RECENT ADVANCES IN THE STUDY OF
FISH EGGS AND LARVAE
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Ultrastructural studies of the morphological variations of the egg surface and envelopes of the African catfish *Clarias gariepinus* (Burchell, 1822) before and after fertilisation, with a discussion of the fertilisation mechanism

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SUMMARY: Much of the existing knowledge of the mechanisms involved in teleost fertilisation is based on a few small model species that have no commercial value. Research is therefore urgently required to address mechanisms involved in fertilisation in species of great commercial value. In this study, the ultrastructural morphological variations in the surface of the egg of *Clarias gariepinus* were recorded before and after fertilisation by using electron microscopy. The outer surface of the unfertilised egg was smooth, whereas the fertilised egg acquired a network of projections on the vegetal hemisphere. Moreover, different patterns of ornamentation on the egg surface were evident. This pattern of ornamentation varied with the progress of embryonic development. The micropyle of the *C. gariepinus* egg consisted of a funnel-shaped vestibule, from the bottom of which a cylindrical micropylar canal extended. The micropylar canal decreased in diameter after completion of fertilisation, forming a micropylar disc. The sperm behaviour on the egg surface was oriented towards any depression on the chorion surface. The chorion of ovulated eggs consisted of one layer. After fertilisation the chorion was differentiated into three layers: the double-layered coat, the zona radiata externa and the zona radiata interna. Four protein subunits of the chorion of *C. gariepinus* were identified by SDS-PAGE. IR-spectra obtained from *C. gariepinus* chorion revealed that the vibration of chorion proteins exhibited different weak activities in the IR-spectra with minor difference between pre- and post-fertilisation chorion proteins.

Keywords: ultrastructure, egg envelops, micropyle, African catfish, chorion proteins.

RESUMEN: ESTUDIOS ULTRAESTRUCTURALES DE LAS VARIACIONES MORFOLÓGICAS DE LA SUPERFICIE Y ENVOLTURAS DEL HUEVO DEL PEZ-GATO AFRICANO *CLARIAS GARIEPINUS* (BURCHELL, 1822) ANTES Y DESPUÉS DE LA FERTILIZACIÓN, CON UNA DISCUSIÓN DE LOS MECANISMOS DE FERTILIZACIÓN. – Gran parte del conocimiento existente sobre los mecanismos de fertilización en teleósteos se basa en unas pocas especies modelo, que no tienen valor comercial. Por ello, se requiere investigación dirigida a conocer los mecanismos involucrados en la fertilización de especies de gran valor comercial. En el presente estudio, mediante el uso del Microscopio Electrónico, se obtuvieron imagines de las variaciones en la morfología ultraestructural de la superficie de los huevos de *Clarias gariepinus* antes y después de la fertilización. La superficie externa de los huevos infertilizados era lisa, mientras que la de los huevos fertilizados adquiría una red de proyecciones en el hemisferio vegetativo. Además, fueron evidentes diferentes patrones de ornamentación de la superficie del huevo. Este patrón de ornamentación varió a lo largo del desarrollo embrionario. El micropilo de los huevos de *C. gariepinus* consistió en un vestíbulo con forma de embudo desde la base del cual se extendía un canal micropilar cilíndrico. El diámetro de dicho canal disminuía tras la finalización de la fertilización, formándose el disco micropilar. El comportamiento del esperma en la superficie de los huevos se orientaba hacia cualquier depresión de la superficie del corion. El corion de los ovocitos consistió en una sola capa. Tras la fertilización, el corion se diferenció en tres capas: la capa double-acodada, la zona radiada externa and y la zona radiada interna. Mediante SDS-PAGE se identificaron cuatro subunidades de proteínas en el corion de los huevos de *C. gariepinus*. Los espectros IR obtenidos del corion revelaron que la vibración de las proteínas del corion exhibía diversas débiles actividades en el espectro IR con menores diferencias entre pre- y post-fertilización proteínas del corion.

Palabras clave: ultraestructura, envuelta del huevo, micropilo, pez-gato africano, corion, proteínas.

INTRODUCTION

Teleosts include more than half the vertebrate species (Baldacci *et al*., 2001). A key feature of teleost evolutionary success is their reproductive system, which must be functional in all aquatic environmental conditions. Much of the existing knowledge of the mechanisms involved in teleost fertilisation is based on a few small model species such as zebrafish, medaka and bitterling, which have no real commercial value (Coward *et al*., 2002). Research is required to address mechanisms involved in fertilisation in species of commercial value, which represent a cornerstone of the field of aquaculture and the associated biotechnology, genetic engineering and biodiversity fields.

Interspecific differences in the microstructure of the chorion (egg envelope) of teleost fishes have been recognised for almost 30 years (Merrett and Barnes, 1996). Such differences have been used not only to identify eggs in plankton samples (Merrett and Barnes, 1996), but also as potentially useful taxonomic characters (Gill and Mooi, 1993; Johnson and Brothers, 1993; Britz *et al*., 1995; Chen *et al*., 1999; Britz and Breining, 2000). Chen *et al*. (1999) concluded that the ultrastructural features of the egg envelope were found to be helpful in species identification of the distantly related species, but not of closely related ones. Merrett and Barnes (1996) clarified Marshall's (1973) suggestion that egg envelope ornamentation is a family characteristic.

The chorion structure and its chemical constituents is the end product of different evolutionary trends, adaptation processes and environmental factors (Yamagami *et al*., 1992; Celius and Walther, 1998; Quagio-Grassiotto and Guimuraes, 2003). Hyllner *et al*. (2001) concluded that the chorion proteins from the vertebrate groups of fishes and their amphibian, avian and mammalian counterparts share a common ancestry and form a unique group of structural proteins. Such proteins exhibit dramatic changes during chorion hardening.

Several important questions arise with regard to fertilisation. Where do the instructions for the process of teleost fertilisation lie? What is the language of these instructions? Are these instructions and their language influenced by genetic programs of both parents? To answer these questions, the fertilisation process of a fish must be described in the light of reorganisation of the chorion at fertilisation, its physiological roles, the dynamic changes of the egg cortex, the secretory functions of cortical granules, mechanisms of sperm-egg interactions, and mechanical blocking of polyspermy (Lönning and Hagstrom, 1975; Hart, 1990; Yamagami *et al*., 1992; Griffin *et al*., 1996; Merrett and Barnes, 1996; Linhart and Kudo, 1997; Chen *et al*., 1999; Britz and Breining, 2000; Coward *et al*., 2002).

Literature dealing with *Clarias gariepinus* (Burchell, 1822) chorion and fertilisation and the associated mechanisms is very rare. Only two articles (Riehl and Appelbaum, 1991; Wenbiao *et al*., 1991) are at hand. The present study aimed to elucidate the morphological variations of the chorion of *C. gariepinus* eggs before and after fertilisation. It also aimed to emphasize the process of chorion hardening and its biochemical composition, in addition to the structure and behaviour of spermatozoa on the chorion surface, using transmission and scanning electron microscopy. The micropyle shape and its closure, the micropyle-like depressions, the folded chorion and the polyspermy prevention and fertilisation mechanisms were also considered and discussed in the light of the available information.

MATERIALS AND METHODS

Gamete collection

Mature African catfish, *C. gariepinus* (weight of 900-1500 g) were collected from the River Nile at Assuit, Egypt and transported to the Fish Lab, Zoology Department, Assuit University. The catfish were kept in 100 l glass tanks to be acclimatised for a two-week period at 24-26ºC. The photoperiod was a 12 hour light to 12 hour dark cycle and the catfish were fed on a commercial pellet diet (3% of the body weight/day).

For collection of semen, male fish were anaesthetised with 200 mg/l MS-222 and one of the testes was removed surgically. Alternatively, the fish were killed and whole gonads were removed. Testes were cleaned from the blood by surgical towels. Sperm from the testes was pressed through a mesh fabric into a sterile dry petri dish and used directly for dry fertilisation. For collection of eggs, ovulation was artificially induced. Females were injected intraperitoneally with pellets (gonadotropin-releasing hormone analogue, GnRHa, D-Ala^6, Pro^9 Net) containing 2.5-3.0 mg of water-soluble dopamine antagonist metoclopramide (Interfish Ltd, Hungary) dis-

solved in 0.65% NaCl. One pellet was used per kg body weight. Eleven to 12 h after injection, the fish were stripped and the eggs were collected in clean dry plastic containers.

Light microscope

Pieces of the ripe ovary of *C. gariepinus* were fixed in 10% neutral buffered formalin (pH 7.5), dehydrated in ascending series of ethyl alcohol, and cleared in methyl benzoate. Embedded tissues were sectioned at 3 μm and stained with hematoxylin and eosin (H&E) (Bancroft and Stevens, 1982).

Transmission electron microscope (TEM)

Pieces of the eggs and the testis were immediately fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 24 h at 4ºC. The specimens were washed in 0.1 M cacodylate buffer (pH 7.2) for 1-3 h and then post-fixed in 1% osmium teraoxide for 2 h. The tissue pieces were placed in propylene oxide for 60 min, then in pure Epon 812.

Tissues were sectioned at 1 μm and stained with toluidine blue. Sections were examined by light microscope to identify different representative regions to be sectioned. Ultrathin sections were mounted in copper grids, stained with uranyl acetate and lead citrate (Bancroft and Stevens 1982), and examined with a TEM (JEOL 100, CXII) operated at 80 kv.

Scanning electron microscope (SEM)

Eggs before insemination and after fertilisation were fixed with 5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4, 4ºC) for 24 h. They were post fixed with 1.5 osmium tetraoxide for 2 h and washed four times with 100 mM phosphate buffer (pH 7.4). Some eggs were cut into halves with a fine razor. After slow dehydration with an ethanol series, the eggs were dried at 30-40ºC, glued to stubs coated with 20 nm of gold, and viewed with SEM (GAOL, GSMS 400 LV) at 15 kv.

Gel electrophoresis of chorion proteins

The isolated chorion of the fertilised and unfertilised eggs of *C. gariepinus* were dissolved in buffer containing 150 mM NaCl, 20 mM Tris, 10 mM EDTA, and 1% SDS with boiling at 100ºC for 5 min and centrifugation to remove undissolved remnants. The chorion was also dissolved in the same buffer with 8 M urea for another run because one-dimensional SDS-PAGE using 10% acrylamide was performed according to the procedure of Laemmli (1970) and according to Swank and Munkres (1971) for SDS-Urea-PAGE. Gradient SDS-PAGE (5-20%) was prepared according to Hames (1981). The low molecular weight standards (Pierce, USA) were run concurrently and the protein molecular mass was determined using Gel-Pro Analyser package (Media Cybernetics, 1998).

