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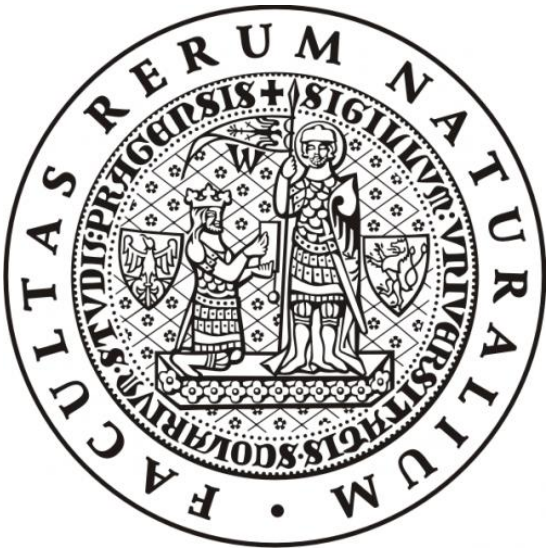


# Development of surface and body musculature of the bird schistosome *Trichobilharzia regenti*

Ph.D. Thesis

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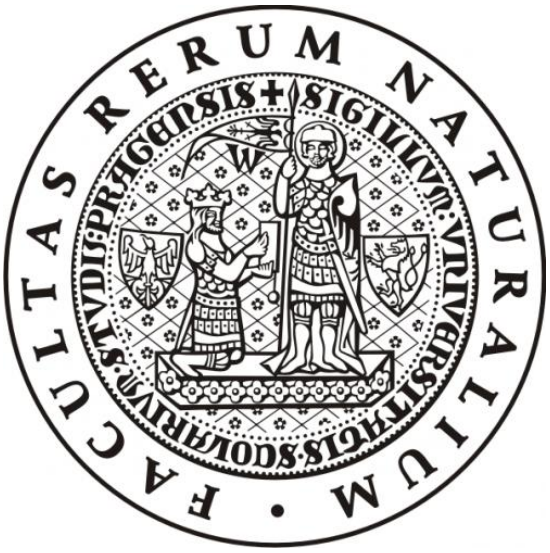


# Vývoj povrchu a tělní svaloviny u ptačí schistosomy *Trichobilharzia regenti*

Dizertační práce

**Mgr. Jana Bulantová**

Školitel: **Prof. RNDr. Petr Horák, Ph.D.**

Prague 2012

**Declaration**

Herewith, I declare that the present thesis summarizes the results of experimental work done by my own or in collaboration with co-authors of the presented original papers. All the other data cited from literature and used in the introductory part are referred to in the list of references. The thesis has not been used as a final work towards any other university degree.

**Prohlášení**

Prohlašuji tímto, že předkládaná práce je souhrnem výsledků dosažených mnou nebo ve spolupráci se spoluautory přiložených článků. Závěrečnou práci jsem zpracovala samostatně a uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Prague…… 2012 Mgr. Jana Bulantová

I declare that the essential data presented in the thesis are results of experiments and ideas of Jana Bulantová. Jana has substantially contributed to the experimental work as well as the writing of the manuscripts.

Prohlašuji, že podstatná část experimentálních výsledků i teoretických závěrů prezentovaných v této práci byla dosažena Janou Bulantovou. Jana se významně podílela na výzkumu modelového organismu i na sepsání přiložených publikací.

Prague……2012 Prof. RNDr. Petr Horák, Ph.D.

**Poděkování**

Na tomto místě bych chtěla poděkovat hlavně svému školiteli Prof. RNDr. Petru Horákovi, Ph.D. za cenné rady a připomínky, kterými významně přispěl ke zdárnému průběhu celého mého studia. Děkuji mu i za trpělivost, se kterou toleroval moje nadšení pro ostatní parazitologické směry, které s tématem dizertační práce souvisely často jen velmi, velmi okrajově.

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**ABSTRACT**

Description of *Trichobilharzia regenti* as a new species of nasal bird schistosome in 1998 was only the first step in our knowledge of this extraordinary parasite. Natural definitive hosts of *T. regeni* are anseriform birds, but infective larvae – cercariae – are able to penetrate also into mammalian hosts including humans. There they are causative agents of hypersensitive skin immune reaction called cercarial dermatitis or swimmer's itch.

Contrary to other schistosomes, miracidia of *T. regenti* hatch directly inside the definitive host tissue. Schistosomula migrate through the nervous system of vertebrates and, together with adult worms, they have predominantly extravascular localization in definitive hosts. Adult worms have a short lifespan and low degree of sexual dimorphism, connected with lower dependence of adult females on long-term contact with males.

During the life cycle, *T. regenti* can be found within three different environments (freshwater, tissue of intermediate molluscan host and tissue of vertebrate host). Each of the seven developmental stages has a different role in the life cycle which corresponds with different organization of various organ systems.

The introductory part of the thesis is focused entirely on ontogenetic changes of surface ultrastructure and body musculature of particular stages of *T. regenti* and other (especially human) schistosomes. The attached publications are then concentrated on tegumental transformation of schistosomula *in vitro*, changes in arrangement of body musculature during *T. regenti* life cycle and localization of dominant antigenic structures on the surface and in the body of cercariae, schistosomula and adult worms of *T. regenti*.

Due to close taxonomical relationship and similar life cycle, many characteristics of surface tegument and body musculature of *T. regenti* resemble those of human schistosomes. Mechanism of gradual transformation of the cercarial head organ to the oral sucker of schistosomula, or presence of radial muscle fibers in cercarial body and subsequent increase of their number in schistosomula and adult worms have newly been described for *T. regenti*. Based on photodocumentation from accessible articles, the above mentioned changes of muscle organization concern not only *T. regenti*, but presumably also the entire family Schistosomatidae.

The most apparent differences between the teguments of human schistosomes and *T. regenti* can be found in adult worms. While in human schistosomes the male tegument has usually more complicated topography compared to females, both genders of *T. regenti* show similar tegumental ultrastructure.

**ABSTRAKT**

Popis nového druhu ptačí schistosomy *Trichobilharzia regenti* v roce 1998 byl prvním krokem k poznání tohoto výjimečného parazita. Přirozenými hostiteli *T. regenti* jsou vrubozobí ptáci; infekční larvy – cerkárie – jsou však schopné náhodně penetrovat i do savčích hostitelů včetně lidí, u kterých pak způsobují hypersenzitivní kožní imunitní reakci zvanou cerkáriová dermatitida.

Na rozdíl od ostatních schistosom se miracidia *T. regenti* líhnou z vajíček přímo ve tkáni definitivních hostitelů. Schistosomula migrují nervovou soustavou obratlovců a stejně jako dospělí červi se v tělech definitivních hostitelů vyskytují takřka výhradně extravaskulárně. Dospělí červi se dožívají pouze nízkého věku a je u nich jen málo rozvinutý pohlavní dimorfismus. S tím souvisí i nižší závislost dospělých samic na trvalém kontaktu se samci.

Během životního cyklu se *T. regenti* vyskytuje ve třech odlišných typech prostředí (voda, tkáň mezihostitelského plže, tkáň obratlovčího hostitele). Každé ze sedmi stadií plní v životě parazitického červa odlišnou funkci, a tomu odpovídají i rozdíly v uspořádání různých orgánových soustav jednotlivých stadií.

Úvodní část dizertační práce je zaměřena výhradně na porovnání výsledků studia ontogeneze povrchu a tělní svaloviny u jednotlivých stadií *T. regenti* a ostatních druhů schistosom, zejména těch lidských. Předložené publikace se pak detailněji zabývají *in vitro* transformací tegumentu schistosomul *T. regenti*, změnami v uspořádání tělní svaloviny během životního cyklu *T. regenti*, a lokalizací imunodominantních antigenů na povrchu i v tělech cerkárií, schistosomul a dospělců *T. regenti*.

Vzhledem k taxonomické příbuznosti a podobnému životnímu cyklu se *T. regenti* v mnoha charakteristikách povrchového tegumentu i podpovrchové svaloviny podobá lidským schistosomám. Zcela nově byl u *T. regenti* popsán proces postupné přeměny hlavového orgánu cerkárií na ústní přísavku schistosomula, nebo distribuce radiální svaloviny v tělech cerkárií a její nárůst u schistosomul a dospělců. Na základě fotodokumentace z dostupných publikací lze ale předpokládat, že výše zmíněná pozorování změn v uspořádání svaloviny se netýkají pouze *T. regenti*, ale celé čeledi Schistosomatidae.

Nejvýznamnější rozdíly mezi tegumentem lidských schistosom a ptačí schistosomy *T. regenti* jsou patrné u dospělých červů. Zatímco u lidských schistosom mívá tegument samců oproti samičímu složitější topografii, u *T. regenti* si je ultrastruktura tegumentu u obou pohlaví vzájemně velmi podobná.

# 1. INTRODUCTION

Schistosomes are ranked among the most important parasitic helminths with a significant pathological effect on millions of people in tropical areas worldwide. Except for human schistosomes, the family Schistosomatidae also contains numerous representatives, which parasitize in adulthood a wide range of other worm-blooded vertebrate hosts. Among them, aproximately 40 species of bird schistosomes belonging to the genus *Trichobilharzia* can be found (Blair and Islam 1983).

Although members of the genus *Trichobilharzia* are bird schistosomes, and their natural definitive hosts are birds, infective larvae (cercariae) can infect also mammals including humans. Cercariae penetrating into the host skin from water milieu are responsible for subsequent local inflammatory immune reaction known as cercarial dermatitis or swimmer's itch. These symptoms are reported from many countries all over the world, including those in temperate zone (Kolářová *et al.* 1999). The number of outbreaks in new regions increases during the last years, and cercarial dermatitis can therefore be regarded as an emerging disease (Horák and Kolářová 2011).

The above mentioned host immune reaction can entrap and eliminate parasites in the skin (Kouřilová *et al.* 2004), but not all parasites are always destroyed. Under certain circumstances some of them may continue with their migration (Horák *et al.* 1999, Hrádková and Horák 2002). While migration in specific host is usually terminated by adulthood of parasites, larvae penetrating into an accidental host develop slower without reaching maturity (Blažová and Horák 2005). In species where the migratory way leads through vital organs the infection can cause serious problems to the host.

This is also the case of *T. regenti*. This species belongs to a small group of schistosomatid species where definitive localization of adult worms is in the nasal tissue (Horák *et al.* 1998a), and it is the only one where a strong affinity of larvae to the nervous system was confirmed (Horák *et al.* 1999). After penetration of *T. regenti* cercariae into the host skin, they transform to schistosomula and migrate through the peripheral nerves to the central nervous system. They feed on nervous tissue and destroy it mechanically by migration. This can be manifested by neuromotor disorders or leg paralysis in the specific avian as well as accidental mammalian hosts (Horák *et al.* 1999, Kolářová *et al.* 2001, Kouřilová *et al.* 2004).

*Trichobilharzia regenti* as a potentially dangerous pathogen with confirmed distribution in many European regions (Jouet *et al.* 2010) is an attractive target for research.

