

## Ultrastructural studies of sperm penetration in the egg of the European catfish, *Silurus glanis*

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### Abstract

The process of sperm penetration into the egg of the European catfish *Silurus glanis* L. was investigated by scanning and transmission electron microscopy. Unfertilized ovum membrane under the internal micropylar aperture shows a critical depression with undulation and short microvilli corresponding to a specialized sperm entry site structure. Twenty seconds after insemination, the fertilizing spermatozoon fused with the egg at this specialized structure, without formation of an earlier fertilization cone, which had been confirmed in eggs of other fish. A cytoplasmic eminence developed however, immediately under the fused sperm head and then disappeared with time after fertilization to form a circular area around the fused sperm head, which in turn became embedded in the circular area with shortening of the flagellum. The micropyle in the catfish egg consisted of a micropylar vestibule and a canal, the internal micropylar canal being wide enough to permit the penetration of only one spermatozoon.

**Keywords:** Micropyle, egg, sperm penetration, catfish, electron microscopy.

*Études de l'ultrastructure de la pénétration de spermatozoïde dans l'ovule du poisson-chat européen, Silurus glanis.*

### Résumé

Le suivi des étapes de la pénétration du spermatozoïde à l'intérieur de l'ovule du poisson-chat européen *Silurus glanis* L. a été étudié au microscope à balayage et au microscope électronique à transmission. La membrane de l'ovule non fertilisé présente au niveau de l'ouverture interne du micropyle une dépression marquée d'ondulations et de courtes microvillosités correspondant à une structure de site caractéristique d'entrée de spermatozoïde. Vingt secondes après l'insémination, le spermatozoïde fertilisant fusionne avec l'ovule au niveau de cette structure spécialisée, sans qu'il y ait eu formation au préalable de cône de fertilisation, comme cela a été constaté chez les autres poissons. Une proéminence cytoplasmique se forme cependant, juste sous la tête du spermatozoïde fusionnant avec la membrane puis disparaît après la fécondation pour former une zone circulaire autour de la tête du spermatozoïde qui s'enfonce finalement dans cette zone circulaire avec le reste du flagelle. Chez le poisson-chat, le micropyle de l'œuf consiste en un vestibule micropylaire et un canal, le canal micropylaire interne étant suffisamment large pour permettre la pénétration d'un seul spermatozoïde.

**Mots-clés :** Micropyle, œuf, pénétration du spermatozoïde, poisson-chat, microscopie électronique.

## INTRODUCTION

The eggs of most teleosts are surrounded by a relatively thick vitelline envelope (VE), and possess a micropyle at the animal pole. A fertilizing spermatozoon gains access to the egg surface only by passing through the micropyle. It has been considered that the diameter of the inner aperture of the micropylar canal contributes to the prevention of polyspermy in fish eggs, since usually the diameter is only slightly larger than that of the sperm head (Ginsburg, 1987; Kuchnow and Scott, 1977; Brummett and Dumont, 1979; Kobayashi and Yamamoto, 1981; Gilkey, 1981; Hart and Donovan, 1983; Billard *et al.*, 1986; Linhart *et al.*, in press). The inner aperture of the micropylar canal in the common carp egg is, however, wide enough to admit several spermatozoa at once (Kudo, 1980, 1982a). Nevertheless, carp eggs are monospermic. This means that there may be at least one factor which acts to prevent polyspermy in addition to the structural factor of micropylar diameter. Indeed, cortical alveolus exudates in the perivitelline space of fertilized or artificially activated fish eggs have the ability to agglutinate fish spermatozoa (Ginsburg, 1961; Kudo, 1988). It is unlikely that such agglutinated spermatozoa would be able to fertilize mature eggs successfully. Furthermore, a specialized conformation on the egg surface immediately beneath the micropylar canal has been reported as the direct site of sperm-egg contact during normal fertilization in eggs of several fish species (Brummett and Dumont, 1979; Kudo, 1980, 1982b, 1983, 1991; Hart and Donovan, 1983; Ohta and Iwamatsu, 1983). In this context, the site may be a specialized target organ with specific receptor for a fertilizing spermatozoon, and its ultrastructural features are appreciably differentiated among species (Kudo, 1982b). Therefore, comparison of the ultrastructure of the sperm entry site among eggs of various fish species is of considerable interest.