Infrared spectroscopy analysis

The chorion of fertilised and unfertilised eggs of *C. gariepinus* were isolated under dissecting microscope and washed thoroughly several times with distilled water in a sonicator to remove the newly formed embryo and other remnants. The chorion was air-dried. Infrared spectra were recorded on the infrared spectroscopy SHIMADZU (IR-470) according to Iconomidou *et al.* (2000) with modification.

Terminology of the chorion

There is considerable variation in the nomenclature used to describe the external membrane of teleost eggs. Commonly used terms for this outer covering include zona radiata, zona pellucida, chorion, radiate membrane, egg membrane, primary membrane, vitelline membrane, vitelline envelope, egg envelope, egg shell and egg capsule. In the present work, the term chorion is used according to Yamagami *et al.* (1992).

RESULTS AND DISCUSSION

The ripe testes and sperm structure

The testes of *C. gariepinus* in a ripe stage displayed various stages of active spermatogenesis. The spermatozoa of *C. gariepinus* were tightly packed in the lumen of the testes lobules (Fig. 1A). Spermatozoa consisted of a head, midpiece and very long tail (Fig. 1B). The head region contained the nucleus, which consisted of variable electron dense granular chromatin materials. The head region was relatively triangular or rectangular. The midpiece

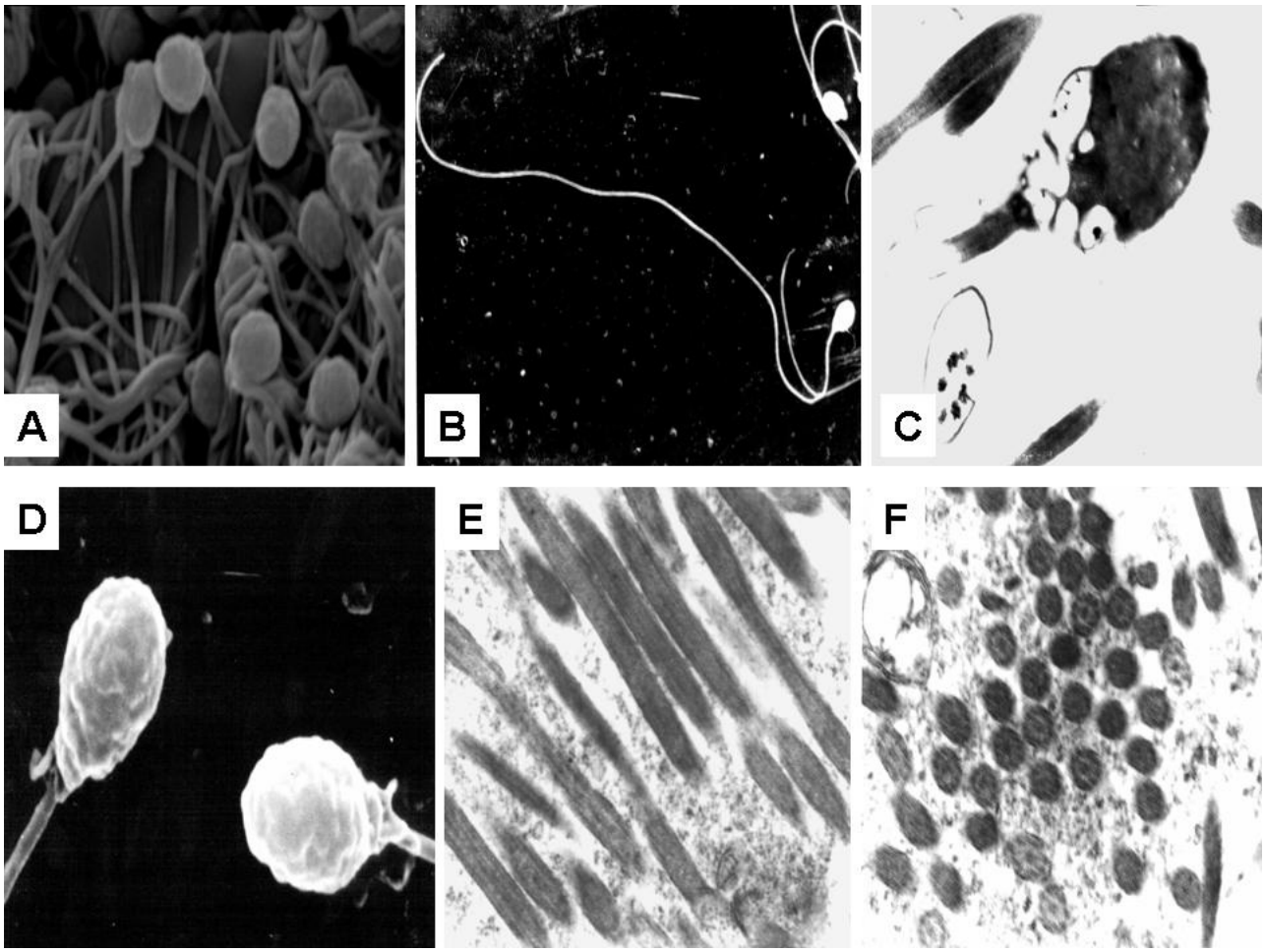

FIG. 1. – Spermatozoa in the lobules of the rip testis of *Clarias gariepinus* (*A*: ×10000). The structures of the spermatozoa as revealed by SEM (B, C) in semen (×3500) and TEM (×20000). The irregularity of sperm head and midpiece were evident with a very long tail (B) and a midpiece process (D: ×20000). E, F represent transverse and longitudinal sections of the flagellum (×20000).

had an inverted conical shape forming an oval shaped structure with the head. The head-midpiece surface showed irregularity (surface with irregular folding, Fig. 1D). The irregular inverted conical shape of the midpiece reflected an increased number of mitochondria. The midpiece had a reticular structure around the flagellum (Fig. 1C), in which the mitochondria were distributed separately. These findings were in contrast with those reported by Mansour *et al*. (2002), who stated that several single mitochondria were fused with each other and formed a complex chondriosome. It was difficult to postulate such a chondriosome structure with the reticulate structure of the midpiece of *C. gariepinus*. Figure 1 (E, F) showed other related structures, especially the flagellum structure in cross and longitudinal sections. The topography of spermatozoa of *C. gariepinus* seemed to be adjusted to the diameter of the inner aperture of the micropylar canal. This condition was indicated by Linhart and Kudo (1997) as contributing to the prevention of polyspermy. Also, the topography of the head-midpiece region provided binding facets for the attachment of the sperm on the chorion surface by a ligand-receptor mechanism.

The ripe ovary and the oocyte

The ripe period of *C. gariepinus* gonad was characterised chiefly by migration of the nucleus toward the animal pole (Zaki *et al*., 1986). The ovarian ripe oocyte of *C. gariepinus* had four distinct layers: an outermost follicular layer (outer theca + inner granulosa layer), a median zona radiata (the chorion), and an inner oolemma or oocyte plasma membrane (Fig. 2A, B). Zaki *et al*. (1986) confused the terminology of the layers surrounding the oocyte since they referred to the outer layer (zona granulose) as chorion and the inner layer as zona radiata (with clearly discernible pores). Rizkalla (1960) referred

to 5 layers encircling the ovarian ripe oocyte of *C. gariepinus*: theca folliculi, membrane proporia folliculi, the follicular epithelium, the definitive membrane proper (zona radiata with faint striation) and the zonoid layer (peripheral cytoplasm). The incomplete second layer of Rizkalla (1960) was not observed in the present work. The chorion of the ovarian ripe oocyte was a single layer (sections shown in Fig. 2C, D, and E). Al-Absawy (2004) reported a single-layered chorion of ovarian oocyte of *Trachinotus ovatus* observed by light microscope. A chorion with more than one layer has been recorded in the ovarian oocyte of some other teleost fishes. The ovarian mature oocyte of *Carassius auratus* had a three-layered chorion (Cotelli *et al.*, 1988). The egg envelope of the full-grown oocyte before fertilisation in viviparous species of Goodeidae was composed of one to three layers: filamentous-zona radiata interna and an electron dense zona radiata externa covered by an additional flocculent layer (Riehl and Greven, 1993). Other oviparous teleosts exhibited a division of the zona radiata into two or more layers (Schoots *et al.*, 1982). The chorion thickness of viviparious fishes was considerably reduced in comparison with that of related oviparious species (Riehl and Greven, 1993).

Egg surface

The outer surface of the unfertilised egg of *C. gariepinus* exhibited no prominent elongated projections (Fig. 3A), whereas the fertilised egg acquired a network of projections 1 h after fertilisation on the vegetal hemisphere (Fig. 3C-F). On the vegetal pole, such projections formed a base-like circle (Fig. 3F), which might represent an adhesive apparatus for contacting substratum. At high magnification (×7500), some of these elongated projections had a dentate lateral process (Fig. 3D). The network projections seemed to be inserted in the chorion. White spots were recorded within the network projections (Fig. 3C, E). Such network projections and their related white spots were unique for *C. gariepinus* fertilised egg, since no *Clarias*-related studies referred to such variability. Moreover, no chorion-related studies on other teleost fishes