Till now, *T. regenti* was studied in detail mainly in the stage of cercaria and schistosomulum, i.e. stages that are responsible for infection of vertebrate hosts and pathology connected with intravertebrate migration through the nervous tissue. No or little attention has been paid to the other stages, for which data about ultrastructure of surface and morphology of muscles were incomplete or missing. Contrary to the immunological, pathological, taxonomical, behavioral and biochemical approaches applied to *T. regenti* in previous studies, the introductory part of the thesis presents a complex data about ultrastructure of surface, arrangement of body musculature and changes of these characteristics during ontogenetic development, from egg to adult worm.

Surface layer of schistosomes undergoes dramatic changes during development. At one moment it is adapted to the water environment, at another time to the milieu of molluscan digestive gland or vertebrate host tissue. Parasite surface represents an effective protection against unfavorable conditions outside the body, but it is also the border for transfer of various substances, mainly between host/parasite tissues or male/female worms.

Musculature of body wall underlies surface and is responsible for active movements of the worms. Radial musculature which is present inside the body of some stages probably helps with maintenance of body shape and allows keeping of internal organs in a stable position. Organs of attachment with highly developed musculature enable attachment, migration and food intake, but can be completely absent in some stages (e.g. in sporocysts).

*Trichobilharzia regenti* exhibits many features different from those in human schistosomes (hatching of miradicium inside the host tissue, migration of schistosomulum through the nervous tissue, predominantly extravascular localization of schistosomula and adult worms, short lifespan, low degree of sexual dimorphism, different mating strategy, etc.). Comparison of ultrastructural and morphological characteristics of our model organism with human schistosomes could help to understand particular adaptations, used by schistosomes with various life strategies.

## 1.1. Life cycle of *Trichobilharzia regenti*

Life cycle of *T. regenti* includes seven stages and two hosts (fig. 1); this resembles other schistosomes (Horák *et al.* 1998a). However, important differences were described for our model organism. Contrary to the most known human schistosomes, *T. regenti* infects primarily anseriform bird hosts (see review by Skírnisson *et al.* 2012) in which it migrates through the nervous tissue outside the blood vessels. Different life strategy leads to adaptations which are evident in e.g. morphology, lifespan, tissue affinity during migration, mating behavior, mode of egg hatching etc. (Bourns *et al.* 1973, Horák et al. 1999, Horák *et al*. 2002, Loker and Brant 2006, Jones *et al.* 2008).

*Trichobilharzia regenti* belongs to a small group of nasal schistosomes which in adulthood inhabit nasal tissue of avian and mammalian hosts (Kolářová 2007). This is also the place where eggs are produced by females. Undifferentiated eggs of *T. regenti* are deposited extravascularly in the nasal mucosa. Within a few days, the first larvae called miracidia are formed inside the eggs which become highly immunogenic for the host and induce formation of granuloma (Chanová and Horák 2007).

Mature miracidia are able to hatch while they are still in the nasal tissue (Horák *et al.* 1998a, Chanová and Horák 2007). This fact distinguishes *T. regenti* from human schistosomes, because hatching of their eggs is stimulated mainly by decrease of osmotic pressure after discharging the eggs with feces or urine from host body to the water environment (Kassim and Gilbertson 1976, Kusel 1970, Xu and Dresden 1990). For *T. regenti* miracidia this indicates possible role of additional hatching stimuli other than osmotic pressure, e.g. light, which was mentioned also in hatching biology of *T. ocellata* by Meuleman *et al.* (1984).

Natural definitive hosts of *T. regenti* are anseriform birds, represented under laboratory conditions usually by ducks *Anas platyrhynchos* f. *domestica*. Anseriform birds have a close affinity to water reservoirs. Their bills, nostrils and nasal mucosae are in frequent contact with water, thus the hatched miracidia can escape from the nasal tissue directly into the water environment. They swim nimbly using their cilia anchored in the surface ciliary plates. Miracidia of *T. regenti* show positive phototaxy and negative geotaxy (Horák *et al.* 1998a) which enables them to reach microhabitat with suitable snail host. Presumably, miracidia are also able to recognize various chemical substances indicating vicinity of specific snail host, as reported for e.g. *T. ocellata* (Kalbe *et al*. 2000, Kock 2001). After miracidia analyze suitable snail host by chemoreceptors, they penetrate into its tissues using apical

papilla with glands openings, and transform to the stage of mother sporocyst (Horák *et al.* 2002).

Intermediate hosts of *T. regenti* are represented by pulmonate freshwater snails of the genus *Radix* from the family Lymnaeidae (Horák *et al.* 1998a). Process of miracidial transformation to the mother sporocyst involves loss of ciliary plates and formation of syncytial surface layer called tegument. As described for e.g. human schistosomes the tegument originates from interciliary ridges of miracidia that expand simultaneously with rejection of ciliary plates (Sobhon and Upatham 1990).

Mother sporocysts with tegument bearing numerous prolonged microvilli migrate to the head-foot region of the snail, and their germinal cells situated in the posterior part of body give rise to daughter sporocysts.

Daughter sporocysts differ from mother sporocysts mainly by presence of spines in the anterior part of body. That is in agreement with data published for human as well as bird schistosomes (Neuhaus 1952, Meuleman *et al*. 1980). Daughter sporocysts of *T. regenti* migrate to the snail hepatopancreas where they settle and produce numerous cercariae arisen from germinal cells (Horák *et al.* 2002).

Mature cercariae emerge from daughter sporocysts inside the snail tissue. They migrate to the surface of snail body using hemolymphatic vessels and leave the snail host by penetrating usually the region of mantle or body extremities (Horák *et al.* 2002). As in the case of miracidia, cercariae are free-living organisms with limited energetic reserves, so they must find vertebrate host within a short time. Cercariae in water environment actively react mainly to optical and mechanical stimuli that may indicate presence of a suitable host.

In a close proximity to the potential host, cercariae of bird schistosomes recognize also chemical substances from the host surface which stimulate attachment of cercariae to the host skin by acetabulum (Feiler and Haas 1988, Haas 1994). Subsequently, cercariae start to penetrate into the host using head organ with openings of penetration glands. During this process and shortly after it, cercariae lose their tails and surface glycocalyx coats and rebuild the outer tegumental membrane from trilaminate to heptalaminate, as known also for other schistosomes (McLaren and Hockley 1977, Hockley and McLaren 1973, Wiest *et al.* 1989).

These changes lead to the formation of the first intravertebrate stage – schistosomulum. After a short migration in the skin, schistosomula of *T. regenti* continue through the peripheral nerves to the spinal cord and brain (Horák et al. 1999, Hrádková and Horák 2002) to the place of their definitive localization in nasal mucosa. Here the adult worms mature, find their sexual partner, mate and produce eggs (Chanová and Horák 2007).

If cercariae of *T. regenti* enter accidental hosts (mammals, including humans), migration of transformed schistosomula can be observed for a limited period in the skin, and also in the nervous tissue as known from natural hosts (Hrádková and Horák 2002). In the case of repeated infections, schistosomula are usually entrapped and destroyed already in the skin. This causes inflammatory reaction known as cercarial dermatitis or swimmer's itch (Kouřilová *et al.* 2004, Kolářová *et al*. 2012). However, schistosomula never reach adulthood in mammalian host.

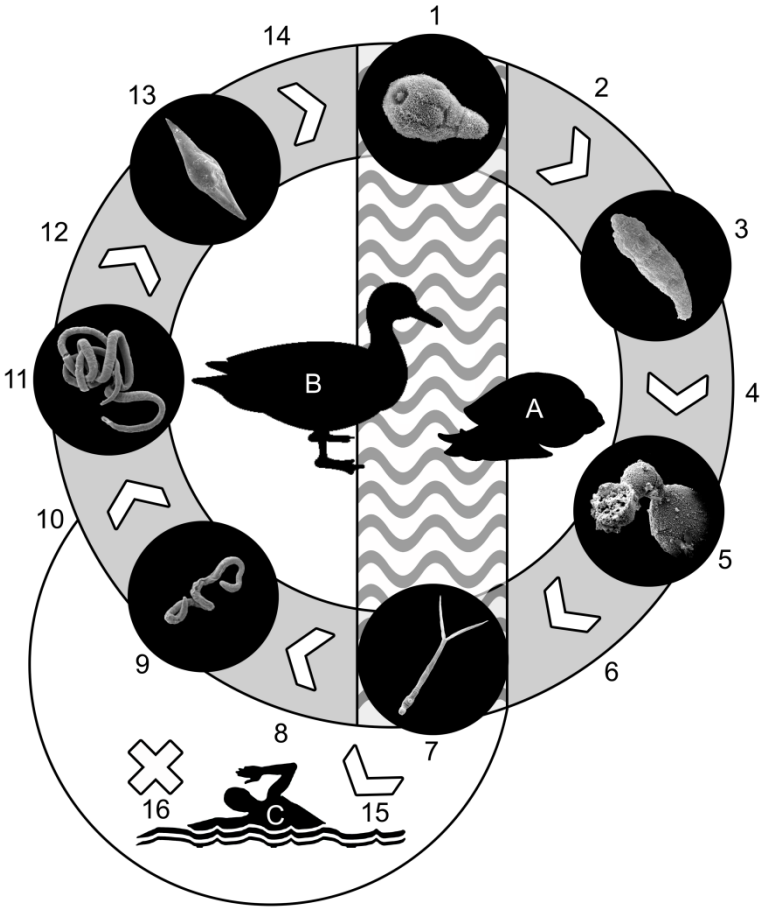



**Fig. 1 – Life cycle of *Trichobilharzia regenti*.**

Miracidia **(1)** hatch in the nasal tissue of the duck host **(B)** and escape to the water environment (waved field). There, they find and penetrate intermediate snail hosts of the genus *Radix* **(A)** and transform **(2)** to mother sporocysts **(3)** where asexual development of daughter sporocysts **(4)** takes place. Each daughter sporocyst **(5)** is a source of numerous cercariae, escaping **(6)** from the snail host to the water environment (waved field). Swimming cercariae **(7)** penetrate the definitive duck host and transform **(8)** to the stage of schistosomulum **(9)**. It migrates through the nervous tissue **(10)** and reaches adulthood in the nasal tissue of the duck host. There, the adult worms **(11)** mate **(12)** and produce eggs **(13)**. Within the nasal tissue of duck host, development of miracidia inside the eggs takes place **(14)**. If cercariae **(7)** penetrate accidental host **(C)**, they transform to schistosomula **(15)**, survive for a limited period and subsequently die **(16)**.

## 1.2. Developmental stages

The life cycle of schistosomes comprises seven developmental stages which are introduced in particular chapters. Basic morphology and detailed ultrastructure of surface layer and body musculature are summarized for schistosomes in all developmental stages and compared to our model organism *T. regenti*.

