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Description of Eggs and Larval Stages of *Fasciola*, Light and Scanning Electron Microscopic Studies

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Abstract: Light Microscopy (LM) and Scanning Electron Microscopy (SEM) were used to study the egg and miracidium of *Fasciola gigantica* and redia, cercaria and metacercaria from field infected *Lymnaea (cailliaudi) natalensis* snails, to analyze the differences between eggs and intra-molluscan stages of *F. hepatica* and *F. gigantica* in a zone of sympatry. The egg of *F. gigantica* has an umbilicus-like invagination at the posterior end of the egg shell. The emerged miracidium has an elongated conical body that has a broad anterior end and tapering posterior end. The surface was found to be covered with varied lengths of cilia except regions of lateral connection of epidermal plates. The redia of *Fasciola* sp. has a caudal papilliform process. Tail of cercaria was found to be provided with two fin folds. Steps of encystation of the cercaria were described as a variable morphological change in cercarial body and cyst wall.

Key words: *Fasciola*, eggs, larval stages, light microscopy, scanning electron microscopy

INTRODUCTION

Fascioliasis is a worldwide problem caused by the liver fluke (Dalton, 1999; Hurtrez *et al*., 2001). In Egypt, this re-emerging disease is caused mainly by *Fasciola hepatica* and *F. gigantica* (Lotfy *et al*., 2002). It is a serious disease of herbivorous animals (Torgerson and Claxton, 1999), leading to huge economic losses in live-stock production, while human infection has long been seemed to be accidentally. Fascioliasis has recently appeared as an outstanding health problem in Egypt, causing severe illness of human liver (Farag *et al*., 1979; Farag, 1998), which is unfortunately suffering also from schistosomiasis (Esteban *et al*., 2003). Studies on fasciolosis are therefore required to understand the epidemiology of this re-emerging disease (Hurtrez *et al*., 2001).

Many studies have been undertaken in medical and veterinary parasitology, as well as in molecular biology (Dalton, 1999). Surprisingly, there is still much to be discovered in morphology of the larval stages for a better understanding. The larval stages of *F. hepatica* had been widely studied (Andrews, 1999), while those of *F. gigantica* have been only partly described. However, it is extremely important to have reliable criteria to distinguish the two species in zones of sympatry, like in Egypt (Lotfy *et al*., 2002). A lot of studies deal with the

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measurements and shape of the eggs of *Fasciola* sp. Eggs of *Fasciola* consist of a fertilized ovum with vitelline cells surrounded with proteinous shell. Eggs of *F. hepatica* are operculated with characteristic yellowish brown colour (Andrews, 1999). They are not readily differentiated from eggs of *F. gigantica* (Chen *et al*., 1990). The miracidium of *F. hepatica* (130×28 μm) comes out of the egg and swims at great speed. It has a conical shaped body covered with cilia (Malek, 1980). Formation of sporocyst, the different generation of rediae, morphologically and the conditions to produce cercariae, the structure and development of cercariae and steps of encystation had been studied (Koie *et al*., 1976; Malek, 1980; Spithill *et al*., 1999). Metacercariae have a big importance for parasitologists so the shape, structure, the size, the life spane, the encystation and excystation steps and infectivity were studied (Robert, 1950; Dinnik and Dinnik, 1956; Dawes, 1959; Kendall, 1965; Wilson and Draskau, 1976; Koie *et al*., 1976, 1977; Malek, 1980; Beaver *et al*., 1984; Yadav and Gupta, 1988; Chen *et al*., 1990; Rakotondravao *et al*., 1992; Abrous *et al*., 1998; Srimuzipo *et al*., 2000). Koie *et al*. (1977) have studied the redia of *F. hepatica* but they had not recorded any process at the posterior end, although Krejci and Fried (1994) reported papilliform process at the posterior end of the rediae of *Echinostoma caproni* and *E. trivolvis*. Belding (1965) described the cercariae of *Fasciola*, but they have not recorded any fin folds like structure. Koie *et al*. (1977) reported that the tail of *F. hepatica* cercaria is provided with dorsolateral folds. Krejci and Fried (1994) reported fin folds but they are two in dorsal position in both *Echinostoma caproni* and *E. trivolvis*, although they have one ventral fold and two ventral folds, respectively.