Mature eggs of several fish species are known to respond to sperm penetration by forming an earlier fertilization cone at the sperm entry site before initiation of the cortical reaction (Kudo, 1980, 1983; Kobayashi and Yamamoto, 1981; Kudo and Sato, 1985). The fertilization cone also contributes to the establishment of polyspermy blocking, the cone plasma membrane showing no fusion with that of supernumerary spermatozoa (Kudo, 1980, 1991; Kobayashi and Yamamoto, 1981; Kudo and Sato, 1985). The formation of the earlier fertilization cone appears to be unrelated to whether the diameter of the internal aperture of the micropylar canal is wide enough to permit the penetration of only one or several spermatozoa at once, but little information is available to shed light on the relationship between the earlier cone formation and the establishment of polyspermy blocking, except for eggs of a limited number of fish species.

Therefore the purpose of the present study was to obtain background information on the ultrastructure

of events associated with fertilization phenomena in *Silurus glanis* eggs.

## MATERIAL AND METHODS

The induction of spermiation, ovulation and fertilization, along with material fixation were carried out at Prerov Fish Farm at Hodonin and the Research Institute of Fish Culture at Vodnany in Czech Republic.

Five- to six-year-old catfish (*Silurus glanis*) of both sexes were stimulated to induce spermiation or ovulation by intramuscular injection of homogenate of carp hypophysis (5 mg/kg). The catfish were then kept separate in tanks containing running water at 23–24°C. The oxygen content of the water ranged from 6.4 to 8.0 mg per litre. Twenty-four or 48 h after injection, sperm were obtained from male catfish under anesthesia in a solution of 2-phenoxyethanol (1 : 1 000) by pressing both sides of the catfish abdomen, and collected into a Luer syringe (20 ml) containing 10 ml of an immobilizing medium for European catfish spermatozoa (200 mM NaCl, 30 mM Tris, pH 7; Saad *et al.*, 1988). Sperm from individual catfish were mixed and kept in glass jars in a refrigerator at 4–6°C until use for the experiments. The final sperm concentration was measured with a Bürker cell.

Mature ova were obtained from two female catfish by the same method as that for obtaining sperm. Insemination was carried out by mixing a batch of 30–60 mature ova with 100 µl diluted fresh sperm ( $0.21 \times 10^9$  spermatozoa per ml) in a dish 5 cm in diameter, and the inseminated ova were then treated with an activating solution (17 mM NaCl, 5 mM Tris, pH 7) at 23°C. The inseminated eggs still kept at 23°C were fixed at various time intervals with 0.1 M cacodylate-buffered 2.5% glutaraldehyde containing 3% sucrose (pH 7.2). Uninseminated mature ova were also fixed in the same way. For transmission electron microscopy (TEM), the eggs were washed overnight with three changes of the same buffer containing 5% sucrose, and post-fixed for 2 h in ice-cold, 1% cacodylate-buffered osmium tetroxide containing 3% sucrose. After dehydration in a graded ethanol series, the eggs were embedded in Epon. Ultrathin sections were observed using an electron microscope after double staining with uranyl acetate and lead acetate.

For scanning electron microscopy (SEM), the fixed eggs were immersed for 2 h in a solution containing equal volumes of 2% solution of sucrose, sodium glutamate, glycine, and arginine hydrochloride. They were then washed repeatedly for more than 30 min in a 5% solution of sucrose, and subsequently immersed in a 5% solution of sucrose containing 0.1% tannic acid for 2 or 18 h. After washing in a 5% solution of sucrose and subsequent postosmication, part of the vitelline envelope (VE) or fertilization envelope in the animal polar region was removed mechanically using a pincette with sharply tapered ends during dehydration

in 70% ethanol, thereby facilitating observation of the egg surface in the micropylar region. Following further dehydration in an ethanol series, the eggs were transferred to amyl acetate, and dried in a critical point drying apparatus with liquid CO<sub>2</sub>. The dried specimens were mounted on metal stubs, coated with platinum by ion-sputtering, and examined with a SEM.