**Particular stages characterized in the text:**

- Egg
- Miracidium
- Mother sporocyst
- Daughter sporocyst
- Cercaria
- Schistosomulum
- Adult worm

### 1.2.1. Egg

Schistosomatid eggs have a thin colorless or lightly brown colored egg shell without any operculum. Eggs of different schistosome species vary in size, shape or presence of protruding spines, knobs or filaments, which are believed by some authors to serve as an anchor protecting the egg against the blood flow. These morphological differences are frequently used for simple, but reliable differential diagnosis, especially in human schistosomiasis (Sobhon and Upatham 1990). In the case of bird schistosomes of the genus *Trichobilharzia*, which is the most numerous within the family Schistosomatidae, shape and size of eggs are usable as a tool for quick identification of sympatrically occurring species with different egg morphology (e.g. *T. ocellata* and *T. franki*). However, some species of bird schistosomes produce similar eggs (e.g. *T. salmanticensis* and *T. parocellata*), thus egg morphology does not allow reliable species determination of *Trichobilharzia* representatives in all cases (Horák *et al.* 2002).

The most important human schistosomes are known as blood-dwelling parasites adults of which inhabit vascular system of their definitive hosts, and this is also the place where eggs are laid by females (Loker and Brant 2006). Definitive localization of *T. regenti* adults is in the connective tissue of nasal mucosa outside the blood capillaries (Chanová and Horák 2007), although some studies reported both, intra- as well as extravascular localization of adult worms (Horák *et al.* 1998a, Kolářová *et al.* 2001). First eggs can be detected in nasal mucosa from day 15 post infection (p.i.), maximum number of deposited eggs was recorded on day 22 p.i. Eggs are deposited extravascularly one by one in small clusters of 5–12 specimens. They are localized mainly within the connective tissue near to the cartilage of nasal conchae, in the close vicinity of adult females. With the length of patent period of trichobilharziasis, the eggs disperse throughout the entire nasal mucosa in reaction to the host cell immunity (Chanová and Horák 2007). Each female of *T. regenti* contains in one moment only one intrauterine egg (Horák *et al.* 1998a), and this resembles the situation in e.g. *Schistosoma mansoni* (Loker 1983), but differs from some oriental schistosomes females of which lay eggs in groups of tens (Loker 1983, Sobhon and Upatham 1990). Egg production of *T. regenti* seems to be lower than that in human schistosomes for which various authors refer to hundreds of eggs laid per day by one female of *S. mansoni* (Michaels and Prata 1968), or describe production of more than two thousand of eggs/day per female in *S. japonicum* (Loker 1983, Fan and Kang 2003).

Morphological characteristics of *T. regenti* eggs were described in detail in the original paper by Horák *et al.* (1998a). Non-operculated, colorless and thin shelled eggs of *T. regenti* belong to the bigger ones among schistosomes (Loker 1983, Sobhon and Upatham 1990). They have elongated fusiform shape (fig. 2A, 2B), and the widest part with developing miracidium inside is situated almost in the middle of the egg. Two processes protrude from the opposite poles of the egg. While the longer process has rounded end, the opposite one is shorter with a thin curved projection on its tip (Horák *et al.* 1998a).

Modern techniques used in electron microscopy, e.g. slam freezing, high pressure freezing (HPF) and subsequent freeze substitution (FS) allow ultrastructural and developmental characterization of schistosome eggs without artifacts caused frequently by impermeability of egg shell (Neill *et al.* 1988, Jones *et al.* 2008). While eggs of human schistosomes were intensively studied as an important pathological agent, only poor data were published about eggs of bird schistosomes in general, or *T. regenti* in particular. Based on our unpublished results from ultrastructural and immunocytochemical study of *T. regenti* eggs, there exist many features similar to those described by various authors for human schistosomes (Neill *et al.* 1988, Ashton *et al.* 2001, Michaels and Prata 1968, Jurberg *et al.* 2009, Ford and Blankespoor 1979, Jones *et al.* 2008); they will be discussed below.

According to studies on human schistosomes, surface of the egg shell is covered by a microspinous layer (Ford and Blankespoor 1979, Neill *et al.* 1988). This layer was observed also in our unpublished TEM (transmission electron microscopy) study of *T. regenti* eggs (fig. 2C). In SEM (scanning electron microscopy), however, the outermost surface of *T. regenti* eggs was coated only with numerous blunt protuberances (frame of fig. 2A) which correspond in size and distribution with the sharp spines visible in TEM. This is in agreement with the data published by Ford and Blankespoor 1979, Hockley 1968 and Race *et al.* 1971 who studied eggs of several human schistosomes by both TEM and SEM techniques. Thus, it seems that microspinous layer changes its appearance, depending on the method of processing for different electron microscopic techniques. Now it is not clear which one should be marked as artificial.

Proper egg shell of human schistosomes is an electron dense firm layer which is formed in female ootype around vitelline cells and zygote during pre-embryonic stage of egg development. Egg shell is obliquely penetrated by minute cribriform pores (Neill *et al.* 1988, Ashton *et al.* 2001). Numerous pores allow exchange between the inner space of egg and the surrounding host tissue. Antigens secreted by the inner envelope of the egg permeate through these pores onto the egg surface, where they are entrapped within microspinous layer (Ashton

*et al.* 2001) and cause intense focused granulomatous immune response in surrounding host tissue. This reaction facilitates escape of the eggs from capillaries or tissues to the lumen of affected organs and outwards from the host. The same reaction is also responsible for serious pathology of schistosomiasis when the eggs are disseminated to organs and release immunoreactive substances for up to three weeks before they die (Sobhon and Upatham 1990, Secor and Colley 2005). Pores in the egg shell serve not only for the export of secreted products, but also for uptake of exogenous host nutrients which are necessary for successful development of schistosome eggs (Ashton *et al.* 2001, Jurberg *et al.* 2009). As a consequence, egg size gradually increases (Michaels and Prata 1968). Finally, more than 2/3 of the constituents of the fully mature egg can be derived from external milieu. That would not be possible without elasticity of the egg shell (Ashton *et al.* 2001).

As mentioned above, the eggs of *T. regenti* are deposited within the nasal mucosa (Chanová and Horák 2007). They are definitely the cause of granuloma formations in the surrounding tissue (Chanová and Horák 2007) as documented for human schistosomes. Also increase of egg size during development was observed in *T. regenti* (unpublished). Therefore, the presence of egg shell pores is highly probable in *T. regenti*. Nevertheless, no pores were observed in our preliminary TEM study of *T. regenti* eggs. This discrepancy could be caused by standard chemical fixation and poor preservation of our material. Thus, additional studies by e.g. freezing methods (HPF and FS) are required for final confirmation of presence or absence of egg shell pores in *T. regenti*.

Contrary to relatively stable surface ultrastructure of egg shell with its microspinous layer, the inner content changes rapidly with development of embryo. These changes were sorted by various authors to several phases on the basis of different criteria, e.g., ratio of egg/embryo sizes, occurrence of egg stage specific structures or other attributes of developmental progress (for review see Jurberg *et al.* 2009). Also terminology of some structures inside the eggs differs in various articles. Anyway, consecution of changes which take place inside the developing eggs is the same, independently on terminology.

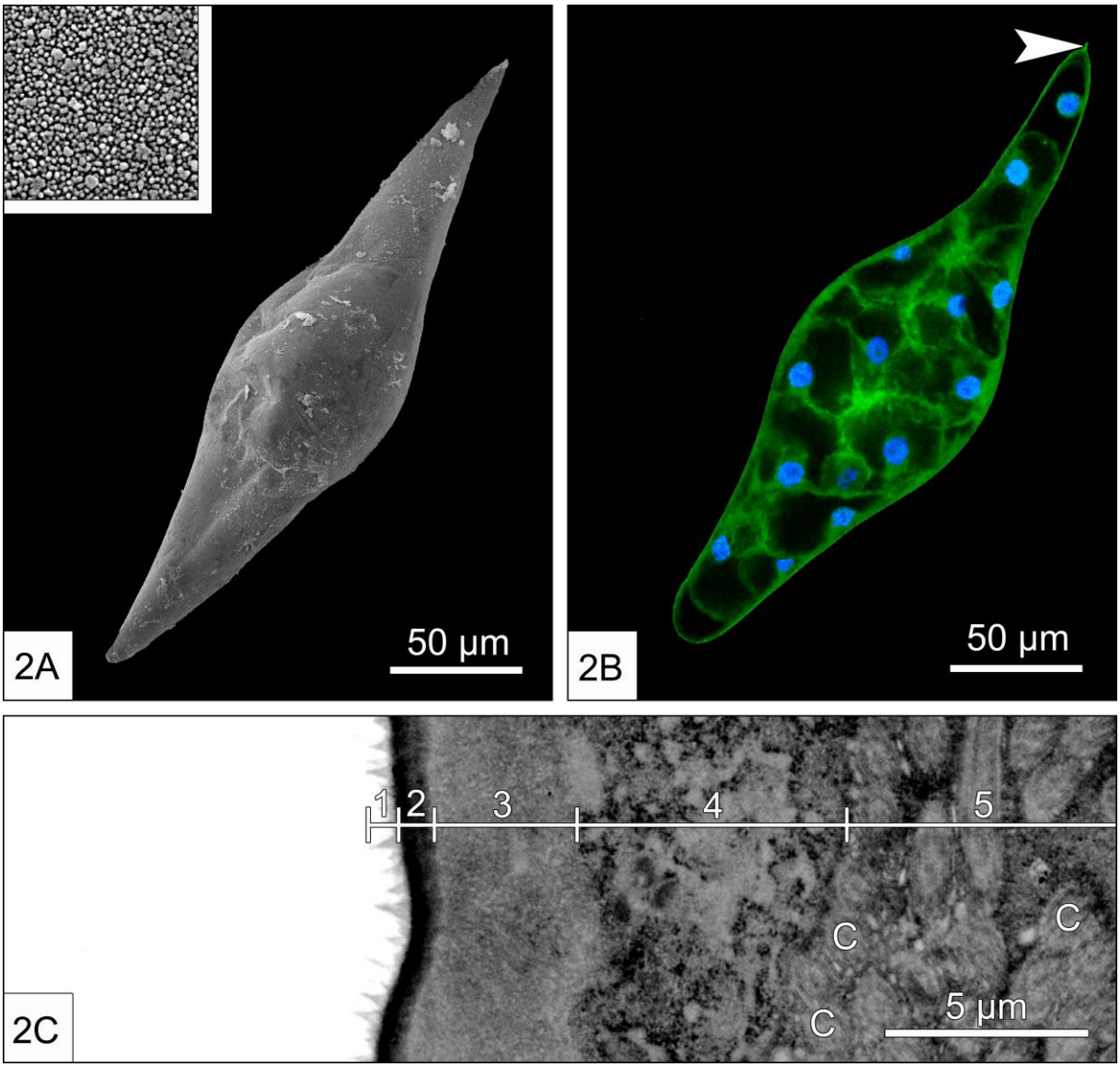
Referring to the results published by Ashton *et al.* (2000) and Jurberg *et al.* (2009), newly deposited eggs of human schistosomes are composed of shell with microspinous layer and 20–45 vitelline cells (vitellocytes) surrounding fertilized zygote. Developing embryo is subsequently surrounded by an outer envelope derived from fused vitellocytes, and several cells detached from early embryo. Subjacent inner envelope comprises of outermost cells of early embryo and surrounds future miracidium in the internal cavity called lacuna (Jurberg *et al.* 2009). The first distinguishable structure of developing miracidium of human schistosomes

is a layer of future epidermal cells and neural mass primordium. In this phase of development, growing embryo already occupies almost all the internal space of the egg. Next progress in embryogenesis is characterized by formation of terebratorium at the apical part of future miracidium, establishment of muscle precursors under the epidermal cells and differentiation of clustered germinal cells within the midposterior part of miracidial body. Terminal phases of development are accompanied with formation of apical gland and afterwards lateral glands, circular and longitudinal muscle fibers and flame cells. Finally, mature miracidium with all organ systems is ready to hatch under appropriate conditions. Jurberg *et al.* (2009).