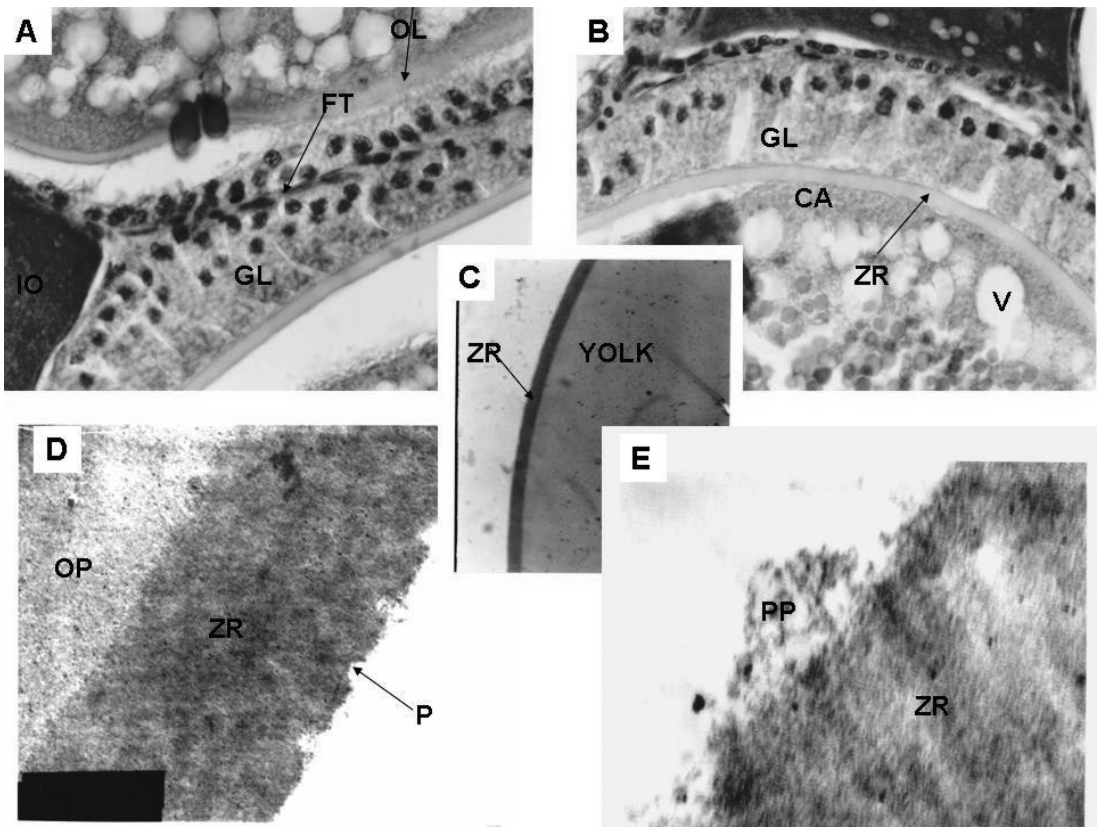

FIG. 2. – The ripe ovary of *Clarias gariepinus* in different cross sections (A, B: ×1000), showing follicular theca (FT), granulose layer (GL), single-layered zona radiata (ZR) and oolemma (OL). CA: cortical alveoli, IO: immature oocyte, V: vacuole. The zona radiata of *Clarias gariepinus* unfertilised egg as seen in semithin (C: ×1000) and ultrathin (D, E: ×10000, 40000 respectively) sections. Note the faint radiated ZR in E. P: pore, PP: pore plug, OP: ooplasm.

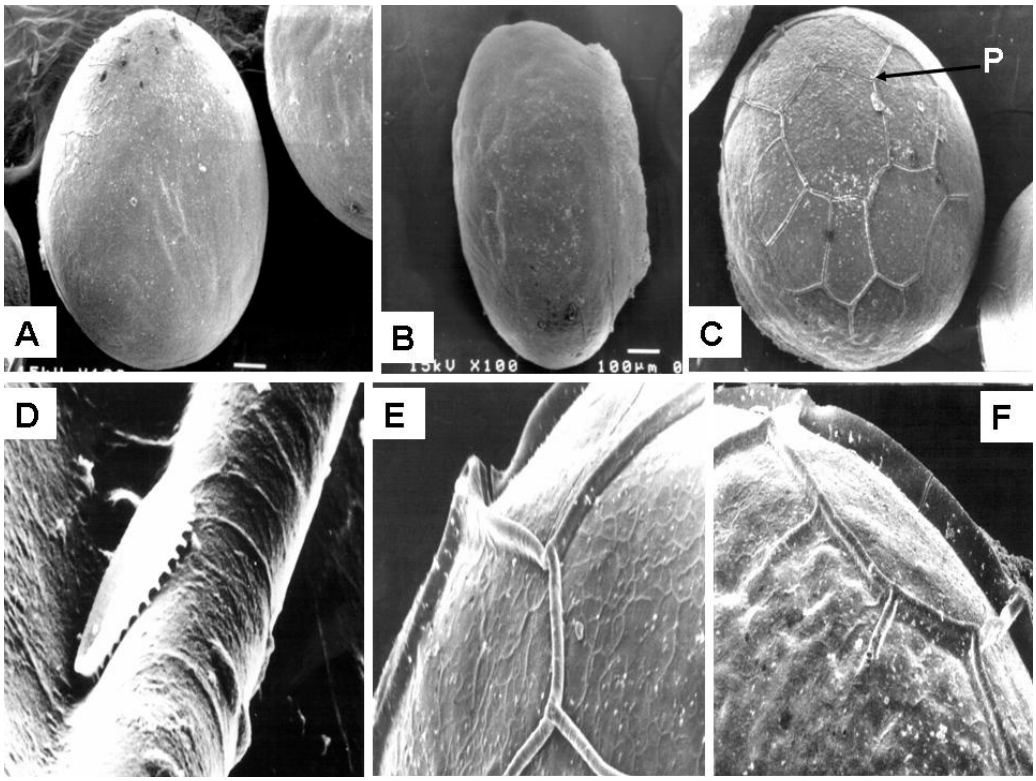

FIG. 3. – The ovulated (A) and fertilised eggs of *Clarias gariepinus* (B, (C+D), F at 1/2-h, 1.5-h, 4-h postfertilisation stages respectively). Network projections (P) on the vegetal hemisphere were formed due to chorion hardening after nearly 1-h postfertilisation (C-F) (A, B, C: ×100; D: ×7500; E: ×500; F: ×350).

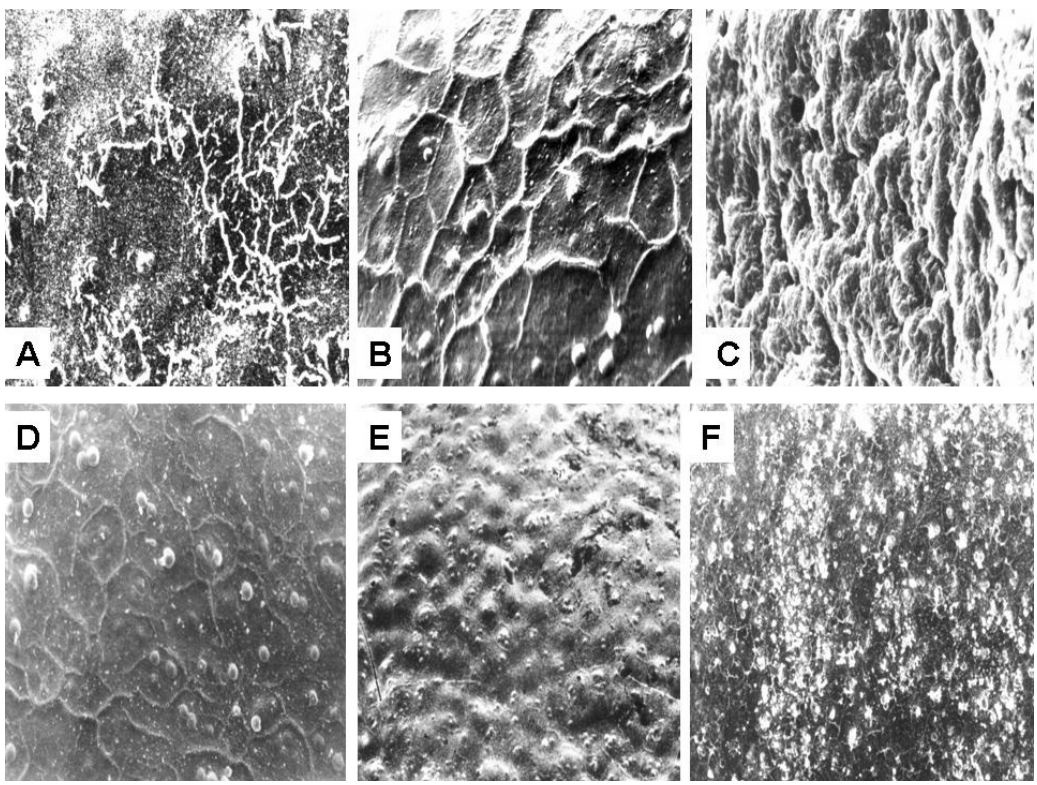

FIG. 4. – Ornamentation recorded on different regions of egg surface of *Clarias gariepinus* at 1/2-h (C, D, E) and 1.5-h (A, B, F) postfertilisation stages (A, B: ×2000; C: ×3500; D: ×1500; E, F: ×750). See text for explanation.

referred to such projections. Different patterns of ornamentation on the egg surface of *C. gariepinus* were evident (Fig. 4). Such ornamentation has been represented as tubercle and/or reticular (Fig. 4A), debris-like dots and batches (Fig. 4B,D), irregularly lobulated ornamentations (Fig. 4C) and partially reticulated debris (Fig. 4F). These patterns were recorded in different regions and at different postfertilisation times. Irregularly distributed pore bulges were recorded 1/2 h after postfertilisation (Fig. 4E). The debris-like dots and the partially reticulated debris might represent the poorly preserved remains of the diffuse mucus layer (Lönning and Hagstrom, 1975; Johnson and Werner, 1986).

The ornamentation varied from the germinal disc region to the vegetal hemisphere (Fig. 5). Moreover, the pattern of ornamentation varied with the progress in embryonic development since hairs and depressions appeared on the animal hemisphere but not on the vegetal one (Fig. 5D). Further changes were recorded at the 30 h stage (Fig.5E). Similar ornamentation patterns have been recorded by many authors working on different teleost species belonging to different taxonomic groups (Johnson and Werner, 1986; Cotelli *et al*., 1988; Merrett and Barnes, 1996; Chen *et al*., 1999; Rizzo *et al*., 2002; Chiou *et al*., 2004). Some of these authors considered the egg surface ornamentation as taxonomic characters at the specific level. The variability in ornamentation of *C. gariepinus* makes their use in species identification difficult owing to their association with the fertilisation and development process. Chen *et al*. (1999) referred to the importance of the outer surface of the chorion in egg identification and phylogenetic study. However, they added that the outer surface of the chorion did not show remarkable differences in microstructure among species in a genus or a family.