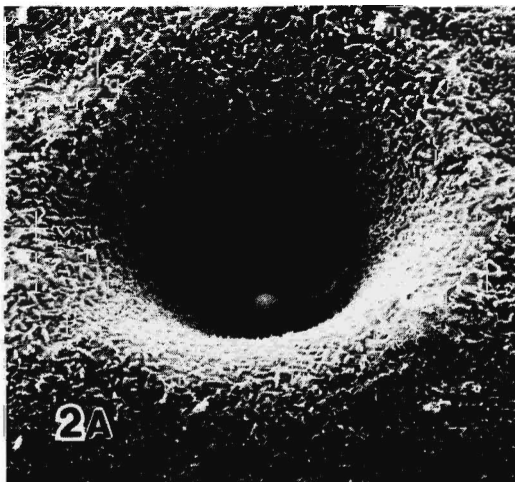
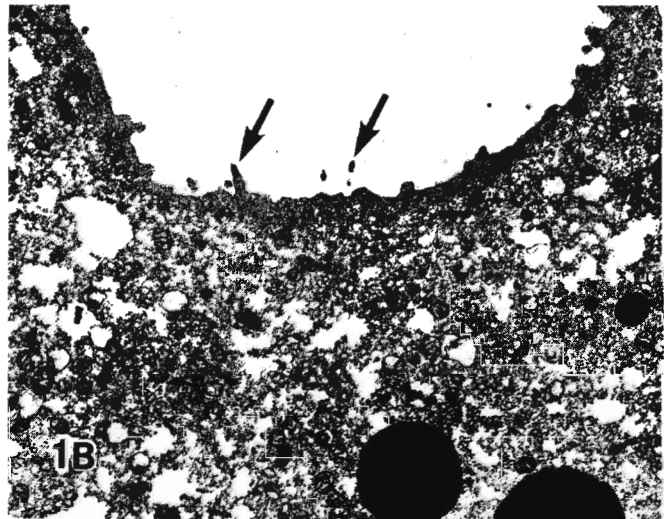
## RESULTS

### Zero time stage

The egg surface under the micropylar region showed a conical depression corresponding to the V-shaped conformation of the VE in this area. This central part of the depression, which appeared to be encircled

by the internal aperture of the micropylar canal, was characterized by an undulation with short cytoplasmic projections or a tuft of 8-12 short microvilli (fig. 1A). This location is the sperm entry site specialized for contact with a fertilizing spermatozoon, and is easily distinguishable from the surrounding egg surface, which is occupied by many low, relatively wide cytoplasmic microridges. TEM revealed a somewhat concave area in the centre of sperm entry site in addition to the short microvilli (fig. 1B).

It is very difficult to observe the micropyle from the VE surface by SEM, because of the jelly coat covering. TEM revealed that the micropyle consisted of a vestibule and a canal, the diameter of the inner micropylar aperture being slightly larger than that of the sperm head (see below fig. 2B).