Basic ultrastructural arrangement of inner content of newly deposited, as well as fully mature eggs described for human schistosomes was confirmed also in the case of *T. regenti*. At the start, individual vitelline cells are present (fig. 2B), and subsequently outer and inner envelope develop around the miracidium (fig. 2C).

As seen from the above data published for human schistosomes, musculature of miracidia inside the eggs starts to develop shortly after differentiation of surface epithelium. Similar situation is expected also for *T. regenti* eggs. In newly deposited eggs, no precursors of future musculature were discovered by fluorescent staining of F-actin, an abundant protein of muscles, and only contours of individual vitellocytes were recorded (fig. 2B). Using DAPI (4',6-diamidino-2-phenylindole) staining and confocal microscopy, we were able to count all nuclei and determine the exact number, size and shape of cells inside the eggs. This labeling could be productively used in future studies of embryogenesis, not only in schistosomes.

Embryonal development of human schistosomes lasts 6–12 days depending on species (Michaels and Prata 1968, Loker 1983, Sobhon and Upatham 1990). The first adults and eggs of *T. regenti* were found in the nasal tissue between day 14–15 p.i. (Chanová and Horák 2007, Kolářová *et al.* 2001) and first mature miracidia inside the eggs were recorded 17 days p.i. (Chanová and Horák 2007). Thus, we suppose that the development of *T. regenti* is more rapid than that of human schistosomes. Referring to Loker and Brant (2006), this can be accompanied with life strategy and lifespan of both organisms. While *S. mansoni* can occupy the body of a human host for several years (Loker 1983), the bird schistosome *T. regenti* spends in its experimental model bird host only a few weeks (Horák *et al.* 1998a). It probably uses the same strategy as was described for *T. ocellata*. This species of bird schistosome is known to reach adulthood and produce eggs very early after penetration of cercariae into the vertebrate hosts. Miracidia probably develop before the nesting bird hosts leave the locality with susceptible snail hosts, and this increases possible transmission success of the parasite (Bourns *et al.* 1973).




**<u>Fig. 2A</u> – Egg of *T. regenti* in SEM.**

Frame shows a detail of surface covered with numerous blunt protuberances corresponding with microspines of microspinous layer visible in TEM **(Fig. 2C)**.

**<u>Fig. 2B</u> – Undifferentiated egg of *T. regenti* in CLSM.**

FITC labeled F-actin is localized in borderline of individual cells, nuclei are labeled with DAPI. Note the apical curved projection at the anterior tip of egg shell **(white arrowhead)**.

**<u>Fig. 2C</u> – TEM of egg shell in *T. regenti*.**

Particular layers are designated by numbered line segments. **1.** microspinous layer, **2.** egg shell, **3.** outer envelope, **4.** inner envelope, **5.** ciliary surface of unhatched miracidium. Cilia are designated with **C**.

### 1.2.2. Miracidium

Generally, miracidia of schistosomes are free-living larvae with a short lifespan (e.g. 20 h at 20 °C is documented for *T. szidati* by Neuhaus 1952). Within this period which is limited by endogenous energy reserves miracidia must find a suitable intermediate snail host, penetrate it and develop to the parasitic stage of mother sporocyst.

In visceral schistosome species, miracidia hatch from non-operculated eggs after their exposure to hypoosmotic water conditions and/or light (Meuleman et al 1984, Xu and Dresden 1990). In the case of *T. regenti*, hatching of miracidia is initiated when the eggs are still entrapped in the nasal tissue (Horák *et al.* 1998a, Chanová and Horák 2007), so there are probably some additional factors involved in hatching stimulation. However, in both cases, miracidia finally escape to the water, where they follow some physical stimuli (light, gravity acceleration) to reach the space where the presence of intermediate host is presumable. Subsequent orientation of miracidia towards snail host is provided by detection of chemical substances which are excreted/secreted from snail body and serve for miracidia as an attractant (e.g. Kalbe *et al.* 1997). Miracidia reaching their intermediate hosts penetrate directly through the skin, or use natural openings as mouth or rectum (Loker 1978, Xia and Jourdane 1991).

Multifunctional surface of miracidium represents the main barrier against hypoosmotic pressure, enables locomotion in water environment, detection of signals from external milieu and, particularly, comprises basement for future neodermis of subsequent developmental stages (Hockley 1973, Pan 1980, Sobhon and Upatham 1990).

Fast oriented swimming of miracidia is enabled by numerous surface cilia which are anchored within the ciliary plates. Miracidia of schistosomes have four tiers of ciliary plates (Eklu-Natey *et al.* 1985, Horák *et al.* 2002). Their arrangement can be recorded as numbers separated by colons. This formula can be common for more genera (e.g. 6:9:4:3 is valid for *T. regenti*, *S. japonicum* and *S. mekongi*), thus the number of ciliary plates is useless for species determination. But there exist apparent differences in shape of ciliary plates and/or their mutual position between some species of schistosomes (Eklu-Natey *et al.* 1985, Sobhon and Upatham 1990).

An individual ciliary plate of schistosome miracidia can be characterized as a cell with anucleate superficial part bearing long ciliae alternating with short microvilli (fig. 3A). It is connected with subepidermal cyton by thin cytoplasmic bridge passing through lamina basalis and body wall musculature (Hockley 1973, Pan 1980). This arrangement seems to be

universal for schistosomes, but differs significantly from that in miracidia of e.g. *Fasciola hepatica* where flattened nuclei are present directly inside the surface part of ciliary plate which lacks connection with internal cells of miracidium (Wilson 1969). These differences can be associated with the mode how both organisms reject ciliary plates during penetration into the molluscan host. While miracidia of *F. hepatica* throw away their ciliary plates in the external water milieu, schistosomes reject them after penetration into the snail, thus ciliary plates can be observed inside the host tissue (see Hockley 1973).

For *S. mansoni* miracidia, only one type of ciliary plates was described (Hockley 1973, Pan 1980). Their cytoplasm is filled with numerous mitochondria stacked near the basal membrane, and with dense bodies of round or oval shape which occur in the upper layer of ciliary plates (Bash and DiConza 1974, Meuleman *et al.* 1978). In *S. japonicum* and *S. mekongi*, two different subtypes of ciliary plates were documented (Sobhon and Upatham 1990), but information about the number and arrangement of both subtypes of ciliary plates in miracidial body is not available. Ultrastructurally, both types differ mainly in the content of various cell structures. Subtype 1 has numerous cilia, mitochondria and lipid droplets, but it contains only few light ovoid granules, contrary to subtype 2 where ratio of the above mentioned structures is inversed. Authors believe that ovoid granules are transported to the surface of ciliary plates where they are exocytosed and their content forms mucinous surface coat (Sobhon and Upatham 1990). Surface coat on miracidium resembles ultrastructurally glycocalyx of other free-living stage of trematodes, namely cercariae (see chapter 1.2.5.). Although the exact chemical nature of material from miracidial surface coat remains unknown, we can hypothesize that it has a role in protection of miracidium against hypoosmotic water conditions, attachment to the intermediate host surface or it represents a layer which facilitates penetration into the invertebrate host skin.

In our unpublished TEM observation of *T. regenti* miracidia, we were able to distinguish surface coat (fig. 3B) as well as variability in cytoplasmic content of individual ciliary plates. The main difference between particular ciliary plates was observed in the number of light ovoid granules (fig. 3A, 3B). First type of plates has only a few granules organized in one row above subjacent layer of mitochondriae, but the second type was fully filled with numerous granula which apparently bulge the surface of ciliary plates. However, we are not convinced that the variability is caused by different function of various plates, but more probably, it reflects asynchronic release of granula from cytons.

Space between individual ciliary plates is filled with intercellular (or epidermal) ridges. The only exception is documented for the first row of ciliary plates where all plates fit

tightly to each other (Eklu-Natey *et al.* 1985, Horák *et al.* 1998a). Contrary to the cells of ciliary plates, intercellular ridges of schistosome miracidia are present in a form of syncytium. Moreover, syncytial character is preserved also among interconnected submerged cytons of intercellular ridges. (Pan 1980, Sobhon and Upatham 1990). Membranes of all structures incorporated in (receptors, excretory ducts openings) or connected with (ciliary plates) intercellular ridges are bound to the syncytial membrane by septate desmosomes (fig. 3A). Intercellular ridges have no ciliae. Their surface is covered only with short sparse microvillar projections. Apparently prolonged extensions of ridges were observed at the borderline with ciliary plates in *S. mansoni* and *T. regenti* (fig. 3A), but not in oriental schistosomes. Cytoplasm of ridges in their superficial part contains mainly pale spherical granules, glycogen particles and mitochondria (Basch and DiConza 1974, Pan 1980). Subtegumental cytons are filled with nuclei and cisternae of endoplasmic reticulum and Golgi apparatus and they are probably source of accumulated spherical granules released into the superficial layer through cytoplasmic bridges (Pan 1980).

Area between ciliary plates of the third row is perforated by pair of openings belonging to the excretory system. Another important region located in intercellular ridges is between the first and second tiers of ciliary plates where various sensory organs (lateral papillae, uniciliated and multiciliated pits) are abundant. The number and position of these receptors vary in different species (Eklu-Natey *et al.* 1985). Apparent differences between the number and arrangements of miracidial surface receptors were recorded also between two bird schistosomes, *Trichobilharzia regenti* and *T. szidati* (Houžvičková unpublished). These features might contribute to differentiation of miracidia of various *Trichobilharzia* species and should be characterized further.