So, the aim of this study is to analyze the differences between eggs and intra-molluscan stages of *F. hepatica* and *F. gigantica* in a zone of sympatry.

MATERIALS AND METHODS

The sexually mature *Fasciola* were collected from different slaughterhouses in Qena governorate in Egypt. The study has been done within a period between 1993-1997. The collected flukes were washed several times in saline solution to be free from the debris; dissecting needle was used in extraction of intrauterine eggs. 50-100. According to Drury and Wallingten (1980), the eggs were fixed in 10% neutral formalin and mounted in Kaiser's glycerol-jelly (aqueous media). A considerable amount of eggs (more than 50) were put in small Petri dish in an incubator at 28°C for hatching. The obtained miracidia were studied with light microscopy.

L. (cailliaudi) natalensis snails were collected, according to Frandsen (1983) from suitable places and carried in plastic containers to the laboratory, identified and counted. Emergence of cercariae was induced by placing groups of snails under indirect sunlight or artificial illumination in glass containers with dechlorinated tap water for half an hour, according to Frandsen and Christensen (1984). In case of emergence of cercariae, the snails were subsequently transferred individually to small beakers and the above scheduled procedures were repeated. According to Khalifa (1972) and Hassan (1987), biological characters and structure were studied for identification of these cercariae. According to Abdel-Ghani (1958), Willomitzer (1974) and Schillhorn-Van-Veen (1980), the shell of infected snails was crushed gently and the fleshy part was dissected. The different intramolluscan stages were transferred to a glass slide for detailed examination. For light microscopic studies, miracidiae were fixed in 5% neutral formalin, cercariae, rediae and metacercariae were fixed in 10% neutral formalin for 2 h. Staining was done according to Drury and Wallington (1980) using Kirkpatrick's carmalum staining method. Differentiation, dehydration, clearing

and mounting were carried out. Preparation of samples for scanning electron microscope was done as follows: eggs or larval stages of *Fasciola* spp. were fixed in 5% glutaraldehyde and dropped in sodium cacodylate buffer (pH 7.3) for 48 h. The samples were washed 3 changes in the same buffer. Postfixation was done by adding 1% osmium tetroxide for 2 h. The samples were washed again in the same buffer 3 times. Dehydration was done in ascending concentrations of ethanol. The excess alcohol was withdrawn after passing from water to amylacetate. The specimen was placed in a chamber where the liquid carbon dioxide is used to substitute for amylacetate, then heated to 35°C to separate carbon dioxide from the specimens and then mounted on holders. Sputter coating (using gold) was done immediately after critical point. It took about 2-3 min and then the specimens were used for examination with scanning electron microscope.

RESULTS

F. gigantica Eggs

Eggs are large yellowish and operculated with thin shell. It has a distinct, flat operculum and an umbilicus-like invagination at the posterior end of the shell (Fig. 1). Using SEM in studying the eggs has shown the outer surface of the egg shell to be smooth and devoid of any microspines and highly conspicuous umbilicus-like invagination on the shell in the opposite side of the operculated end (Fig. 2 and 3). This umbilicus is sometimes containing some fine debris taking different shapes in SEM photos. This debris may give a false impression of a knob in some eggs seen by light microscopy.