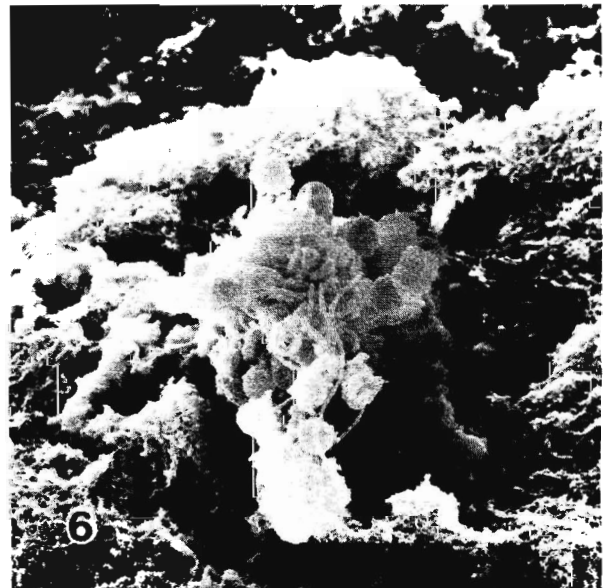
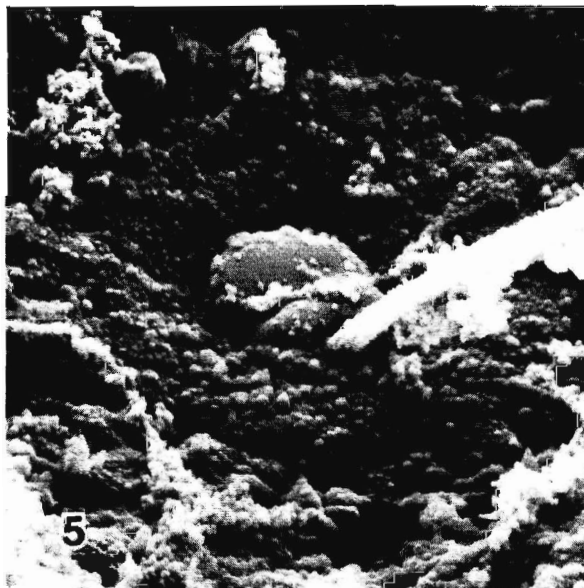
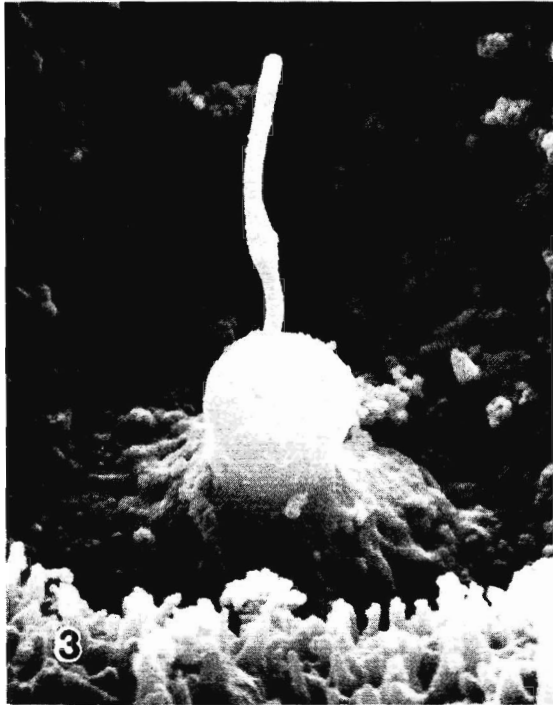
**Figure 1.** – A sperm entry site revealed by SEM (1A) and TEM (1B) at the zero-time stage "from inseminated ova". It consists of a tuft of 12 short cytoplasmic projections microvilli (arrow), A  $\times$  2 100 (1 cm = 4.76  $\mu$ m), B  $\times$  6 400 (1 cm = 1.56  $\mu$ m).

**Figure 2.** – A sperm-egg contact at the sperm entry site, which is located in the central portion of the conical depression. A fertilizing spermatozoon is located in the conical depression (2A), and such a fertilizing spermatozoon has fused with the egg cytoplasm (2B), 20-second stage, A  $\times$  1 600 (1 cm = 6.25  $\mu$ m), B  $\times$  4 600 (1 cm = 2.17  $\mu$ m).

### Twenty-second stage

Twenty seconds after insemination, one fertilizing spermatozoon had reached the sperm entry site located

at the bottom of the conical depression (*fig. 2A*), and the sperm head plasma membrane fused with the egg plasma membrane (*fig. 2B*); simultaneously, a cytoplasmic eminence was seen under the fused



**Figure 3.** – A fertilizing spermatozoon fused with an egg. A cytoplasmic eminence is evident immediately under the fused sperm, implying fusion at the anterior part of the sperm, so that the flagellum extends from the top area of the sperm. One-minute stage,  $\times 10\,300$  ( $1\text{ cm} = 0.97\ \mu\text{m}$ ).

**Figure 4.** – A fertilizing spermatozoon fused with an egg at the two-minute stage. A plate-like circular area appears around the sperm head, with recession of the cytoplasmic eminence,  $\times 10\,300$  ( $1\text{ cm} = 0.97\ \mu\text{m}$ ).

**Figure 5.** – Further progression of sperm penetration into an egg at the two-minute stage. Most of the sperm head has become embedded,  $\times 15\,500$  ( $1\text{ cm} = 0.65\ \mu\text{m}$ ).