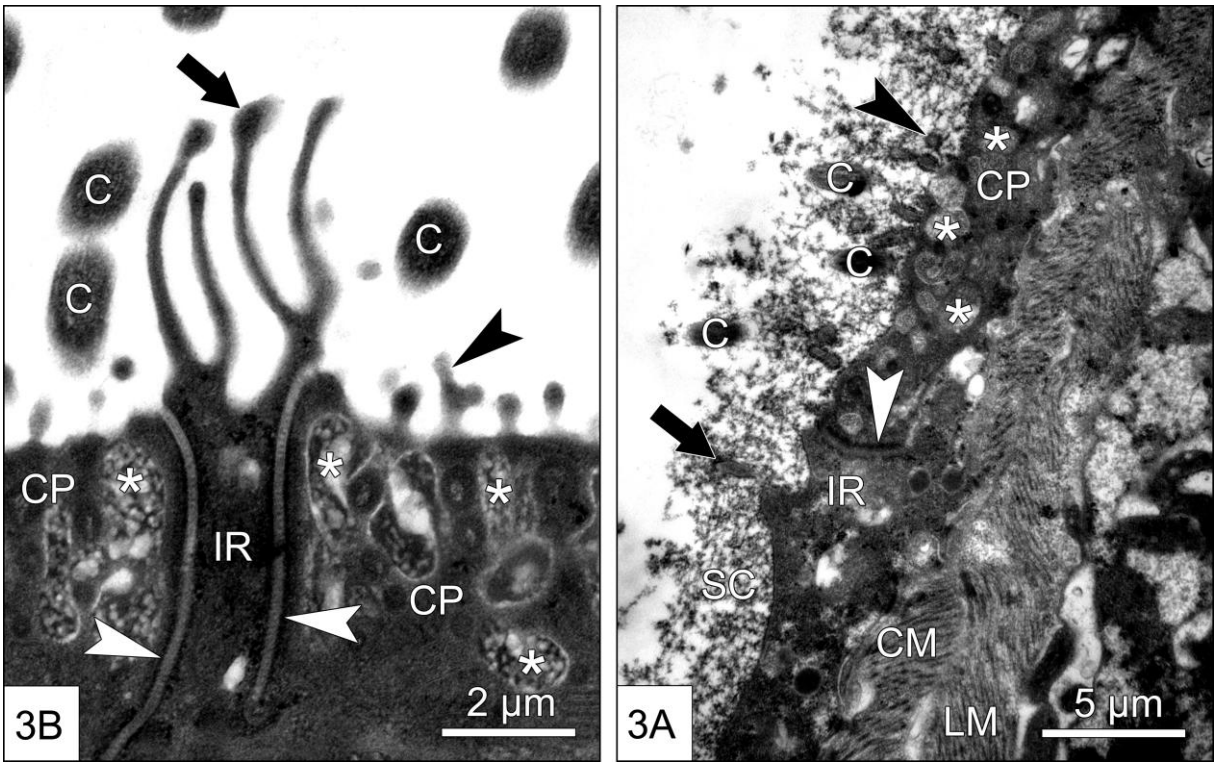
Anterior end of miracidia culminates in apical papilla called terebratorium. This unique structure is involved in miracidial attachment to and penetration of the snail host. Ultrastructurally, it is composed of anastomosing microfolded epithelium with sponge-like appearance. Eklu-Natey et al (1985) distinguish two types of terebratorium of human schistosomes. First type with "rosette" pattern was observed in *S. haematobium* and *S. intercalatum*. Second type with "honeycomb" pattern was documented for *S. mansoni* and *S. japonicum*, and based on our unpublished data also for *T. regenti* (fig 4A, 4B). Variability in patterns of terebratorium could be connected with specific place used by miracidium for penetration. These speculations remain to be verified in both human as well as bird schistosomes.

Sensoric function of terebratorium is provided by several uniciliated and multiciliated pits (fig. 4A, 4B), mutual position of which can differ in various schistosome species. Terebratorium is also the place where apical and lateral glands open and release secretory granules with a role in penetration of miracidium into the molluscan host (fig. 4B). Together with the number and position of surface receptors of intercellular ridges, shape and arrangement of ciliary plates, the above mentioned features of terebratorium could serve in some cases (not only in schistosomatids) as an important tool for species determination (Wirkel and Bogitsh 1974, Pan 1980, Sobhon and Upatham 1990 Horák *et al.* 2002).

Hatching from eggs, escaping from host nasal tissue in the case of *T. regenti*, oriented swimming in water reservoir and penetration into the intermediate host – all these activities of miracidia are closely connected with functioning of musculature. The arrangement of miracidial musculature in human schistosomes corresponds with that of *T. regenti* (Bahia *et al.* 2006, Bulantová *et al.* 2011, Collins *et al.* 2011). Miracidia lack internal muscular organs like intestine or reproductive system, thus the only one apparent muscle fibers are organized under the surface where they form so called body wall musculature (Bahia *et al.* 2006, Collins *et al.* 2011). Miracidial muscles are arranged in two layers. Outer circular musculature of miracidia is comprised of thin muscle fibers which form nearly compact layer without distinct gaps. It has appearance of a muscular sac with only one opening under the terebratorium where circular muscles are absent.

Subjacent longitudinal musculature has entirely different structure. Individual muscle fibers are compressed into ribbon-like structures, anchored in the area of terebratorium as six distinguishable separated wide ribbons (Bahia *et al.* 2006, Bulantová *et al.* 2011). Very early, each muscular belt divides into two individual ribbons leading towards the rear (Collins *et al.* 2011). Closely before posterior end of miracidia, muscular ribbons join laterally. Individual ribbons of longitudinal muscles are separated by wide gaps which have the same or larger width as ribbons themselves (Bahia et al 2006, Bulantová *et al.* 2011).

Such organization of longitudinal musculature is preserved only in miracidia, and diffuses to individual muscle fibers during intramolluscan development of mother sporocysts (Bahia *et al.* 2006, Bulantová *et al.* 2011). This could indicate importance of longitudinal muscle bundling in the stage of miracidium for the activity of ciliary plates which are responsible for oriented swimming in the water environment.

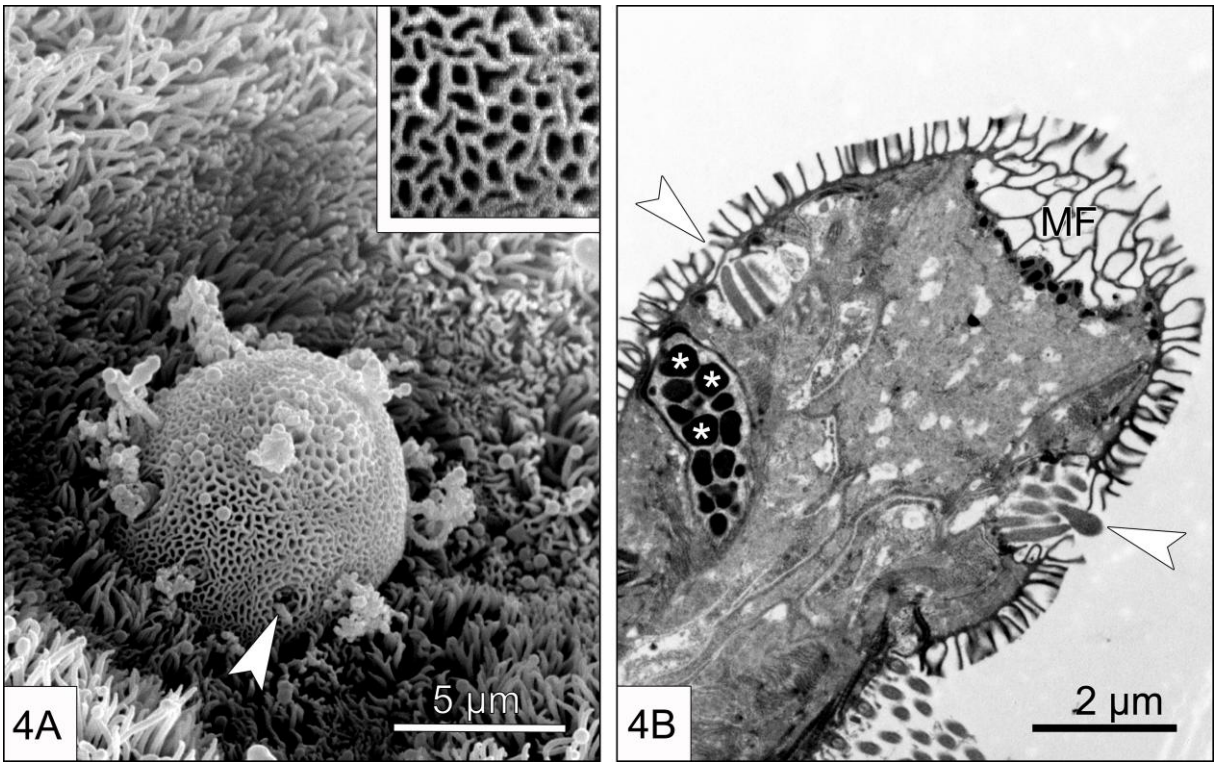



**<u>Fig. 3A</u> – Detail of miracidial surface in *T. regenti*, TEM.**

Two ciliary plates **(CP)** filled with ovoid granules **(white asterisks)** are bordered by septate desmosomes **(white arrowheads)** from intercellular ridge **(IR)**. Ciliary plates bear cilia **(C)** and small microvilli **(black arrowhead)**. Microvilli on margins of intercellular ridges **(black arrow)** are considerably prolonged.

**<u>Fig. 3B</u> – Miracidium of *T. regenti*, TEM.**

Ciliary plates **(CP)** and intercellular ridges **(IR)** are covered with surface coat **(SC)**. Ciliary plate is separated from intercellular ridge by septate desmosome **(white arrowhead)**. Labels: **C** = cilia, **CM** = circular muscles, **LM** = longitudinal muscles, **black arrowhead** = microvilli of ciliary plates, **black arrow** = microvilli of intercellular ridges, **asterisks** = ovoid granules.




**<u>Fig. 4A</u> – Terebratorium of *T. regenti* miracidium with "honeycomb" pattern, SEM.**

Note the row of sensoric ciliary pits on the base of terebratorium **(white arrowhead)**. Frame: Detail of "honeycomb" pattern of terebratorium.

**<u>Fig. 4B</u> – Terebratorium of *T. regenti* miracidium, TEM.**

Note the anastomosing microfolds **(MF)** on the surface of terebratorium, dark granula **(white asterisks)** inside the opening of lateral gland and two multiciliated pits **(black arrowheads)**. Cilia and microvilli of the first row of ciliary plates are apparent in the lower part of picture.

### 1.2.3. Mother sporocyst

Miracidia, which successfully found their suitable molluscan host, penetrate into the body of the snail and transform there to the stage of mother sporocyst. The entire process of miracidium/sporocyst transformation is situated to the snail subepidermal tissue, closely to the region where miracidium entered the snail host. Later, mother sporocysts with a fully transformed surface migrate to the head-food region of the snail (Basch 1991, Horák *et al.* 2002). Germinal cells, which were established already in miracidial body (fig. 5A), differentiate in developing mother sporocysts to numerous individuals of asexually arisen subsequent generation of daughter sporocysts.

Transformation of miracidia to mother sporocysts includes rejection of ciliary plates from miracidial surface (Basch and DiConza 1974). Shedding of each individual ciliary plate begins by bulging of its surface and movement of mitochondria from basal to more internal position in the center of the plate where they aggregate. Nuclei regularly deposited in submerged cytons of epithelial cells can rarely migrate through cytoplasmic bridges to the epithelial layer of ciliary plates. However, presence of a nucleus in rejecting ciliary plates is very unusual in schistosomes and it was documented only in oriental schistosomes (Sobhon and Upatham 1990).