F. gigantica Miracidium

The emerged miracidium swims rapidly in aimless directions. It has an elongated conical body that has a broad anterior end and tapering posterior end. The surface is covered with numerous cilia, except in lateral connection regions of epidermal plates. These cilia are found

Fig. 1: Light micrograph of egg of *F. gigantica* showing flat small operculum (short arrow) and an umbilicus-like invagination (arrowhead). X 400

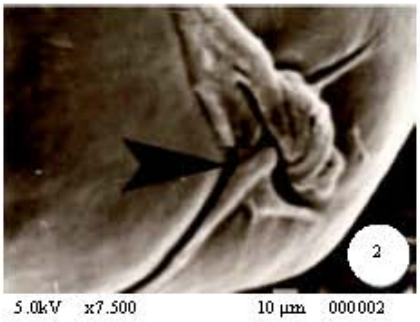

Fig. 2: Scanning electron micrograph of the egg showing an umbilicus-like invagination (arrowhead). X 5.000

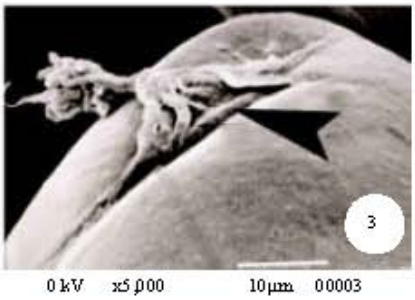

Fig. 3: Scanning electron micrograph of the egg showing another shape of the umblicus-like invagination (arrowhead). X 5.000

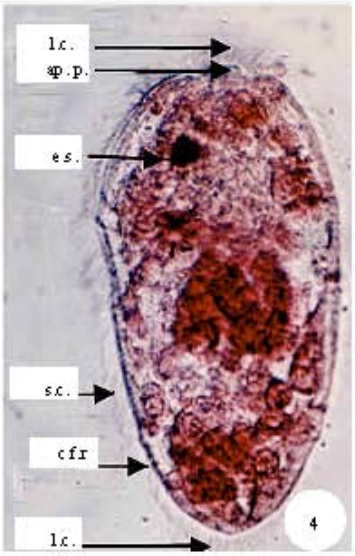

Fig. 4: Light micrograph of miracidium of *F. gigantica* showing apical papilla (ap. p.) and varied length of cilia; anterior and posterior long cilia (l. c.), eye spot (e. s.), short cilia (s. c.) and cilia free region (c. f. r.). X 400

to be characteristically longer on the apical part of the anterior end and the posterior extremity than the cilia on the rest of the body. There is an apical or boring papilla, on which open one apical gland and two pair of penetration glands. There is one pair of eye spots at the right side of midline of anterior part (Fig. 4). The miracidium has one pair of flame cells situated at the end of second third of the body. Each flame cell leads to a tubule which goes down to the lateral side to end in an excretory pore. Germ cells are scattered in the posterior part. Studying the miracidium with SEM has shown the typical pyriform shape of the body. The apical papilla is shown in the middle of the anterior broad part, while the whole surface of the body was illustrating dark pits with variable size (Fig. 5).

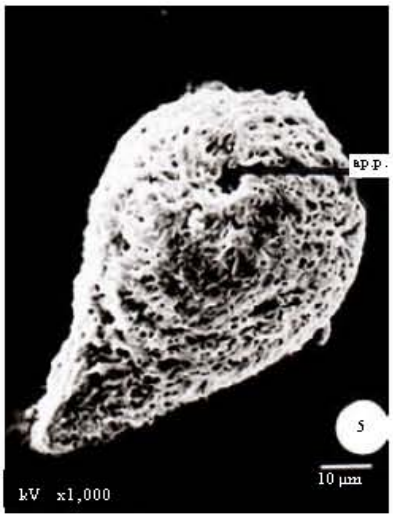

Fig. 5: Scanning electron micrograph of the miracidium showing the conical body shaped, apical papilla (ap. p.) and many pits on the surface. X 1,000