**Figure 6.** – Sperm aggregation at the upper micropylar vestibule. Two-minute stage,  $\times 2\,100$  ( $1\text{ cm} = 4.76\ \mu\text{m}$ ).



Figure 7. – Complete penetration of the fused sperm head at the three-minute stage, except for a small area around the base of the flagellum,  $\times 4\ 100$ .

Figure 8. – Complete penetration of the fused sperm head and progressed penetration of the sperm flagellum at the four-minute stage,  $\times 5\ 200$ .

sperm head. The flagellum extended from the posterior portion of the sperm head.

#### One-minute stage

The depression under the micropyle appeared to be retained, and the whole base immediately under the fused sperm head protruded (fig. 3). The flagellum remained attached to the fused sperm head.

#### Two-minute stage

The whole protrusion immediately under the fused sperm head, as seen in the previous stage, had regressed, leaving a slight trace around the fused sperm head (fig. 4). Further progression of sperm penetration into the egg left only its posterior end exposed (fig. 5). A relatively flat circular area (about  $5.5\text{--}8.0\ \mu\text{m}$  in diameter) appeared around the rest of the fused sperm head as a feature of progressing fusion. The conical depression under the micropyle was apparently retained but shallower than that in the previous stage. No shortening of the fused sperm flagellum was yet evident. Sperm aggregation was frequently observed in the upper part of the micropylar vestibule (fig. 6).

#### Three-minute stage

At this stage, the fused sperm head has become totally embedded in the egg, except for a small area surrounding the base of the fused sperm flagellum (fig. 7). A relatively flat circular area was retained around the embedded sperm.

#### Four-minute stage

The sperm flagellum had penetrated considerably into the egg except for a short length (fig. 8), with some exceptions for relatively long flagella. Around the flagellum, a circular area (about  $8.0\text{--}8.8\ \mu\text{m}$  in diameter) remained extended. It was easy to detect the conical depression even at this stage, and the circular area was located in the bottom of the depression.

#### DISCUSSION

The results indicate that in the vitelline envelope of the egg of the catfish *Silurus glanis* the micropyle has an internal aperture wide enough to permit penetration of only one spermatozoon. Underneath the micropyle a specialized structure, the "sperm entry site", is located in the egg surface and a fertilization cone is formed by a cytoplasmic eminence immediately under the fused sperm head. The ultrastructural features of the sperm entry site in the present eggs are rather similar to those of carp eggs (Kudo, 1982b) and zebra danio eggs (Hart and Donovan, 1983). The presence of the sperm entry site has been confirmed in eggs of all fish species examined to date, with one exception of absence in the eggs of *Oryzias latipes* (Iwamatsu and Ohta, 1981). Presumably, the plasma membrane lining the sperm entry site, particularly that of the cytoplasmic projections, is specialized to provide biochemical or cytochemical characteristics responsible for membrane fusion of both gametes. The importance of egg surface

specialization remains to be clarified using other techniques.

It is known that the eggs of several fish species respond to sperm penetration at the initial stages by forming an earlier fertilization (or growth) cone (Kudo, 1980, 1983, 1991; Kobayashi and Yamamoto, 1981; Kudo and Sato, 1985). The growth of the earlier fertilization cone starts from the top area of the fused sperm head, and eventually reaches the micropylar vestibule (Kudo, 1980, 1985). No such fertilization cone was formed in the species in this study, but the present cone is rather similar ultrastructurally to that in sea urchin eggs (Tilney and Jaffe, 1980) and brittle star eggs (Yamashita, 1983). It is not

yet clear why the fish eggs considered here have no requirement for cone formation corresponding to the earlier cone seen in fertilized eggs of the carp and *Plecoglossus*. There seems to be no relationship between the formation of the earlier cone and the size of the internal micropylar aperture, since the earlier cone in chum salmon eggs is formed irrespective of the diameter of the aperture allowing passage of only one spermatozoon (Kobayashi and Yamamoto, 1981). Lack of formation of an earlier cone may be a rather unique feature of normal fertilization of fish eggs. This aspect will be clarified by further investigation, particularly in relation to the process of fertilization envelope elevation.

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