The micropyle

The micropyle of *C. gariepinus* egg consisted of a funnel-shaped vestibule from the bottom of which a cylindrical micropylar canal extended (Fig. 6A,

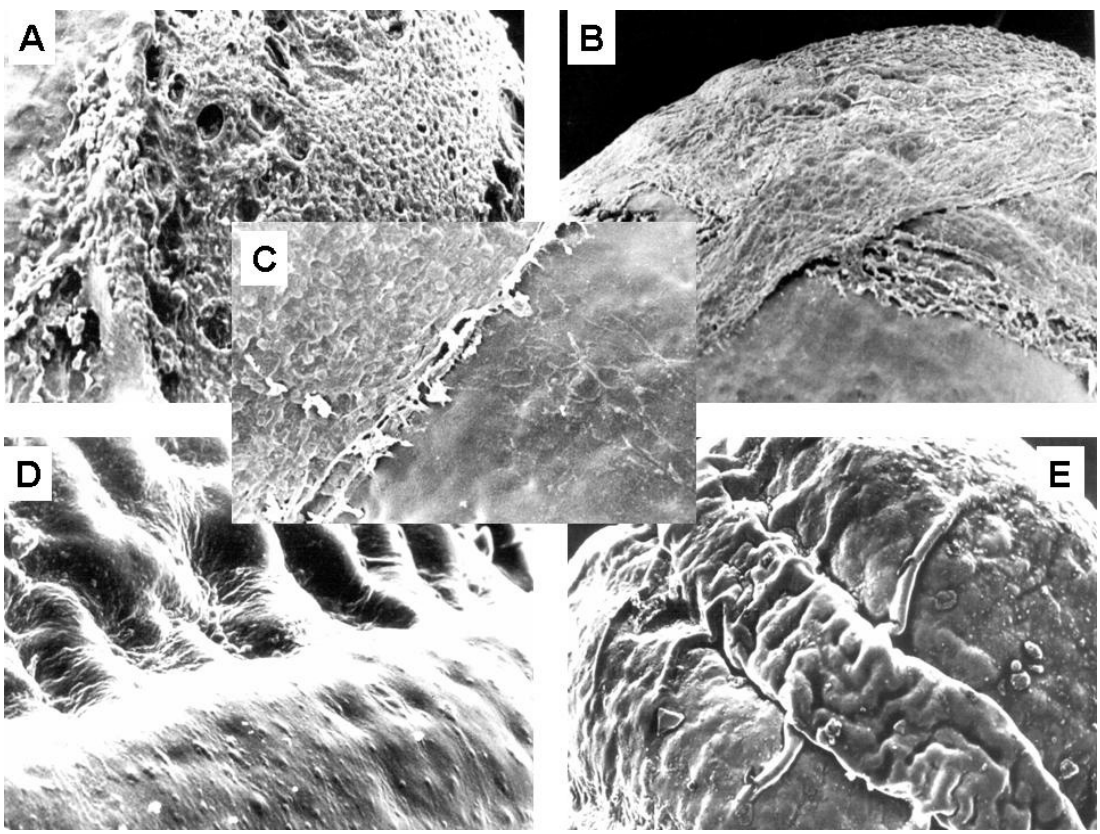

FIG. 5. – Ornamentation on the micropylar disc (A, at 1/2-h postfertilisation), the separation line between animal and vegetal hemispheres (B, C, at 1-h postfertilisation; D, at 9-h postfertilisation) and the region of *Clarias gariepinus* embryo attachment (E, at 30-h postfertilisation) (A: ×350; B, E: ×200; C, D: ×750).

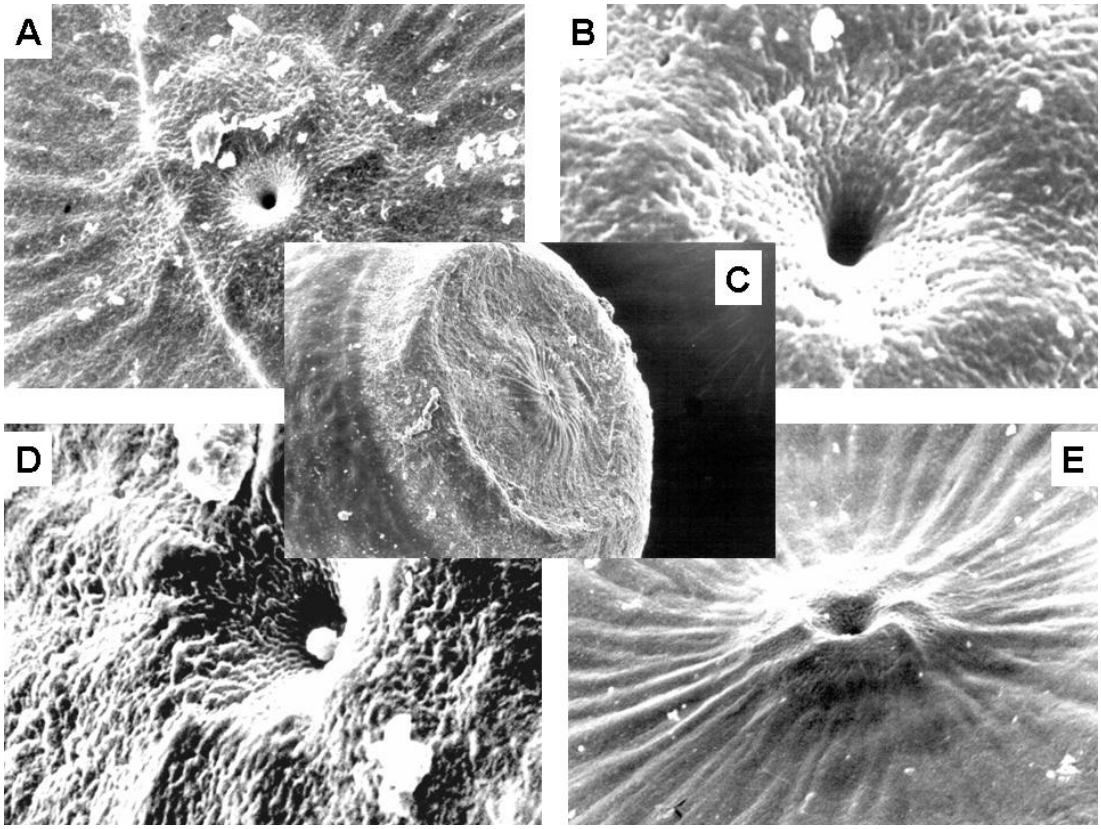

FIG. 6. – The micropylar apparatus of *Clarias gariepinus* at different magnifications (A-B). Note ornamentation specific to the micropylar regions. The spermatozoon of *Clarias gariepinus* inside the micropyler canal (D, at 1/2-h postfertilisation) and the formation of micropylar cone (C,E, at 1/2-h postfertilisation and 1.5-h postfertilisation respectively) (A: ×1500; B: ×5000; C: ×150; D: ×3500; E: ×1000).

B). Such a micropyle was similar to Type III of Riehl and Schulte (1977), inasmuch as no micropyle pit was recorded in *C. gariepinus* eggs. No micropylar pit was found in *Epinephelus malabaricus*, *E. coioides, Sciaenops ocellatus* and *Mugil cephalus* (Li *et al*., 2000). The micropylar canal of *C. gariepinus* eggs apparently decreased in diameter after completion of fertilisation-related ooplasmic changes. The narrowing of the micropyle was involved in the polyspermy-preventing reaction (Hart, 1990, Linhart and Kudo, 1997). Riehl and Schulte (1977) described two other types of micropyles: micropyles with a deep pit and short micropylar canal (Type I) and micropyles with a flat pit and a corresponding longer canal (Type II). In addition, a cylindrical, a conical and a funnel shaped micropyle have been described in *Gadus morhuu marisabli, Mugil cephalus* and *Gleginus navaga* (Mikodina, 1987).

Deung *et al*., (1997, 1999, 2000), Kim (1998) and Kim *et al*. (1993, 1996, 1998 a,b, 1999, 2001) described the micropyle of different species belonging to four families, Cichlidae, Characidae, Cyprinidae and Belontiidae, referring to taxonomic validity of the micropyle (the egg built-in advantage, Brummett and Dumont, 1979). The shape of the chorion around it appeared to facilitate the movement of spermatozoa toward the micropyle. The micropyle represents the initial isolating mechanism for preventing interspecific hybridisation (Chen *et al*., 1999) at least in teleost species, and it is considered to be species-specific (Kobayashi and Yamamoto, 1981).

With development progress, the micropyle of *C. gariepinus* egg continued to narrow with the formation of the micropylar disc (Fig. 6C, E). Riehl and Appelbaum (1991) and Wenbiao *et al*. (1991) referred to this micropylar disc. The development of such a micropylar disc made *C. gariepinus* eggs have a unique characteristic shape (a fur cap) that differs from that of other catfish such as *Silurus glanis* (Kobayakawa, 1985; Riehl and Appelbaum, 1991). The micropylar disc of *C. gariepinus* resembled that of *P. mattereri, S. spiloplema, R. aspera, Cichlasoma nigrofasciatum and Polypeteus* spp (Wirz-Hlavacek and Riehl, 1990; Riehl and

Appelbaum, 1991; Bartsch and Britz, 1997; Rizzo *et al*., 2002). In the absence of data that elucidate the mechanism by which eggs of these species adhere to substratum, Riehl and Appelbaum (1991) concluded that the micropylar disc may play a role in their adhesiveness. Similarly, Wenbiao *et al*. (1991) termed the micropylar disc as the attachment disc. As such, the eggs should be fertilised before their attachment to the substratum (Wirz-Hlavacek and Riehl, 1990). The network of projections on the vegetal hemisphere founding *C. gariepinus* leads to the conclusion that these projections might represent another attachment mechanism. In fertilisation experiments of *C. gariepinus*, the animal pole was usually directed upward. Most catfish, including *C. gariepinus,* possess demersal eggs, which become sticky after encountering water. Catfish eggs adhere to substratum with several other methods (Riehl and Appelbaum, 1991). In *Silurns glanis* and two Japanese *Silurus* species (*S. asotus* and *S. biwaensis*), the eggs adhered with a voluminous layer of jelly (Kobayakawa, 1985; Hilge *et al*., 1987; Riehl and Appelbaum, 1991), whereas the eggs of Japanese *Silurus lithophilus* were not adhesive. The jelly layer coat was also present in the adhesive eggs of other siluriformes, including *Ictalurus* spp and *Chrysichthys* spp, in addition to *Silurus* spp. (Sato, 1999; Rizzo *et al*., 2002) and in non-adhesive eggs of siluriformes including *Paulicea luetkeni*, *Pimelodus maculatus* and *Conorhynchus conirostris* (Sato, 1999; Rizzo *et al*., 2002). The adhesive apparatus of *C. gariepinus* was formed of a large number of tiny attaching filaments, which were embedded in a certain cement substance (Riehl and Appelbaum, 1991). Such tiny structures were observed in the present work as microvilli extending from the outer surface of the chorion in embryonic stages before hatching (Fig. 5E).