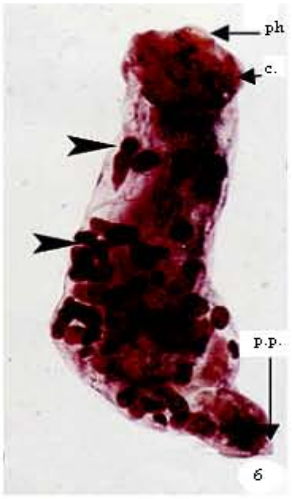

Fig. 6: Light micrograph of mother redia of *Fasciola* sp. showing the muscular pharynx (ph.), collar (c.), germ cells (arrow heads) and a caudal papilliform process (p. p.). X 401

Description of Larval Stages of *Fasciola* sp. Obtained from Field Infected Snails

Redia

Mother redia has an elongated, flat body with an anterior projecting circular ridge or collar and ended with caudal papilliform process (Fig. 6). It has a muscular pharynx followed by a simple sac-like intestine (gut). The mother redia contains undifferentiated structures and germ cells. Mother redia measures 1.47 - 1.86 (av 1.65) mm in length and 0.39-0.59 (av 0.47) mm in width. The gut length varied between 0.68-0.95 (av 0.81) mm in length, (it occupy about two thirds of the total body length). Daughter redia (Fig. 7) has a long, cylindrical body but hasn't collar and the caudal papilliform process is inconspicuous. It has two posterior processes (lappet) at the beginning of the posterior third of the body. It contains developing cercariae and germ cells. The alimentary canal begins with mouth, which leads to suctorial pharynx, followed by a short and simple gut. There is a birth pore at the anterior end through it the developed cercariae emerge. Daughter redia measured 1.26-3.01 (av 1.65) mm in length by 0.16-0.37 (av 0.29) mm in width. It has a short gut that measures 0.42-1.02 (av 0.66) mm in length (occupy about one-third of the total body length). SEM study of the daughter redia revealed the conspicuous suctorial pharynex with rounded muscular walls (Fig. 8, 9).

Cercaria

The emerged cercaria in water swims actively in aimless direction. It has a large heart shaped body and simple long tail. The body has a characteristically thick wall (Fig. 10) and is surrounded by minute spines all over its surface. The body length ranged between 0.15-0.21 mm (mean 0.18 mm) in length and 0.18-0.24 mm (mean 0.20 mm) in width. The tail length is nearly three times as the body, as its length ranges between 0.56-0.7 mm (mean 0.61 mm) its width was 0.35-0.056 mm (mean 0.048 mm). The ventral sucker is larger than the oral sucker. The former varied from 0.028-0.039 mm (mean 0.033 mm), while the latter 0.037-0.048 mm (mean 0.041 mm). The rudiment of the alimentary canal consists of a mouth followed by pharynx surrounding the oesophagus that leads to intestine. The latter bifurcates into two simple branches that extend around the ventral sucker to a level below the posterior border of the ventral sucker. The genital primordium is dumble-shaped with two

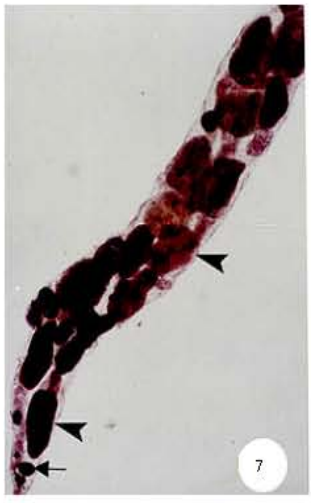

Fig. 7: Light micrograph of daughter redia of *Fasciola* sp. showing developing cercariae (arrowheads) and germ cells (short arrow). X 40

