli – like structures (Figs. 1b, d, e). Round or oval accessory pores of various sizes were distributed

regularly in the pit, around the micropyle canal (Fig. 1e). The outer terminus of the micropyle, measuring about 18.55 μm in diameter. The micropyle was of type 2. In this type, micropyle has a flat pit and a long canal. The surface of the zona radiata (chorion) was smooth with the large number of regularly distributed lipped oval pores at a density of 213 per $400 \mu\text{m}^2$ and pore openings were $0.155\text{--}0.235 \mu\text{m}$ in diameter (0.20 ± 0.02) (Fig. 1f). Some other morphometric measurements for ultrastructures of ripe eggs are given in Table 1.

Table 1: Microstructure characters of *Cyprinodon tenuiradiatus* eggs (means \pm SD)

Egg diameter (μm)	1153.09 ± 65.55
Micropyle region diameter (μm)	47.29 ± 4.38
Diameter of micropyle pit (μm)	18.41 ± 0.27
Pore openings diameter of micropyle pit (μm)	0.49 ± 0.14
Distance between pores inside pit of micropyle (μm)	0.55 ± 0.20
Diameter of superficial pore openings (μm)	0.20 ± 0.05
Distance between superficial pores (μm)	0.65 ± 0.12
Number of pores in pit of micropyle ($25 \mu\text{m}^2$)	25
Number of pores around of micropyle pit ($25 \mu\text{m}^2$)	53
Number of pores in edge of micropyle pit ($25 \mu\text{m}^2$)	23

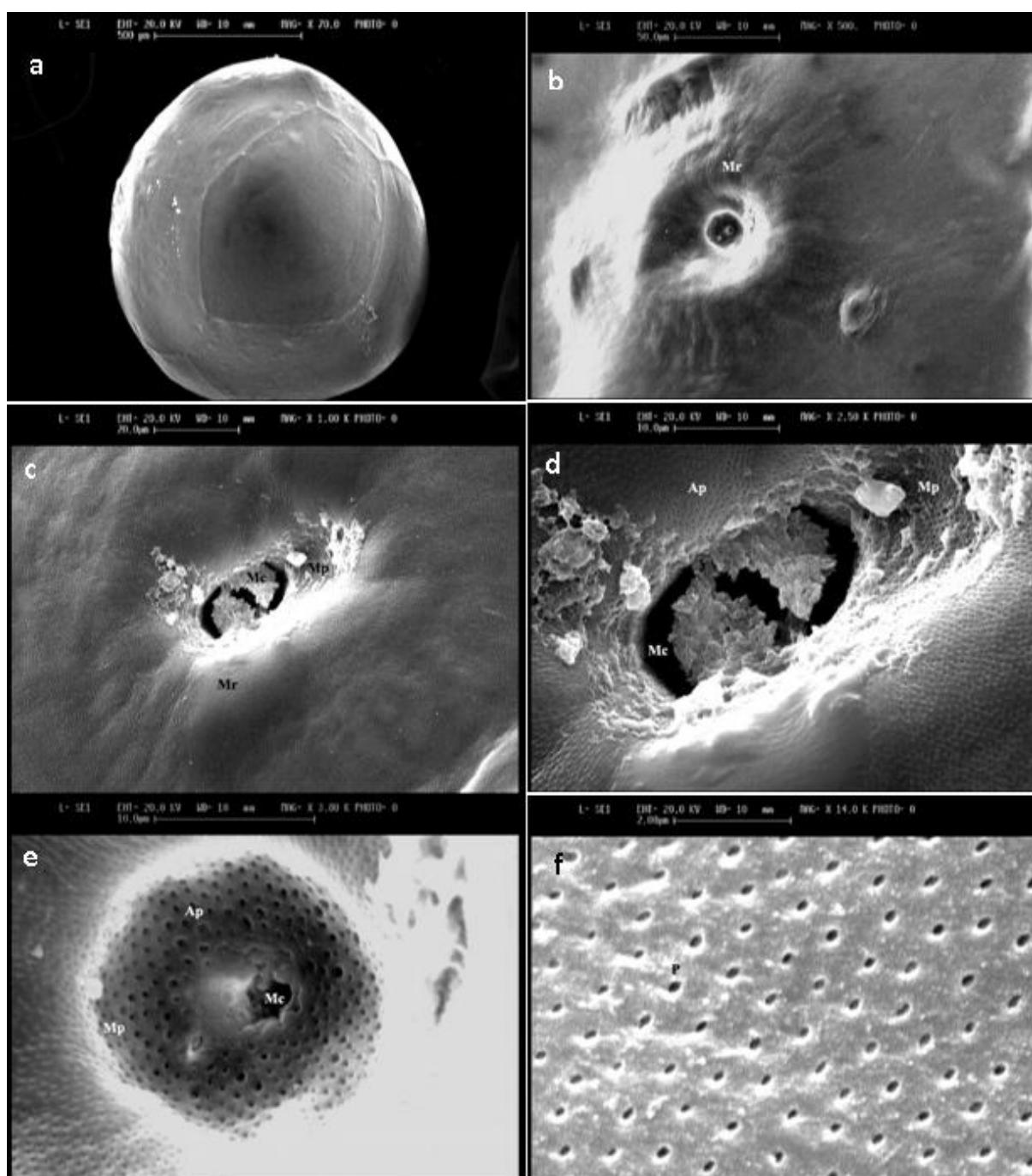


Figure 1: Scanning electron microscopy of egg surface structures of *Cyprinodon tenuiradius*: (a) whole view of egg; (b) micropyle region; (c) micropyle region and oval micropylar canal; (d) higher magnification of micropyle region showing oval micropylar canal and accessory pores in pit around of it; (e) higher magnification of micropyle showing round or oval accessory pores in pit around micropylar canal; (f) Regular distribution of lipped oval pores on the egg surface. Ap, accessory pore; Mc, micropyle canal; Mp, micropyle pit; Mr, micropyle region; P, pore