Numerous small vacuoles appear between the rejecting ciliary plate and subjacent lamina basalis. Later, small vacuoles fuse to one big vacuole separating all basement of ciliary plate from the rest of miracidial body. Finally, septate desmosomes connecting ciliary plates to intercellular ridges disintegrate and the plates are rejected as small balls with ciliae protruding from their external surface (Voge and Seidel 1972, Basch and DiConza 1974, Samuelson *et al.* 1984, Sobhon and Upatham 1990). Rarely, the rejected ciliary plates could be observed with cilia infolded by the balled plate, as was documented for *T. regenti* transformed *in vitro* (Houžvičková and Skála – personal communication). Naturally, miracidia of schistosomes penetrate the snail and lose their ciliary plates once inside the intermediate host (Basch 1991). Unusual balling of some ciliary plates observed for *T. regenti* miracidia transformed to the mother sporocysts *in vitro* could be accidental, because of lack of mechanical stimuli provided by snail tissue *in vivo*. Atypical way of balling of some ciliary plates could also be connected with certain types of ciliary plates which differ in minute ultrastructural characteristics. Anyway, the main principles of ciliary plate rejection are in *T. regenti* similar to those described for human schistosomes.

Simultaneously with the rejection of ciliary plates, intersticial mass of intercellular ridges expands to fill the space after the rejected ciliary plates, and forms a continuous surface layer of syncytial neodermis = tegument (Basch and Diconza 1974, Sobhon and Upatham 1990). Formation of the new surface is essential for survival and next development of sporocysts inside the host tissue. It provides adaptation to internal milieu of snail where mother sporocysts actively inhibit host defense mechanisms of specific snail host, and enables nutrition uptake through the tegumental surface. Time required for complete transformation of miracidium to mother sporocyst is highly dependent on temperature, but in e.g. *S. mansoni* it takes 3 hours *in vitro* (Basch and Diconza 1974, Samuelson *et al.* 1984).

Tegument of newly arisen mother sporocysts is formed from a thin anucleate syncytial layer and is connected with submerged cytons by cytoplasmic bridges (Smith and Chernin 1974, Meuleman *et al.* 1980). Sensoric receptors as ciliated pits of the former intercellular ridges disappear, while terebratorium and lateral papillae become only less apparent in early mother sporocysts (Koie and Frandsen 1976). Surface of tegument starts to form numerous, densely arranged, short, and sometimes branched microvilli (Basch and DiConza 1974). This arrangement rapidly increases surface area which can be in direct contact with the surrounding snail tissue in order to acquire nutrients from the intermediate host (Cheng 1963, Smith and Chernin 1974). Disintegration of surface receptors, as well as multiplication and growth of tegumental microvilli (fig. 5B) were observed also in our model organism and confirm similarity of intramolluscan development of human and bird schistosomes.

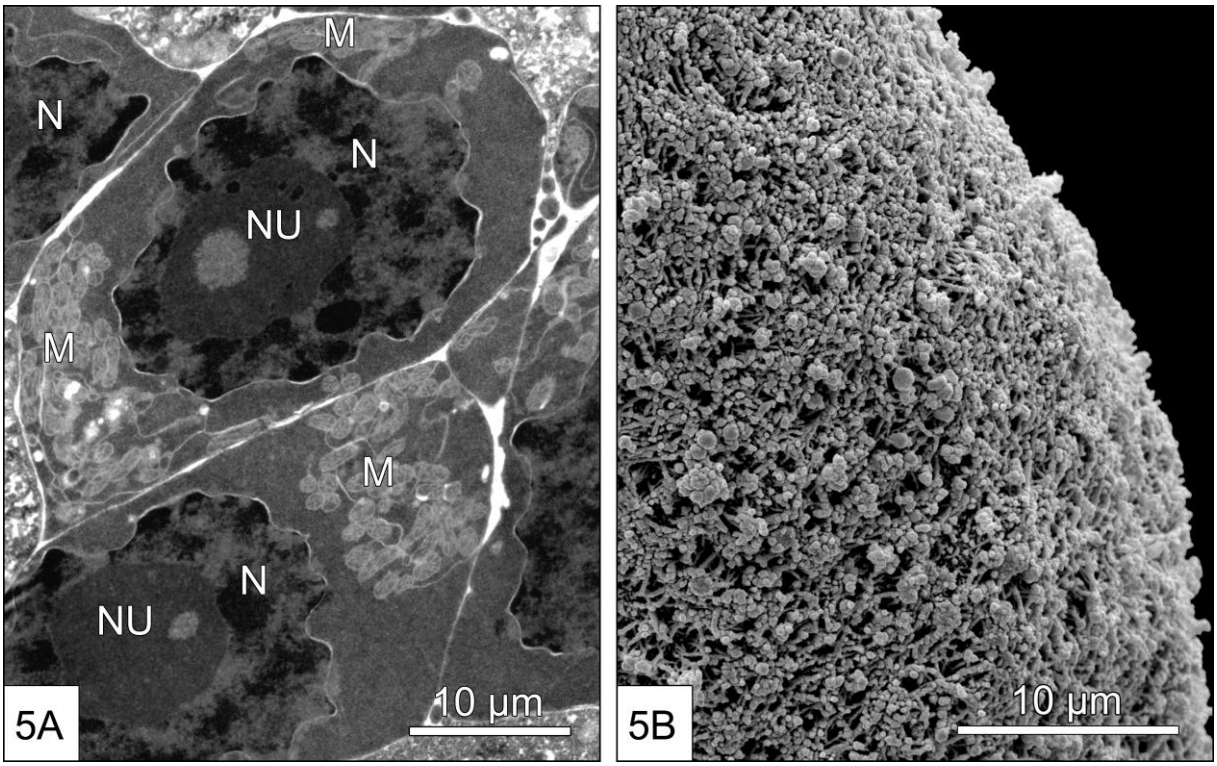
Cytoplasm of the tegument is in mother sporocysts filled with large membrane-bound vesicles, granular endoplasmic reticulum, glycogen particles, lipid droplets and sparse mitochondria. Surface layer of tegument is supported by thin lamina basalis and fine muscles. Subtegumental cells (cytons of tegumental syncytium) form long projections, surrounding in this way individual germinal balls and embryonal daughter sporocysts. Further, these projections fuse to form a compact embryonal envelope called primitive epithelium (Meuleman and Holzmann 1975, Meuleman et al 1980, Sobhon and Upatham 1990).

Research on mother sporocysts produced *in vivo* is a complicated matter, because of low chance that the tiny sporocysts will be found inside the snail tissue. Therefore, a lot of work has been performed on mother sporocysts transformed *in vitro*. Nevertheless, these data could be accompanied with possible artifacts, predominantly in e.g. length of microvilli and ultrastructure of tegument lacking a direct contact with host tissue. To prove this presumption, comparative study of sporocysts acquired from *in vitro* and *in vivo* conditions should be performed.

Motoric activity of mother sporocysts decreases with time post transformation. This is reflected also by decreasing massiveness of body musculature during growth and development of the worm. Body wall musculature of *S. mansoni* mother sporocysts was described by Smith and Chernin (1974) as a complex of isolated fibers with no clear orientation, forming a loose reticulum. Meuleman *et al.* 1980 mentioned that musculature in sporocysts of *S. mansoni* is no longer used for locomotion and becomes an irregular net of muscle fibers. Anyway, transversally crossed muscle fibers observed in *S. mansoni* (Bahia *et al.* 2006) or *T. regenti* (Bulantová *et al.* 2011) by confocal microscopy indicate, that muscles preserve their regular arrangement from the stage of miracidium, and sporocysts exhibit a limited locomotion ability within the snail tissue. Possible peristaltic movements provided by body wall musculature of mother sporocysts can also be accompanied with release of mature daughter sporocysts outwards to the snail tissue.

Bahia *et al.* (2006) disclosed that musculature of early mother sporocysts is represented by outer circular muscles which are preserved from the stage of miracidium, but newly ordered in doublets. Subjacent longitudinal muscles were, compared to those in miracidia, less apparent. The same authors found also two newly created actin rich areas within the anterior and posterior ends of mother sporocyst.

According to our observation of *T. regenti*, musculature of mother sporocysts is strictly organized as a transversal net, composed of outer circular and underlying longitudinal muscles. Compared to data about *S. mansoni*, no doubling of circular muscle fibers was observed. Presence of actin rich area was observed only in the apical end, under the former miracidial terebratorium. (Bulantová *et al.* 2011). The main noticeable difference between the stage of miracidium and the fully developed mother sporocyst consists in progressive unfastening of longitudinal muscular ribbons into the individual, uniformly organized muscle fibers. Terminal phase of development of body wall musculature in mother sporocysts is characterized by increasing gaps between muscles, accompanied with simultaneous growth of parasite.




**<u>Fig. 5A</u> – Germinal cells inside the young mother sporocyst of *T. regenti* from the snail host 5 days p.i., TEM.**

These cells differentiate and give birth to the next stage – daughter sporocyst. Note apparent nuclei **(N)**, nucleoli **(NU)** and groups of mitochondria **(M)** inside the cytoplasm of germinal cells.

**<u>Fig. 5B</u> – Surface of *T. regenti* mother sporocyst from the snail host 5 days p.i., SEM.**

Numerous densely arranged surface microvilli provide direct contact with the surrounding host tissue, and allow nutrition uptake from the snail host.

### 1.2.4. Daughter sporocyst

Daughter sporocysts of schistosomes develop inside the body of mother sporocysts, leave them by penetration of their body wall (Ivanchenko *et al.* 1999) and migrate from head food region of the snails to their digestive gland (hepatopancreas) or ovotestis (Basch 1991, Horák *et al.* 2002). In those organs they grow and produce numerous cercariae or other generation of daughter sporocysts (Basch 1991). Mother as well as daughter sporocysts are sack-like and thin walled organisms with reduced musculature. Both generations of sporocysts are highly adapted to nutrition uptake from the snail tissue, and also for asexual production of next developmental stages. While mother sporocysts fill only minute part of host organism, daughter sporocysts can fill substantial part of the volume of host body. This is caused by the total number of specimens, but also by the terminal size of daughter sporocysts which are fully filled with numerous developing cercariae (Meuleman *et al.* 1980, Sobhon and Upatham 1990, Basch 1991).

Early stage of daughter sporocysts comprises tightly packed cluster of proliferated germinal cells enveloped by a thin layer of primitive epithelium. This thin envelope originates from extensions of subtegumental cells of mother sporocysts being in direct contact with the host tissue. That enables transport of nutrients taken up from the hemolymph to the close vicinity of developing embryo inside mother sporocysts (Meuleman *et al.* 1980, Sobhon and Upatham 1990). Development of daughter sporocysts continues with formation of tegumental basement from superficial cells of the embryo. Afterwards, these epithelial cells coalesce into syncytium, lose their own nuclei and connect to subjacent cells by cytoplasmic bridges. Surface membrane undulates and forms numerous long microvilli (fig 7) which can be branched in some species (Sobhon and Upatham 1990, Basch 1991). Later, surface of mature daughter sporocysts can subsequently corrugate to form wide villi (Sobhon and Upatham 1990).