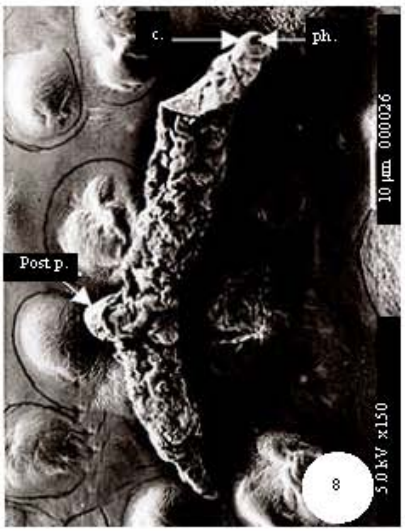

Fig. 8: Scanning electron micrograph of daughter redia showing pharynx (ph.), collar (c.) and a posterior process (post. p.). X 150

Fig. 9: Scanning electron micrograph of the anterior end of daughter redia showing the muscular pharynx (ph.) and collar (c.). X 750

unequal rounded or oval masses connected together with a longitudinal bar. It is located between the upper surface of the excretory vesicle and the upper border of the ventral sucker. The body is full of numerous cystogenous glands. Due to the thick body wall and the densely deposited cytogenous glands, it was difficult to visualize the excretory system and flame cell formula. Study of the cercaria with SEM has shown that the body is concave ventrally (Fig. 11) and the tail is provided with two fin-like processes at its lateral sides (Fig. 12).

Fig. 10: Light micrograph of cercaria of *Fasciola* sp. showing the general characters. X 100

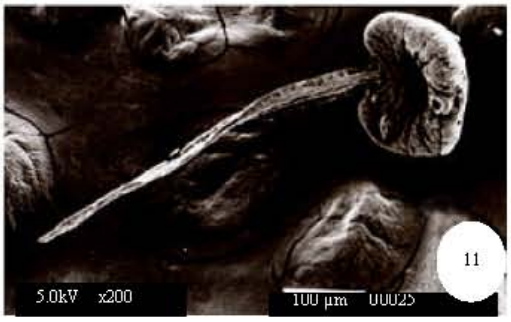

Fig. 11: Scanning electron micrograph of the cercaria, ventral view showing the general shape and ventral concavity of the body. X 200

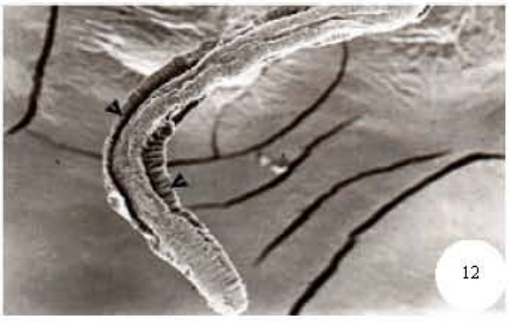

Fig. 12: Scanning electron micrograph of tail of cercaria showing two fin folds (arrowheads). X 500

Cercarial Encystation

Encystation of cercariae occurred in the following steps:

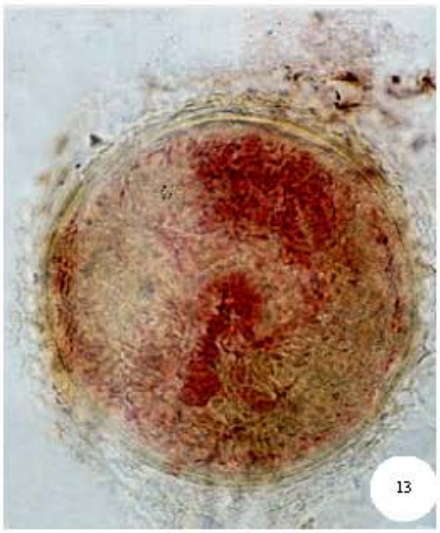

Fig. 13: Light micrograph of recently formed metacercaria of *Fasciola* sp. X 400

Fig. 14: Scanning electron micrograph of the metacercaria, showing the general shape and the felt-work filaments. X 350