The spermatozoa of *C. gariepinus* move along the surface of the chorion and enter the micropyle or any micropyle-like depression in a directed fashion (Fig. 7). Receptors of various sources or motility stimulating factors of *C. gariepinus* must be involved in aggregating spermatazoa. Linhart and

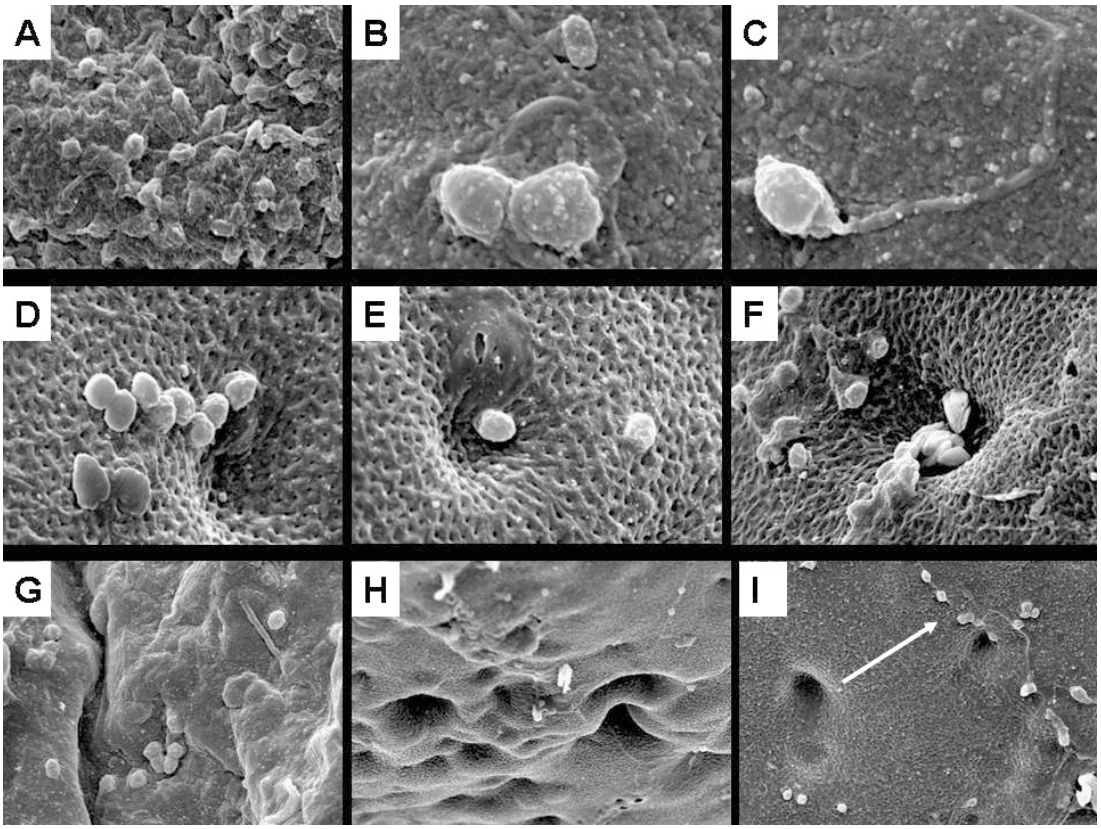

FIG. 7. – Different spermatozoa trapped on the surface of *Clarias gariepinus* eggs in increased number (A) with different white secretion on their heads and tails (B, C). Different spermatozoa of *Clarias gariepinus* swarmed and trapped or embedded in the outer surface of the micropyle-like depressions at the vegetal pole (D-F). Note the pore outer surface of the chorion in this region. The micropyle-like depressions and the folded chorion at the vegetal pole of *Clarias gariepinus* eggs (G-I) with spermatozoa trapped and directed in lines (arrow). (A-I) 30 seconds postfertilisation (A,I: ×2000; B,C: ×15000; D,E: ×7500; F: ×5000; G: ×3500; H: ×1500).

Kudo (1997) reported that the multiplicity of micropyles recorded in some fishes, such as Acipenserid species, was less favourable to the prevention of polyspermy than that of other fish eggs that possess only one micropyle. They also referred to the uncertainty of how the multi-micropylar eggs responded to stimulus of fertilisation by forming a cytoplasmic process underneath several micropyles of the same egg by polyspermy or other mechanisms. Accordingly, the multiplicity of micropyle-like depression of *C. gariepinus* eggs was more beneficial for trapping sperm without the formation of cytoplasmic processes. Brummett and Dumont (1979) stated that the main block to polyspermy in the teleost was inherent in the morphology of the chorion with its single point of entry. Cosson *et al*. (2002) reported that for spermatozoa, the interface trapping mechanism was a very efficient means of increasing their concentration on a surface of the egg instead of their being dispersed in a three-dimensional volume. They also added that this mechanism was highly efficient and crucial for species in which spermatozoa had a very short period of motility to reach the micropyle. Our findings were also corroborated by the observations of experimentally induced polyspermic eggs by Iwamatsu and Ohta (1978) and Ohta (1985), who reported that spermatozoa can bend and enter the teleost egg at locations other than the site of sperm entry. Moreover, in their studies of polyspermic and monospermic fertilised eggs of *Oryzias,* Iwamatsu and Ohta (1978, 1981) described folds of the egg surface that rapidly engulf the sperm in a "Cave-like pit" before the fusion of egg and sperm plasma lemmae, which occurred some 20 s later. The sperm behaviour of *C. gariepinus* on the chorion surface, micropylar area, micropyle-like depression and folded chorion (Fig. 7) indicate that spermatozoa are oriented inherently towards any depression on the chorion surface in addition to their trapping mechanism on the chorion fibres (Fig. 8A, B).

The chorion structure and fertilisation

The ovarian or the ovulated chorion of *C. gariepinus* consisted of one layer under the light

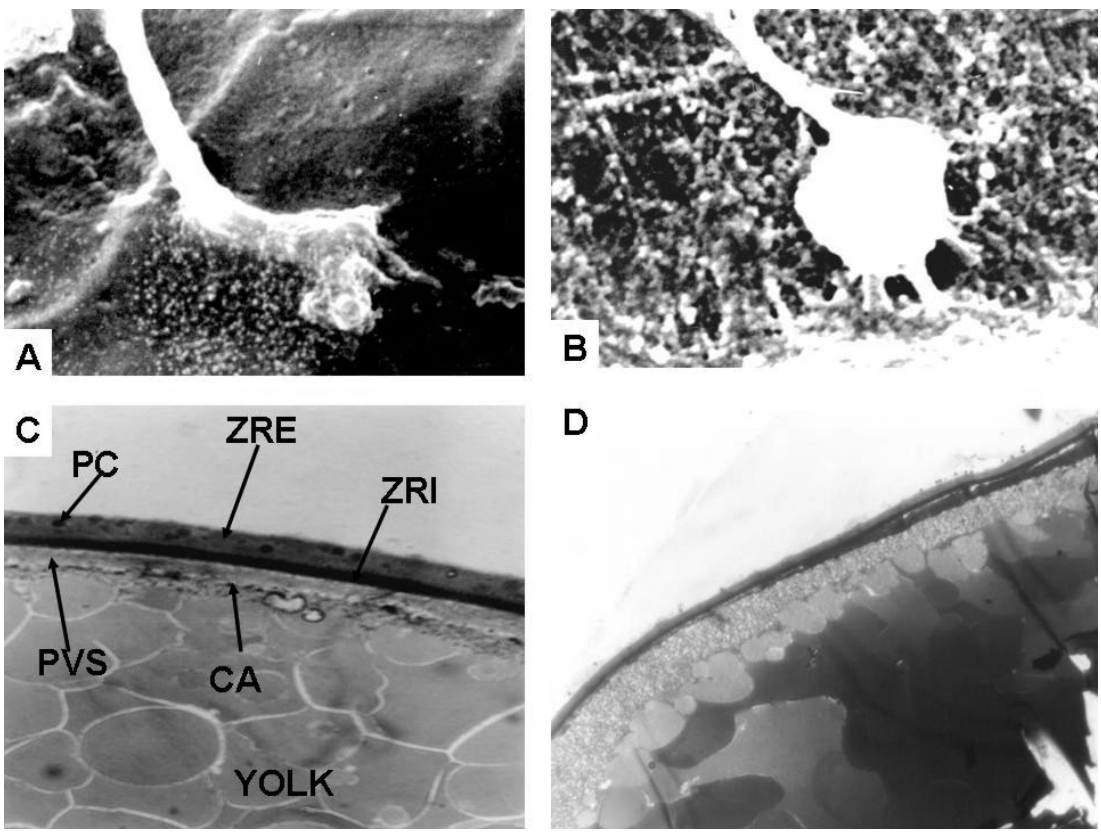

FIG. 8. – Spermatozoon embedded by head on the egg surface (A: ×10000) of *Clarias gariepinus* and one or more extension (B: ×20000) with the chorion surface microvilli (A at 1-h, B at 10 sec postfertilisation). Semithin sections of the fertilised eggs of *Clarias gariepinus* showing differentiation of the single-layered zona radiata (ZR) into zona radiata externa (ZRE) and interna (ZRI) after fertilisation (C: ×1000). Note the decreased thickness of the chorion in D (×1000). CA: cortical alveoli, PC: pore canal, PVS: perivitelline space.