Architecture of surface and body wall seems to be similar in mother and daughter sporocysts. However, Smith and Chernin (1974) observed *S. mansoni* sporocysts and showed that mother sporocysts have longer and more branching microvilli than daughter sporocysts. According to data from oriental schistosomes and our observation of *T. regenti*, however, the length, density and branching of microvilli depend significantly on age of individual larvae and differ also between species (Sobhon and Upatham 1990). Therefore, these structures should not be considered as a reliable characteristic for distinguishing mother and daughter sporocysts.

Tegument of daughter sporocysts contains numerous uniciliated nerve endings and, contrary to mother sporocysts, also posteriorly directed spines in apical region. These tegumental spines are ultrastructurally similar to those of cercariae. They have been documented for human as well as bird schistosomes (Smith and Chernin 1974, Meuleman *et al.* 1980, Neuhaus 1952), including *T. regenti* (Bulantová unpublished) (fig. 6A).

We can only hypothesize about the function of spines that are most apparent in newly emerged daughter sporocysts. Data about biology of young daughter sporocysts are insufficient, but it is probable that their tegumental spines could have a role in liberation of daughter sporocysts from the internal space of mother sporocyst. Spines could also facilitate migration through the snail tissue, or serve as an anchor in the definitive localization of daughter sporocyst.

Simultaneously with progressive development of tegument of daughter sporocysts inside mother sporocyst, primitive epithelium formed by mother sporocysts around next intramolluscan stages of the worm degenerates (Smith and Chernin 1974, Meuleman *et al.* 1980, Sobhon and Upatham 1990). Cytoplasm of tegument and subtegumental cells of daughter sporocysts contains the same organelles as documented for mother sporocysts. Namely, there are mitochondria, endoplasmic reticulum, membrane-bound vesicles, numerous large lipid droplets (fig. 7) and glycogen particles. In addition, tegument of young daughter sporocysts contains dense bodies. Formerly, these bodies were supposed to be involved in formation of tegumental spines. According to recent data, dense bodies from tegumental cytoplasm of mature, but still not emerged daughter sporocysts contain lytic enzymes which enable release of daughter sporocysts from the body of mother sporocyst in case that no birth pore exists. (Smith and Chernin 1974, Meuleman *et al.* 1980, Sobhon and Upatham 1990, Ivanchenko *et al.* 1999).

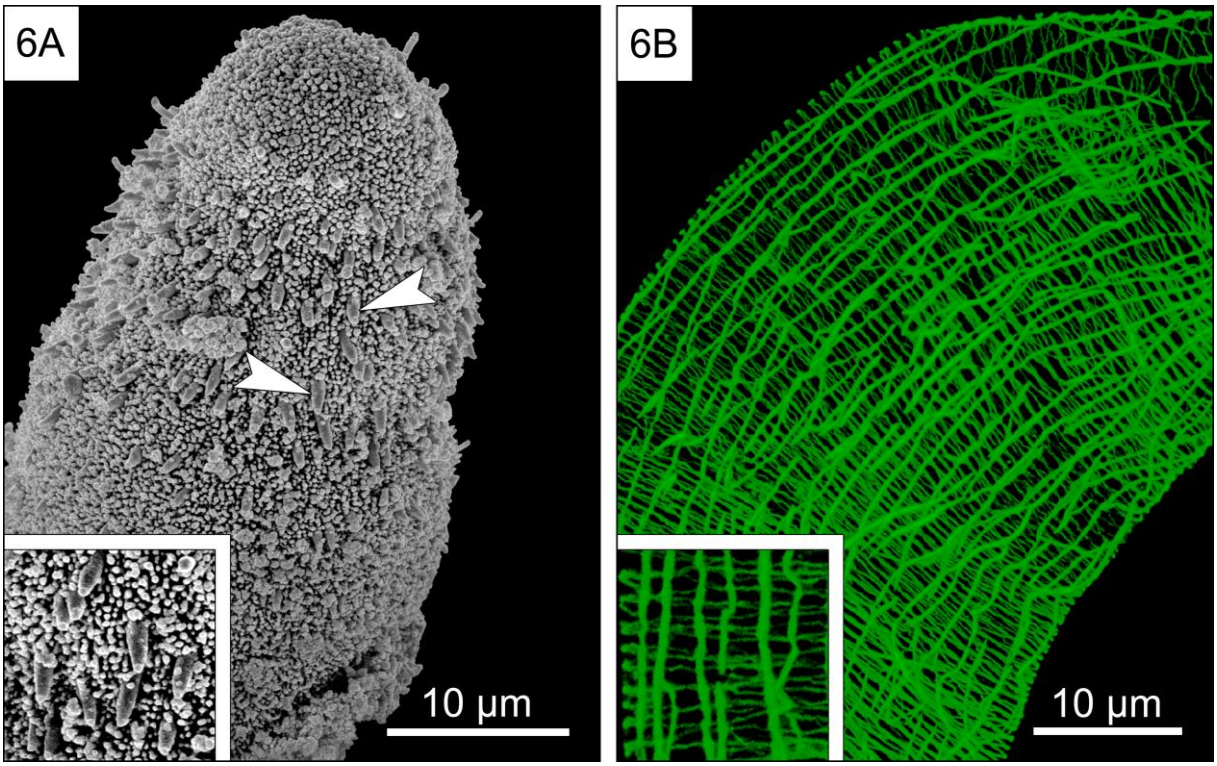
In some species of human schistosomes, organization of subtegumental cells can be more complicated if compared to that in mother sporocysts. While in *S. mansoni,* only one type of subtegumental cells was described for daughter sporocysts (Hockley 1973, Meuleman *et al.* 1980), Sobhon and Upatham (1990) distinguish two types of subtegumental cells of *S. japonicum* – the large round cells and the additional subjacent cells with spindle-shaped nuclei and very long cytoplasmic processes. In addition, large round subtegumental cells of *S. mekongi* daughter sporocysts can be divided into two groups mainly according to the characteristics of nuclei. Third cell type corresponds with subjacent cells described above for *S. japonicum* (Sobhon and Upatham 1990). The same authors established that subtegumental cells of oriental schistosomes with long cytoplasmic processes are able to coalesce around

embryonic cercariae and form envelope of primitive epithelium as described for mother sporocysts. Although only one type of subtegumental cells was described in *S. mansoni* (Meuleman and Holzmann 1975), process of formation of primitive epithelium around embryonic cercariae is the same as for oriental schistosomes with two or three types of subtegumental cells.

Anyway, body wall of sporocysts comprises complicated labyrinth of subtegumental cells with numerous thin and long projections dynamically arising and disappearing around the internal embryonal content. Therefore, detailed characterization and correct interpretation of its morphological features based on 2–D sections is difficult. In addition, ultrastructural characteristics can differ between various generations of daughter sporocysts that can be present in the snail tissue simultaneously. Observation can also vary in different regions of one sporocyst, thus sometimes we are not able to recognize, if similarity or disparity between two schistosome species is caused by real situation, insufficient amount of serial sections or investigated specimens. However, general construction and development of tegument in *T. regenti* daughter sporocysts is in agreement with the data known for human schistosomes, especially for *S. mansoni*. Based on the uniformity of intramolluscan development of schistosomes, the above data are probably common for all schistosomes, with only minute differences.

Ultrastructural studies of musculature in daughter sporocysts of human schistosomes usually refer to "not clearly arrayed net of fibers" (Smith and Chernin 1974). Based on the observation of whole mounted daughter sporocysts of *T. regenti* in confocal microscope (Bulantová *et al.* 2011), it was shown that musculature in early emerged daughter sporocysts resembles that in mother sporocysts after unfastening their longitudinal muscle bundles. In both generations of sporocysts, thin circular and longitudinal muscle fibers are densely arranged during the early phase of development, but spaces between individual fibers increase with growth of the organism. Nevertheless, muscle fibers preserve their well organized transversally crossed appearance for the rest of life (fig. 6B) (Bulantová *et al.* 2011).

Similarly to mother sporocysts, daughter sporocysts use their body wall musculature for locomotion within the snail tissue. Daughter sporocysts migrate from the position of mother sporocyst (head-food region) to their definitive localization in ovotestis or hepatopancreas (Basch 1991, Horák *et al.* 2002). Hypothetically, contractions of body wall musculature can serve also for release of mature cercariae to the outside of daughter sporocysts.




**<u>Fig. 6A</u> – Apical end of *T. regenti* daughter sporocyst from the snail host 10 days p.i., SEM.**

Note apparent spines **(white arrowheads)** protruding over the layer of microvilli. Frame: The same situation in detail.

**<u>Fig. 6B</u> – Body wall musculature of *T. regenti* daughter sporocyst from the snail host 20 days p.i., CLSM, FITC labeled F-actin in muscle fibers.**

Note the regular arrangement of thin circular and thicker longitudinal muscle fibers. Frame: Circular musculature (horizontal fibers) and longitudinal musculature (vertical fibers) in detail.