- Just after attachments to a suitable object, or even in water (if this was not available) and while the tail is still lashing vigorously from side to side, a thin irregular wall starts to be secreted around the body from the cystogenous glands
- Tail becomes separated from the body while the cystogenous glands secretions become more accumulated in several layers usually four around the more or less rounding-up body. At that stage, many of the internal structures of the cercarial body were easily seen through the cyst wall particularly the suckers and the genital primordium (Fig. 13)
- The wall becomes more and more thick and the cercarial body becomes curled on itself in the cyst, which become slightly smaller in size but with a well developed cyst wall. At that stage it is difficult to differentiate any of the internal structures of the body

Metacercaria

Metacercaria has a double thick cyst wall. The recently formed metacercaria has larger diameter than the mature one where the former diameter ranging between 0.224-0.272 mm (mean 0.257 mm) and the latter's diameter ranging between 0.215-0.256 mm (mean 0.235 mm). Non-infected *L. natalensis* snail swallowed a few number of metacrecariae, which were later on passed out in a linear shape mixed with the snail faeces (Fig. 13). Study of the metacercaria with SEM has shown that the outer layer consists of felt work filaments (Fig. 14).

DISCUSSION

Koie *et al.* (1976) had not reported the umbilicus-like invagination in the eggs of *F. hepatica* when they study these eggs with stereoscan microscope, but Allam (1992) had been differentiate between the eggs of *F. gigantica* and *F. hepatica* according to the measurements. Krejci and Fried (1994) mentioned the presence of abopercular knobs of the eggs of *Echinostoma caproni* and *E. trivolvis*. The measurements of *F. hepatica* eggs depend on the host (Abrous *et al.*, 1998; Srimuzipo *et al.*, 2000). The present work included light and scanning electron microscopic studies of eggs and larval stages of *Fasciola*. This study showed that eggs of both species have an umbilicus-like invagination at the posterior rounded end of the shell. Miracidia and all intramolluscan stages and encysted metaceracrial formation. This was shown by SEM pictures (also with light microscope) may be for the first time during the present study. Most of the previous work on miracidia was done on that of *F. hepatica*. It has been also shown that cilia on anterior and posterior borders of the miracidium are particularly larger than on the rest of the body. Also, disconnections of the miracidial cilia were clearly seen. The SEM also illustrated the miracidium. Koie *et al.* (1977) have studied the redia of *F. hepatica* but they had not recorded any process at the posterior end of the rediae, although Krejci and Fried (1994) reported papilliform process at the posterior end of the rediae of *Echinostoma caproni* and *E. trivolvis*. In the present study, mother and daughter redia of *Fasciola* sp. were described by light microscopy and SEM, which recorded presence of caudal papilliform process (conspicuous in mother redia and inconspicuous in daughter redia) in the caudal end of the egg shell. Belding (1965) described the cercariae of *Fasciola*, but they have not recorded any fin folds like structure. Koie *et al.* (1977) reported that the tail of *F. hepatica* is provided with dorso-lateral folds. Krejci and Fried (1994) reported fin folds but they are two in dorsal position in both *Echinostoma caproni* and *E. trivolvis*, although they have one ventral fold and two ventral folds, respectively. During the present study cercariae of *Fasciola* sp. seemed to be not different from previously described forms. However, detail structure of the genital premordium is given. Moreover, SEM has shown a characteristic sochet at the posterior border of the body in which the tail is usually attached. The tail was also shown for the first time in *F. gigantica* cercariae to be provided with two lateral fin folds, which might to have a great help in swimming of the cercariae. Boray and Enigk (1964) and Dixon (1966) described the encystations process, most of their studies were concentrated on the wall of the metacercariae. During the present study the steps of metacercarial encystations and the variable morphological changes in cercarial body and cyst wall during the steps of encystation were described. Moreover, SEM has shown that the outer layer consisted of felt work filaments. Yadav and Gupta (1988) reported no affection on the viability of *F. gigantica* metacercariae ingested by non-infected *L. natalensisi* snails. The present data confirmed this work that the metacercariae were found to be passed unchanged with faeces of the snail.

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