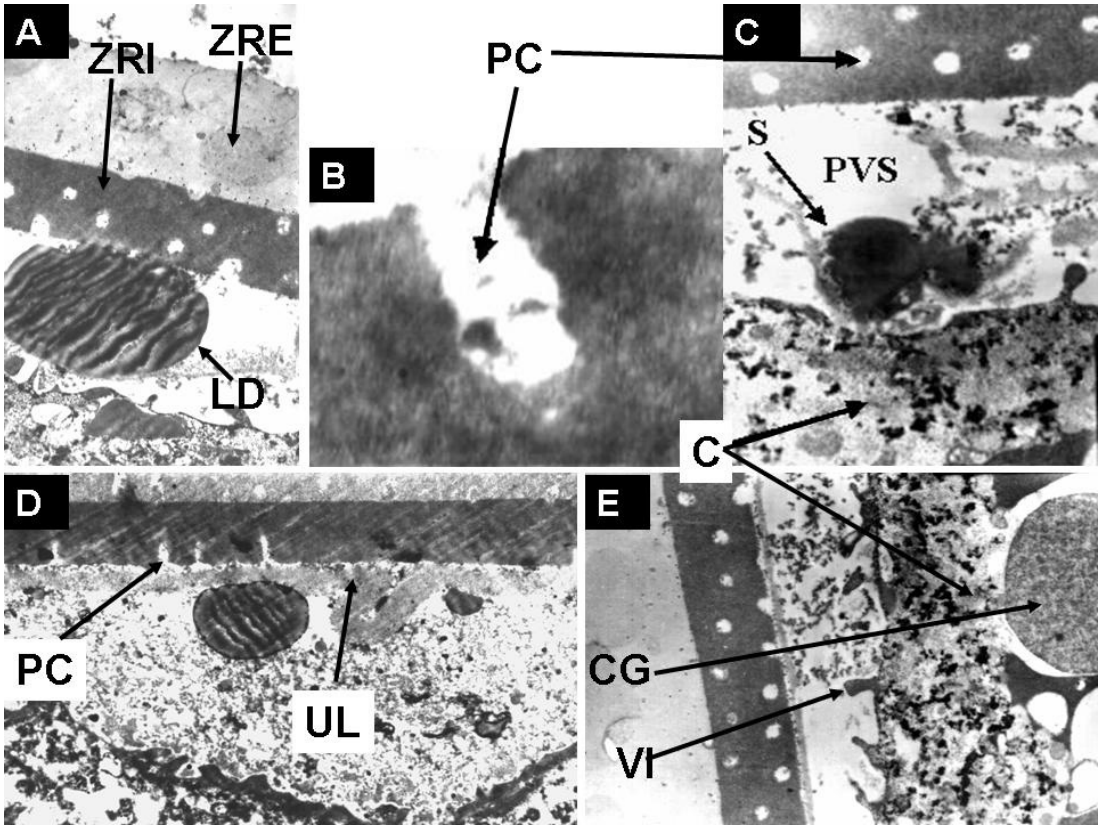

FIG. 9. – Ultrathin sections in the chorion and outer layer of the ooplasm of the fertilised eggs of *Clarias gariepinus* (A: ×6700; B: ×40000; C: ×8000; D: ×4000; E: ×5000) (1-h postfertilisation). PC: pore canal, UL: unknown layer, CG: cortical granule, C: cortex, VI: villii, S: secretion, LD: lipid.

microscope and TEM (Fig. 2C, D). The single-layered chorion of the unfertilised egg of *C. gariepinus* had faint striations (Fig. 2E). Directly after fertilisation, the single zona radiata was differentiated into a zona radiata externa (ZRE) and a zona radiata interna (ZRI) (Fig. 8C, D). At 1/2 h and 1 h after fertilisation, the chorion of *C. gariepinus* was differentiated into three layers (Figs. 9, 10), the double-layered coat (DLC, Figure 10C, E), which might have been involved in the first steps of fertilisation, the middle layer, the zona radiata externa (ZRE) and the innermost layer, the zona radiata interna (ZRI), which was half the thickness of the ZRE. The pore canals were expressed in both ZRE and ZRI. These pore canals and striations were more prominent in ZRI (Fig. 9A-C). Following ZRI, there was an electron opaque layer (Fig. 10). This latter unknown layer invaded the perivitelline space (PVS) and came into contact with the oolemma.

The three layers of the chorion identified by TEM were detected by SEM with elucidation of their pore canals (Fig. 11). The ZRE showed variability in its thickness in comparison with the ZRI on the same egg, especially in the region of the mycropylar disc (Fig. 11D). The ZRE disappeared completely in the micropyle, in the micropylar-like depression and in the vegetable pole (Fig. 11E, F). The lamellar and tubular nature of the ZRI was evident (Fig. 11A, B). The disappearance of the ZRE and the increased tubular nature of the ZRI in the vegetal pole facilitated the embedding of spermatozoa on this region. Griffin *et al.* (1996) reported that for *C. pallasi* eggs the chorion at the animal pole was distinct functionally, structurally and biochemically from the remainder of the surface. The postfertilisation changes of the chorion of *C. gariepinus* reflected the morphological aspect of chorion hardening and fertilisation processes.

Variation in chorion thickness and rigidity with development progress and 2-4 layers, ZRE and ZRI, are common among most fishes (Riehl and Greven, 1993; Kim, 1998; Kim *et al.*, 1993, 1996, 1998a, b, 2001, 2002; Deung *et al.,* 1997, 1999, 2000).

The current postfertilisation morphological changes of the chorion of *C. gariepinus* were associated with cortical reactions and cortical alveoli discharge in the perivitelline space (Fig. 12). Based principally on studies with the eggs of

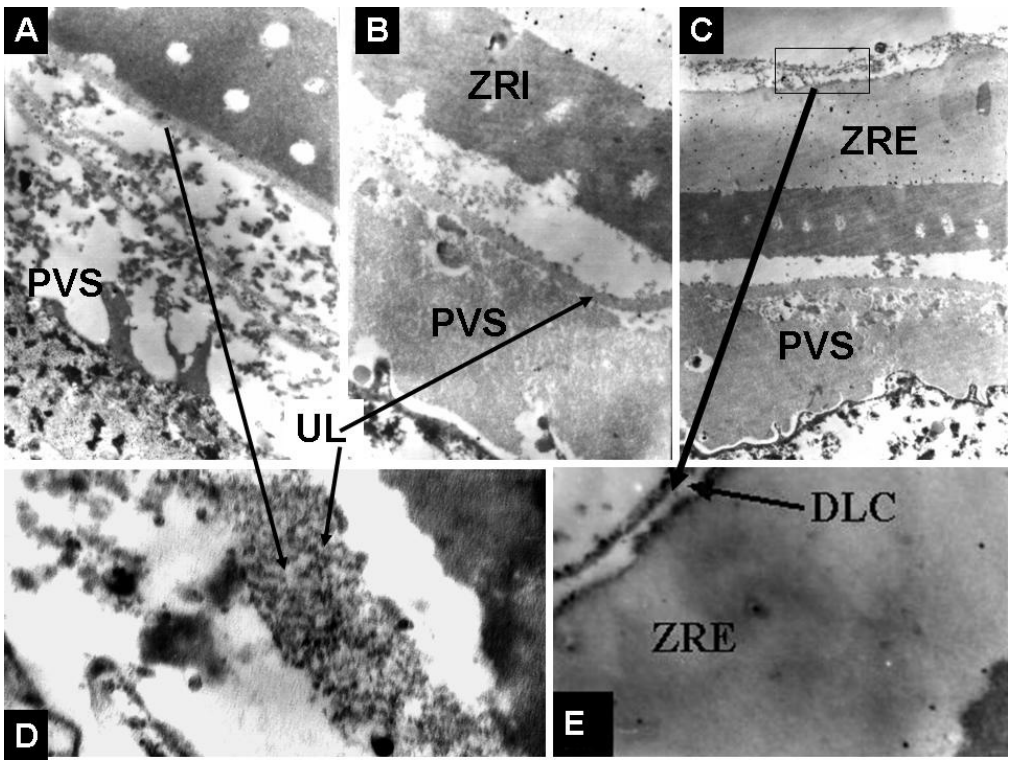

FIG. 10. – Ultrathin sections in the outer layers of the fertilised eggs of *Clarias gariepinus* showing the double-layered coat (mucous coat, DLC) and the unknown layer (UL) and UL-course in relation to ZRI and the plasma membrane (A, B: ×10000; C: ×5000; D: ×40000; E: ×20000) (1-h postfertilisation).

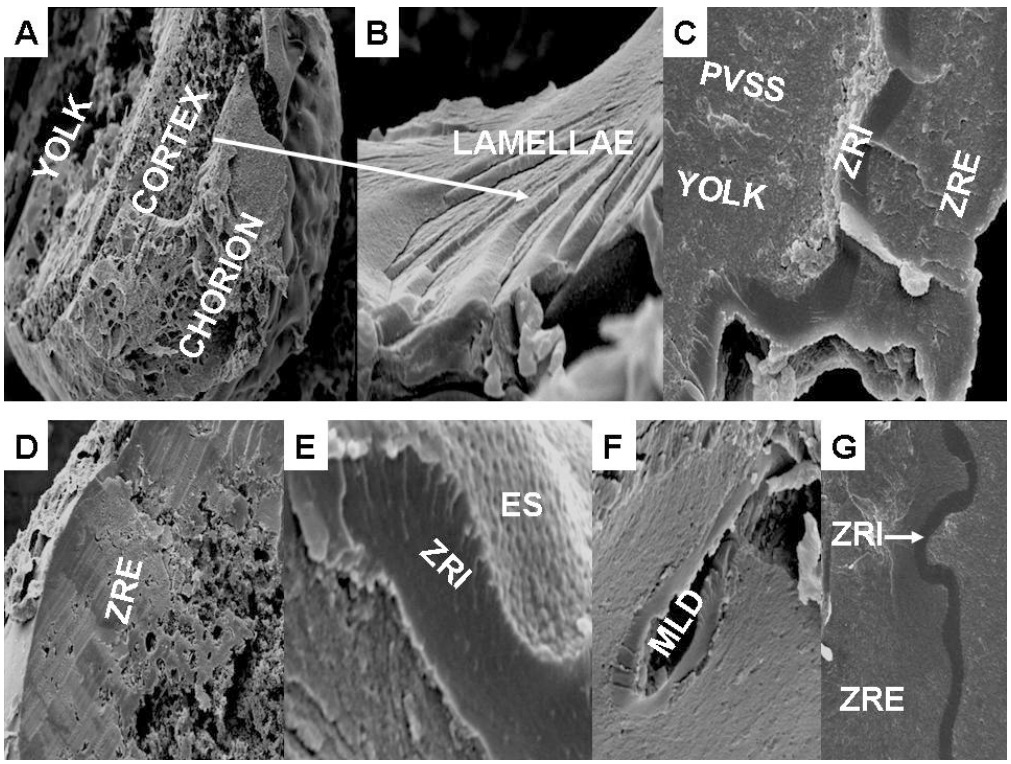

FIG. 11. – Fractured parts of the fertilised eggs *Clarias gariepinus* showing the chorion layers and the cortex. Lamellar nature of ZRI was evident (A: ×150; B: ×5000). The tubular structure of ZRI and to some extent ZRE and secretions in PVS (PVSS) (C: ×2000). Disappearance of ZRE in the vegetal pole and at micropyle-like depression (MLD) (E: ×10000; F: ×2000). Note the tubular nature of ZRI (E). ES: egg surface. The thickness-relationship between ZRE and ZRI especially in the micropylar region (G: ×1500), the great thickness of ZRE in the animal region (D: ×350) and the brushed inner side ZRI (E).