Discussion

Number, shape and size of micropyle and ultrastructere of egg membrane have been used as taxonomic tool in ichthyology (Esmaeili and Johal, 2005). In the present work it was found that the unfertilized eggs of the *C. tenuiradius* possess only one micropyle at the animal polar region. The same is reported in many other fish species. According to Ginsburg (1968) and Lahnsteiner (2003) most fish species have only one micropyle, but a few species may have up to 52 micropyles. Among the cyprinid species, presence of only one micropyle has also been reported in eggs of *Aulopyge huegeli* (Bless and Riehl, 2002) and *Hypophthalmichthys molitrix* (Esmaeili and Johal, 2005). The existence of only one micropyle in eggs of this carp may be one of the mechanisms which prevent polyspermy. During fertilization a single sperm enters the micropyle, then the inner part of the micropylar canal becomes narrower and a plug-like blockage quickly forms on the micropyle to prevent polyspermy (Chen et al., 2007).

Diameter of micropyle is another aspect which is been considered for egg identification. Base on the obtained results, the mean of outer opening diameters of the micropyles canal of Qarah Aghaj botak was about 10.5 μm . These amounts have been recorded 7.5 μm and 9.5 μm for *A. huegeli* and *H. molitrix*, respectively (Bless and Riehl, 2002; Esmaeili and Johal, 2005). The diameter of the micropyle canal was larger than in some fishes such as *Epinephelus malabaricus* (6.36 μm), *Mugil cephalus* (3.55 μm) and near to the same as that of *Epinephelus*

coioides (9.06 lm) (Li et al., 2000). As a fertilizing spermatozoon gains access to the egg surface only by passing through the micropyle, therefore, we concluded that the diameter of the micropyle canal should be slightly larger than that of the sperm head. The size of the micropyle is species-dependent but is usually only wide enough to allow one sperm through at a time. This adaption is crucial in the prevention of polyspermy (Coward et al., 2002). The type of micropyle is another useful character for identification of fish eggs. Riehl and Schulte (1978) described three types of micropyles: type I, micropyles with a deep micropylar pit and short micropylar canal; type II, micropyles with a flat pit and a correspondingly longer canal; type III, micropyles without a pit, only with a canal. Our observations showed that the micropyle of *C. tenuiradius* should be classified as type II, because micropyles were found with a flat pit and a longer canal than type I. In two other cyprinid species, *A. huegeli* and *H. molitrix*, the type of micropyles have been recorded as type I and III, respectively (Bless and Riehl, 2002; Esmaeili and Johal, 2005). According to Chen et al. (1999), the micropyles of the four species of Sparidae including: *Acanthopagrus latus*, *A. schlegeli*, *Pagrus major* and *Sparus sarba* were classified as type III.

Teleostean eggs are known to possess a number of pores or knobs uniformly distributed on the surface of the egg membrane. As stated earlier, many oval pores having lips were scattered uniformly on the egg envelopes but, any special

attachment structures such as microvilli-like structures were not found in the pore opening region or other part of chorion; hence the egg surface is completely smooth. These pores may increase the gas exchange between egg and water. The presence of pores has been reported on the egg envelopes of *Epinephelus malabaricus* and *E. coioides* while only pore-traces have been observed on the egg envelope of *Sciaenops ocellatus* and no pores have been found on the envelopes of *Mugil cephalus* (Li et al., 2000). According to Chen et al. (1999) and Li et al. (2000), pore diameter did not differ significantly for fishes in the same genus, but was significantly different for different genera, even when the genera were in the same family.

Some cyprinid fishes, such as *Rutilus rutilus*, and *Leuciscus frisii meidingeri* (Patzner et al., 1996), *Alburnoides bipunctatus* (Glechner et al., 1993), *Vimba vimba* (Riehl et al., 1993c), have strong attachment extensions on their eggs while others, such as *Alburnus chalcoides mento* (Riehl et al., 1993a), *Squalius cephalus* (Riehl et al., 1993b), *Cyprinus carpio* (Riehl and Patzner, 1994) and *Leucaspis delineatus* (Riehl et al., 1995), do not have any special attachment structures and whose eggs appear completely smooth. *Cyprinion teniradius* is also included in this group.

It seems that *C. tenuiradius* egg shows special characteristics in terms of shape and size of micropyle and absence of special attachment structure. These characteristics may be useful to have clear picture of taxonomic position of *Cyprinion* taxa in Iran when the comparative data are

completed on other related taxa such as *C. macrostomum*, *C. kais* and *C. watsoni*. Hence, study of fine structures of egg envelop in other members of *Cyprinion* is highly recommended.

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