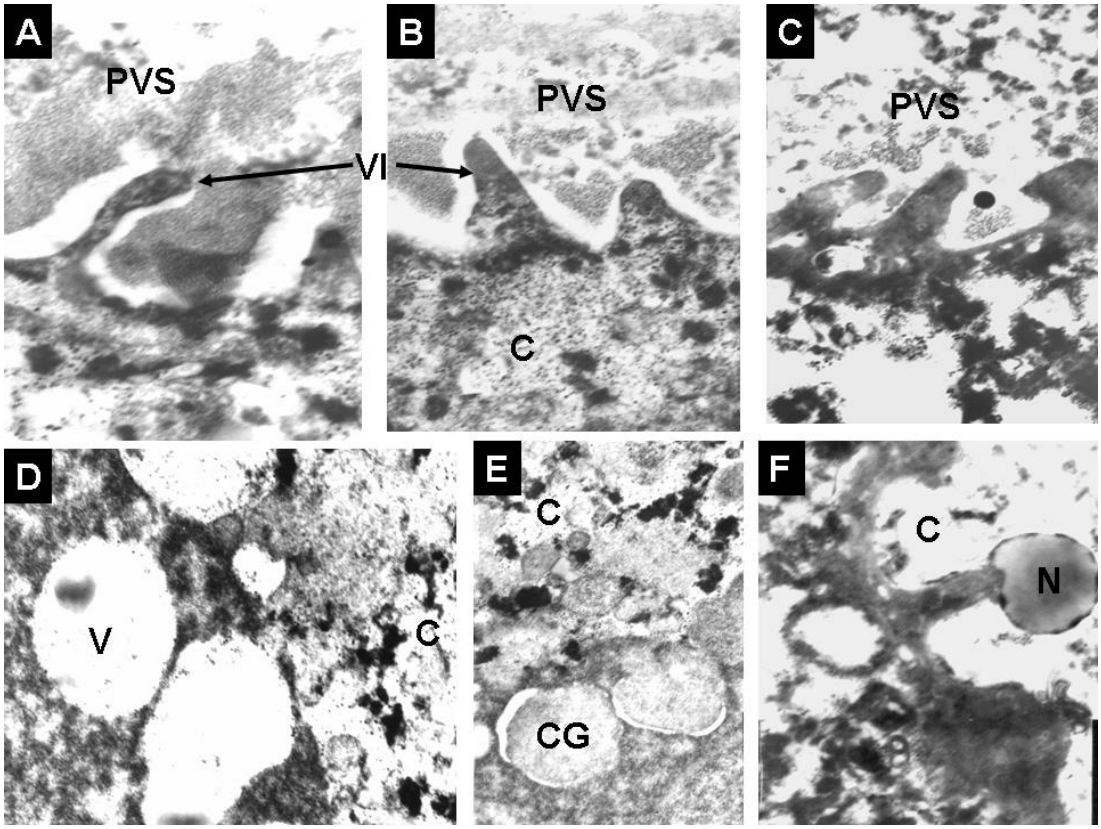

FIG. 12. – Ultrathin sections in the cortex (C) of the fertilised eggs of *Clarias gariepinus* showing cortical alveoli, granules (CG) and their secretion in the perivitelline space (PVS) (A: ×27000; B, C, F: ×20000; D, E: ×14000) (1-h postfertilisation). N: nucleus, V: vacuole, VI: villii.

TABLE 1. – The molecular size of protein subunits of different fish species based on SDS-PAGE.

Fish species	Chorion protein subunits	Author(s)
Oryzias latipes	150, 77-73 and 49 kDa 132, 114, 62 and 61 kDa	Iwamatsu *et al*. (1995)
The cod	78, 54, 47 kDa	Oppen-Berntsen *et al*. (1992)
The pipe fish	109 and 98 kDa	Begovacand Wallace (1989)
The rainbow trout (*Oncorhynchus mykiss*)	60, 55 and 50 kDa	Hyllner *et al*. (1991, 2001)
	110, 64, 56 and 50 kDa	Luchi *et al*. (1991)
	49, 56, 65, 123 kDa	Luchi *et al*. (1996)
The sea bass	170 and 47 kDa	Scapigliati *et al*. (1994)
Epinephelus malabaricus	97 and 76 kDa	Chiou *et al*. (2004)
Oryzias latipes	76, 74 and 49 kDa	Hamazaki *et al*. (1987a,b, 1989)
Carassius auratus	At least 20 proteins (30-250 kDa) (classified into 5 main classes-those of 40-60 kDa region represent the largest class).Glycoprotein components (46, 60, 84, 110 kDa)	Cotelli *et al*. (1988)
Chionodracco hamatus	200, 92, 80, 68, 46 and 40 kDa	Baldacci *et al*. (2001)
Dicentrarchus labrax	44-250 kDa (several peptides)	Scapighiati *et al*. (1994)
Coregonus lavaretus	17-80 kDa (7 peptides)	Scapighiati *et al*. (1995)
Danio rerio	116, 97, 50 and 43 kDa	Bonsignorio *et al*. (1996)
C. pallasi	117&48-54 KDa (90-95% of the polypeptides)	Griffin *et al*. (1996)
Gasterosteus aculeatus	19.4, 36.7, 39.4, 42.9, 46.1, 53 kDa	Deung *et al*. (1999)
Salmo solar	60, 55 and 50 kDa	Oppen-Berntsen *et al*. (1994)
Salmo solar	66, 61 and 55 kDa	Celius and Walther (1998)

echinoderms (Vacquier, 1981), the cortical layer of the egg was considered to be a gel with specialised viscoelastic mechanical properties (Hart, 1990). Moreover, the egg cortex became increasingly contractile after sperm-egg union, causing a twisting movement of oil droplets toward the animal pole; a meshwork of polymerised actin appeared in the egg cytoplasm and microfilaments became highly organised in the microvilli (Vacquier, 1981; Mabuchi, 1983).

TABLE 2. – The chorion protein subunits (bands) identified by SDS-PAGE in the ovulated (OE), hardened OE and post-fertilised (1-minute, 1-hour and 2-hour FE) eggs of C*larias gariepinus*.

Lanes:	MW	1		2		3		4		5	
	MW-marker	Hardened OE		OE		1-minute FE		1-hour FE		2-hour FE	
Bands	MW	MW	%	MW	%	MW	%	MW	%	MW	%
r1	223.0										
r2						133.6	43.1	132.6	47.0	129.7	29.8
r3	110.0	108.9	38.6	108.9	47.8						
r4	81.6										
r5	46.8										
r6						36.4	31.5	35.6	24.6	35.9	35.1
r7	31.8	31.5	32.0	33.7	26.8						
r8						28.3	25.4	28.8	12.0	29.2	15.0
r9	24.8	25.3	16.0	26.9	12.6						
r10	16.5	16.0	13.4	17.0	12.8			17.0	16.4	17.0	20.1

TABLE 3. – The chorion protein subunits (bands) identified by SDS-UREA-PAGE in the ovulated (OE), hardened OE and post-fertilised (2- and 25-minute, 1-, 2- and 3-hour FE) eggs of *Clarias gariepinus* in addition to its semen and sperm proteins.

Lanes:	1	2	3	4	5	6	7	8	9	MW
Bands	Semen	Sperm	3-HFE	2-HFE	1-HFE	25-MFE	2-MFE	Hardened OE	OE	Marker
	Molecular weight									
r1				248.5						
r2	235.3	230.2		231.0						223.0
r3			199.1	188.0	192.8	199.1	200.7	195.9		
r4	183.2	175.3				168.9				
r5	153.0			156.2	159.3		164.1	166.9		
r6		137.1	146.4			133.9		135.9		
r7										100.0
r8	101.8					100.3		95.8	98.8	
r9		92.8		89.8		89.1		89.1		
r10	85.3			86.8		80.3				81.6
r11		74.8						79.3	79.0	
r12		65.5	63.9	65.5	65.0	66.9	65.5	66.7	68.2	
r13	54.6							54.3	54.3	
r14		49.4							48.5	
r15	45.8		46.0	46.2	46.2	46.6	46.6	46.6		46.8
r16		44.9		43.7	43.7	45.1	44.3	44.3	44.5	
r17			43.0		41.6		41.6			
r18	39.9	39.7	40.3	40.8		41.3		41.0	41.3	
r19	35.1	35.1								
r20			31.0		30.8	31.8		31.4		31.8
r21	30.4	29.9		29.9			30.4		29.5	
r22						27.3		26.6		
r23	25.9	25.6	25.4	25.2	25.6	25.6	25.7	25.3	25.2	24.8

Actin and actin-containing filaments have been described in the cortical layer of *Brachydanio* (Wolenski and Hart, 1988) and *Oncorhynchus* (Kobayashi, 1985) eggs. These lectins, as major components in vertebrate cortical granules (Krajhanzl, 1990), are involved in the formation of the egg envelope and in turn in its polyspermy-blocking functions (Quill and Hedrick, 1996). Dong *et al*. (2004) identified a C-type lectin from oocyte of a freshwater fish species *Carassius auratus gibelio*. TEM showed that the cortical cytoplasm of the eggs of *C. gariepinus* contained membrane-limited cortical granules with an internal matrix of varying electron density (Fig. 12). The determination of the chemical and molecular composition of cortical granules is essential to understanding the role of these organelles in fertilisation and early development (Hart, 1990). Cortical reaction seems to be a prerequisite for the chorion hardening process, which has been considered by some authors to be independent of fertilisation (Lönning *et al*., 1984; Davenport *et al*., 1986). There was a precise relationship between sperm behaviour, chorion hardening and cortical reaction of *C. gariepinus*.

Chorion hardening is a process of initiated chain polymerisation of substances within the membrane itself (Hart, 1990) to form insoluble proteins of higher molecular weight (Yamagami *et al*., 1992). The solubility of chorion of unfertilised egg is a requirement for the hardening system or machinery

TABLE 4. – % of the chorion protein subunits (bands) identified by SDS-UREA-PAGE in the ovulated (OE), hardened OE and post-fertilised (2- and 25-minute, 1-, 2- and 3-hour FE) eggs of *Clarias gariepinus* in addition to its semen and sperm proteins.

Lanes:	1	2	3	4	5	6	7	8	9
Bands	Semen	Sperm	3-HFE	2-HFE	1-HFE	25-MFE	2-MFE	Hardened OE	OE
r1				0.8					
r2	3.2	3.7		1.8					
r3			3.2	2.9	3.0	4.2	3.7	5.0	
r4	6.9	3.5				4.0			
r5	4.3			4.7	4.2		3.8	5.6	
r6		6.4	6.5			9.0		4.4	
r7									
r8	16.8					38.6		33.5	62.3
r9		8.1	54.1		59.0		56.3		
r10	13.4			58.2		4.3			
r11		13.0						6.7	2.9
r12		6.5	4.7	3.2	4.7	8.8	6.5	7.7	3.2
r13	12.3							2.3	1.7
r14		11.0							2.2
r15	8.1		4.7	2.2	1.9	2.3	2.2	4.0	
r16		7.5		1.0	1.3	2.4	1.6	2.8	1.5
r17			3.6		2.2		3.4		
r18	11.7	12.1	1.4	2.6		2.2		4.9	2.
r19	3.0	9.2							
r20			12.2		14.6	14.1		14.6	
r21	5.9	5.5		15.0			15.1		19.3
r22						5.3		5.6	
r23	14.2	13.4	9.7	7.8	9.3	4.9	7.4	3.0	4.7

TABLE 5. – The chorion protein subunits (bands) identified by gradient SDS-PAGE in the ovulated (OE), hardened OE and post-fertilised (2- and 25-minute, 1-, 2- and 3-hour FE) eggs of *Clarias gariepinus* in addition to its semen and sperm proteins.

Lanes:	1	2	3	4	5	6	7	8	MW	10
Bands	Sperm	3-HFE	2-HFE	1-HFE	25-MFE	2-MFE	Hardened OE	OE	Marker	Semen
	Molecular weight									
r1	230.6	223.0	249.5	249.5	228.1	246.7	223.0	220.5	223.0	
r2	197.1	190.6				210.8				206.2
r3	168.5						176.2			182.2
r4	134.6	121.7								
r5	111.2	108.5	106.4	103.9		105.4			110.0	
r6		102.9								102.5
r7	99.1	96.8	99.6							
r8	92.7	91.4	94.5	93.6	89.3	92.7				
r9	79.5	84.0	87.2		83.2		84.0		81.6	87.2
r10			73.9	69.8	76.3	77.5	78.3	77.1		70.2
r11	68.0	68.4	64.9	64.6		67.7				
r12				61.0	59.7	61.6	61.6	62.9		
r13	55.0	55.6	57.9		55.0	55.8		55.0		58.8
r14			53.6		51.1	51.1	53.0	51.1		51.7
r15	49.0	49.8		49.8	46.4	45.8	48.5	45.8	46.8	46.7
r16	43.7	45.5					44.7	42.3		43.9
r17	40.5	39.7			38.8					
r18	38.0		38.2	37.8	36.9					36.9
r19	34.9	35.8	34.8	34.4		35.7	35.1	34.6		34.4
r20	33.1	32.0			33.3	32.6	32.0	30.9	31.8	
r21	28.8		30.2			28.3	27.8			27.8
r22		25.7	25.8			26.1		26.7	24.8	25.7
r23		24.0	22.9	24.6	23.6		24.4	24.1		23.1
r24	22.4	22.4		22.3	21.3	22.6	21.9	21.3		
r25	20.9	20.3		21.1	20.1	20.4				20.4
r26		19.4	19.2	19.4	18.9	19.2	19.6	19.2		
r27	18.2	18.2		18.6	17.9		18.3	17.9		18.1
r28	17.3		16.9	17.5			17.3	16.9		
r29			15.9		16.7		16.1		16.5	16.7
r30	14.7				15.2	15.8	15.0	15.5		
r31	14.3		13.9	14.4		14.1		14.4		13.5
r32	12.8	13.0				12.5	13.4	12.7		
r33	12.1		12.3	11.5		11.9	11.9	11.6		
r34	11.2	11.3			11.3	10.8	10.6	10.9		11.4
r35	10.4	10.1		10.5		9.8	10.1			10.4
r36	9.7	9.6				9.3	9.3			
r37	8.9	8.5					8.7			

TABLE 6. – % of the chorion protein subunits (bands) identified by gradient SDS-PAGE in the ovulated (OE), hardened OE and post-fertilised (2- and 25-minute, 1-, 2- and 3-hour FE) eggs of *Clarias gariepinus* in addition to its semen and sperm proteins.

Lanes: Bands	1 1:Sperm %	2 3-HFE	3 2-HFE	4 1-HFE	5 25-MFE	6 2-MFE	7 Hardened OE	8 OE	10 Semen
r1	2.0	2.9	1.1	1.4	2.4	1.6	3.7	1.3	
r2	1.6	3.0				2.8			1.3
r3	3.4						5.2		0.9
r4	4.6	11.9							
r5	3.8	4.5	23.9	20.4		11.7			
r6		4.2							5.8
r7	2.8	5.6	4.9						
r8	5.7	5.1	4.0	19.3	29.2	15.5			
r9	3.9	10.1	5.8		4.4		21.0		4.1
r10			1.8	2.5	13.3	10.0	13.3	36.2	8.5
r11	12.7	5.4	5.8	2.3		4.5			
r12				7.9	3.7	4.7	4.6	4.6	
r13	5.1	2.5	1.4		1.3	2.1		1.6	3.4
r14			4.7		1.8	2.3	2.2	2.3	3.8
r15	4.8	3.1		4.4	2.5	2.8	2.6	2.8	4.8
r16	6.0	2.3					3.5	1.9	8.0
r17	5.3	8.3			5.0				
r18	3.7		13.0	10.5	4.5				4.0
r19	5.0	6.7	8.4	9.6		9.7	7.9	11.7	3.9
r20	3.9	3.0			10.4	8.6	7.6	10.4	
r21	4.2		2.5			1.7	2.4		6.7
r22		2.2	2.3			0.9		1.9	4.5
r23		2.2	7.7	2.5	2.5		1.2	1.5	5.5
r24	5.4	4.6		3.2	4.8	2.4	2.4	2.6	
r25	4.1	2.8		3.5	2.5	4.4			3.0
r26		1.1	2.4	1.0	2.1	6.7	5.2	6.4	
r27	6.4	2.2		1.0	1.8		3.8	3.1	5.7
r28	1.3		1.3	1.5			2.4	2.6	
r29			2.2		1.1		1.6		4.4
r30	1.3				1.2	1.6	2.2	1.9	
r31	0.5		2.9	1.8		0.9		2.9	17.8
r32	0.4	4.2				1.4	1.8	2.0	
r33	0.6		4.0	4.8		1.2	1.8	1.6	
r34	0.5	0.8			5.5	1.2	1.3	0.6	2.2
r35	0.3	0.8		2.5		0.8	1.2		1.7
r36	0.4	0.3				0.6	0.8		
r37	0.2	0.3					0.3		

incorporated in the egg envelope (Quagio-Grassiotto and Guimaraes, 2003). The electrophoretic profile showed interesting similarities among some but not all. The variability reported for teleost chorion (Table 1) might be explained by species difference (Griffin *et al*., 1996). Also, variability might be due to the difficulty of extracting certain polypeptides from the teleost chorion (Oppen-Berntsen *et al*., 1990).

Four protein subunits of the chorion of *C. gariepinus* were identified by SDS-PAGE: three proteins of low molecular weights (MW) and one protein of relatively higher MW that had the highest percentages, 29.8-47.89% (Tables 2-6). Hardening of ovulated eggs resulted in no or minor variation in the MW. Fertilisation influenced the chorion proteins of the first three categories (108.93 to 129.7-133.6; 31.5-33.7 to 35.6-36.4; 25.3-26.9 to 28.3-29.2 kDa). According to SDS-UREA-PAGE, more chorion protein subunits were identified, especially those of higher MW (Tables 3, 4). The chorion protein of higher MW detected by normal SDS-PAGE was 86.8-100.3 kDa and represented the highest percentage, 28.3-52.9%. Two other categories of protein subunits were identified: one with relatively low MW (5.2-80.3 kDa) and another with higher MW after hardening and fertilisation (133.87-248.46 kDa). More protein subunits were also recorded by gradient SDS-PAGE (Tables 5, 6); each was represented by a low percentage. Different analyses resulted in different patterns of protein subunits.

Chemical analysis of chorion protein subunits of *C. gariepinus* from eggs 2 min to 3 h after fertilisation by partial hydrolysis indicated the raw material of the polymerisation process. Polymerisation in influenced by the length in time of chorion hardening. Zotin (1958) indicated that the chorion began to harden from between <1 min of insemination to 2 to

4 h in salmonid and coregonid eggs, whereas Hart (1990) found that maximum hardening of the chorion was reached within 3-7 days in the trout and 1-2 days in the whitefish. For lump sucker (*Cyclopteurs*) and cod (*Gadus*), chorion hardening started shortly after exposure to sea water and reached a maximum resistance by about 24 h (Lönning *et al*., 1984). Hardening in these two salt water species did not require fertilisation (Hart, 1990).

Iconomidou *et al*. (2000) and Hamodrakas *et al*. (1987) indicated that the β-pleated sheet was the molecular conformation of protein macromolecules that constitute the fibrils and of fibrils of the egg shell with a helicoidal architecture. These studies did not deal with the interaction between chorion hardening, fertilisation and progress in development. Further spectroscopic studies must be done to demonstrate a relationship between chorion hardening and the time of development based on different amide ranges.

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