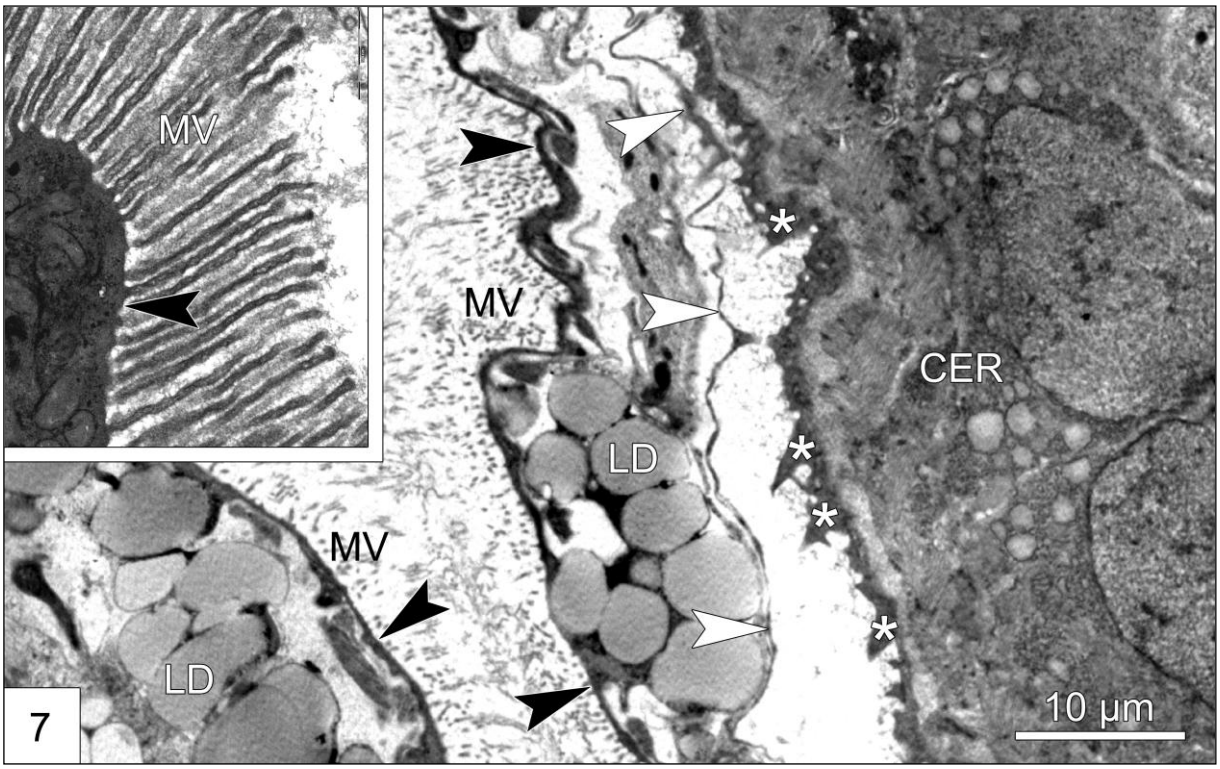



**Fig. 7 – Ultrastructure of *T. regenti* daughter sporocysts inside the snail hepatopancreas 29 days p.i., TEM.**

Note the body wall of daughter sporocysts **(black arrowheads)** with numerous non-branching microvilli on the surface **(MV)** and large lipid droplets **(LD)** in cytoplasm. Thin layer of primitive epithelium **(white arrowheads)** surrounds developing cercaria **(CER)**. Tegumental spines of cercariae are marked with **white asterisks**.

### 1.2.5. Cercaria

Being infective for definitive host, the stage of cercaria is together with adult worms probably the most examined developmental stage. In the case of bird schistosomes, cercariae are important not only as infective stage for natural bird hosts, but also as an agent causing cercarial dermatitis in accidental mammalian hosts including humans.

Cercariae develop in large amounts inside the body of daughter sporocysts. Mature larvae are released to the snail tissue where they migrate to reach external water environment. Free-living cercariae swim to find a suitable vertebrate host before they spend their energy reserves (Basch 1991, Horák *et al.* 2002).

Cercariae of schistosomes have elongated contractible body with long tail terminated by two dorsoventrally flattened furcae. Each is lined with an undulated hem and terminated by small apical fin (Sobhon and Upatham 1990, Horák *et al.* 2002, Dorsey *et al.* 2002). Some groups of schistosomes (among others also members of the genus *Trichobilharzia*) have two pigmented eye spots and, therefore, they are termed "ocellate" cercariae. Apical end of body, called sometimes head organ or anterior organ, carries openings of penetration glands with a number of associated sensory receptors, and basement of future oral opening (fig. 12A). Well developed ventral sucker (acetabulum) is situated in the posterior half of body. Highly flexible tail stem is connected to the end of the body by a thin stalk surrounded by apparent body collar (fig. 9A, 10A).

Origin and development of embryonic cercariae inside daughter sporocysts closely resemble development of daughter sporocysts inside mother sporocysts (see chapter 1.2.4.). Each germinal ball (future cercarial embryo) is enveloped by one or more layers of primitive epithelium derived from thin cytoplasmic processes of subtegumental cells of daughter sporocyst body wall. Cytoplasmic processes merge to form a syncytium. Cercarial tegument originates from joined superficial cells of embryo, in which nuclei gradually disappear. Subsequently, surface syncytial layer is connected to subjacent subtegumental cells by their cytoplasmic extensions, future cytoplasmic bridges (Hockley 1973, Meuleman and Holzmann 1975, Sobhon and Upatham 1990). Finally, the tegument is enriched with tegumental spines and coated with glycocalyx. Simultaneously with completion of cercarial tegument, primitive epithelium inside the daughter sporocyst body disintegrates (fig. 7) (Cheng and Bier 1972, Hockley 1972, Meulemann and Holzmann 1975, Sobhon and Upatham 1990). Mature cercariae escape from the internal space of daughter sporocysts and migrate through the snail tissue to reach external environment.

Surface of newly emerged mature cercariae is coated with layer of highly immunogenic glycocalyx (Dalton *et al.* 1987) produced by cercarial tegument during intramolluscan development (Hockley 1972, Stein and Lumsden 1973). Compact layer of glycocalyx is interrupted only in the area of surface receptors, openings of penetration glands and excretory pores (Hockley 1973). Based on TEM studies, glycocalyx is formed from a net of thin branching filaments that are closely associated with surface tegumental membrane. Rarely (e.g. in *S. mekongi*), only seamless gel-like structure of glycocalyx was observed (Sobhon and Upatham 1990).

Surface coat of glycocalyx effectively protects cercariae against external osmotic changes accompanying migration through the snail tissue and subsequent rapid transition to the hypoosmotic water environment. Other presumable functions were summarized by Hockley (1973) or Sobhon and Upatham (1990), and include adhesivity of cercariae to the vertebrate host skin before its penetration, protection of cercarial surface against aggressive substances from snail hepatopancreas or cercarial penetration gland products, and lubrication of cercarial surface for facilitated escape from snail body or penetration into the vertebrate host skin.

Glycocalyx of cercarial body and tail differs in thickness (Hockley 1973, Dorsey 2002) and chemical composition (Nanduri *et al.* 1991). These findings from human schistosomes were confirmed also for *T. regenti* and other members of the genus *Trichobilharzia* (Podhorský *et al.* 2009) (fig. 8A, 10A). Some basic functions of glycocalyx are probably common to both cercarial parts (body and tail), at least at the time of intramolluscan development/migration and swimming in water reservoir. Functional differences are manifested predominantly during the process of vertebrate host infection. While the body continues in development inside the vertebrate host and gradually transforms its trilaminate outer membrane with immunogenic glycocalyx to heptalaminate membrane with carbohydrate residues, the tail is discarded.

Syncytial layer of tegument covers the entire surface of cercariae and reaches up to the inner space of the oral opening and the pairs of openings of excretory pores. It forms also external parts of some surface receptors and folds which cover openings of penetration glands (Hockley 1973). Simple trilaminate tegumental membrane is only mildly undulated. The same is valid for basal membrane linked to the subjacent lamina basalis by numerous regularly distributed hemidesmosomes (Hockley 1973, Sobhon and Upatham 1990).

Tegument is armed with numerous, posteriorly directed spines, except for the apical area with penetration gland openings, the region around oral opening (fig. 12A), the inner part

of collar at the body/tail junction (fig. 9A) and furcal tips (Dorsey *et al.* 2002). Density of spines decreases from anterior to posterior end of cercariae (for situation in cercarial body compare fig. 9A and 12A). The least density of spines was detected on the tail stem and furcae where also tegumental layer is thinner compared to that of cercarial body (Hockley 1973, Sobhon and Upatham 1990, Dorsey *et al.* 2002). Tegumental spines are anchored to dense plaques on the basal membrane, thus their large part is embedded within the tegument. Protruding tips of spines are covered by surface membrane of tegument and embedded in layer of glycocalyx (Hockley 1973) (fig. 8A). Numerous surface receptors of several types are incorporated into the tegument *via* septate desmosomes. Number and arrangement of sensoric papillae can serve in some species for determination (Podhorský *et al.* 2009).

Tegument of cercarial body lacks nuclei, but contains small mitochondria, dense spherical granules and flattened elongated (or discoid) bodies (Hockley 1973, Hockley and McLaren 1973, Cousin *et al.* 1981, Dorsey *et al.* 2002). Anucleated tegumental syncytium is connected with cytons of subtegumental cells which are situated under the lamina basalis and subjacent body wall musculature. Connection is provided by cytoplasmic bridges, lined with tubular reinforcement (Sobhon and Upatham 1990, Dorsey *et al.* 2002) (fig. 8A).

Subtegumental cells are densely filled with numerous bodies. Only one type of subtegumental cells with two apparently different types of membraneous bodies were described for cercariae of oriental schistosomes (Sobhon and Upatham 1990). According to Dorsey *et al.* (2002), four types of subtegumental cells can be distinguished in cercariae of *S. mansoni* according to their localization within cercarial body, length of cytoplasmic bridge and appearance and function of intracellular bodies. Majority of described subtegumental cells contain membraneous or dense bodies used for future transformation of tegumental membrane inside the vertebrate host. Surprisingly, one type of subtegumental cells contains also granula with biogenic amines and is belived to serve as a sensory cell (Dorsey *et al.* 2002).

Another organ contributing to the content of tegument is the head gland. It lies inside the head organ and is filled with numerous granula resembling those inside penetration glands (Dorsey *et al.* 2002, Ligasová *et al.* 2011). While penetration glands release their content out of the body of cercaria via wide openings in the apical part of head organ, the head gland opens its numerous thin projections directly into the tegumental syncytium. Thus, the mode of content release resembles subtegumental cells rather than penetration glands.

Cercarial tail is used for swimming and it is lost during transformation of cercariae to schistosomula. Its tegument contains only small mitochondria and sparse discoid bodies

which are known also from the tegument of cercarial body, schistosomula and adult worms (Hockley 1973). Wilson and Barnes (1974) suggest that discoid bodies represent source of granular components of tegument, while MacGregor *et al.* (1988) refer to contribution of discoid bodies to formation of surface tegumental membrane. Discoid bodies were believed to be also included in formation of spines, but this presumption was finally rejected (Matsumoto *et al.* 1988). Anyway, presence of these structures inside the tail tegument indicates that discoid bodies are probably not necessarily involved in transformation of trilaminate cercarial membrane to heptalaminate membrane of schistosomula.

Cercaria is the most motile stage during the development of schistosomes, and its musculature is similar in both, human and bird schistosomes. Musculature of cercarial body comprises thin outer circular muscle fibers and subjacent thick bands of longitudinal muscles, organized into dense muscular sheath (fig. 8A). Underlying layer of muscle fibers is called diagonal musculature and it is developed only in the forebody. Individual fibers aggregate into close pairs separated from each other by wide gaps. Paired fibers from left and right side of cercarial body cross each other in the angle of 120° and resemble sparse net. (Mair *et al.* 2003, Bulantová *et al.* 2011). Presence of these muscles only in forebody suggests their presumable importance during the process of penetration into the vertebrate host.

Cercarial body is reinforced also by sporadic radial muscle fibers, leading through the cercarial body from the innermost muscle layer to primordia of future internal organs (fig. 13A). This type of musculature was newly established in cercariae and becomes abundant in subsequent intravertebrate stages of schistosomes (Bulantová *et al.* 2011). No equivalents of these muscle fibers were described for miracidia or sporocysts of any schistosome examined. This is evidently caused by the fact that miracidia and sporocysts are simple sack-like larvae without any specialized digestive or reproductive systems inside and only minute internal organs (e.g. flame cells) need to maintain stable position in the worm body.

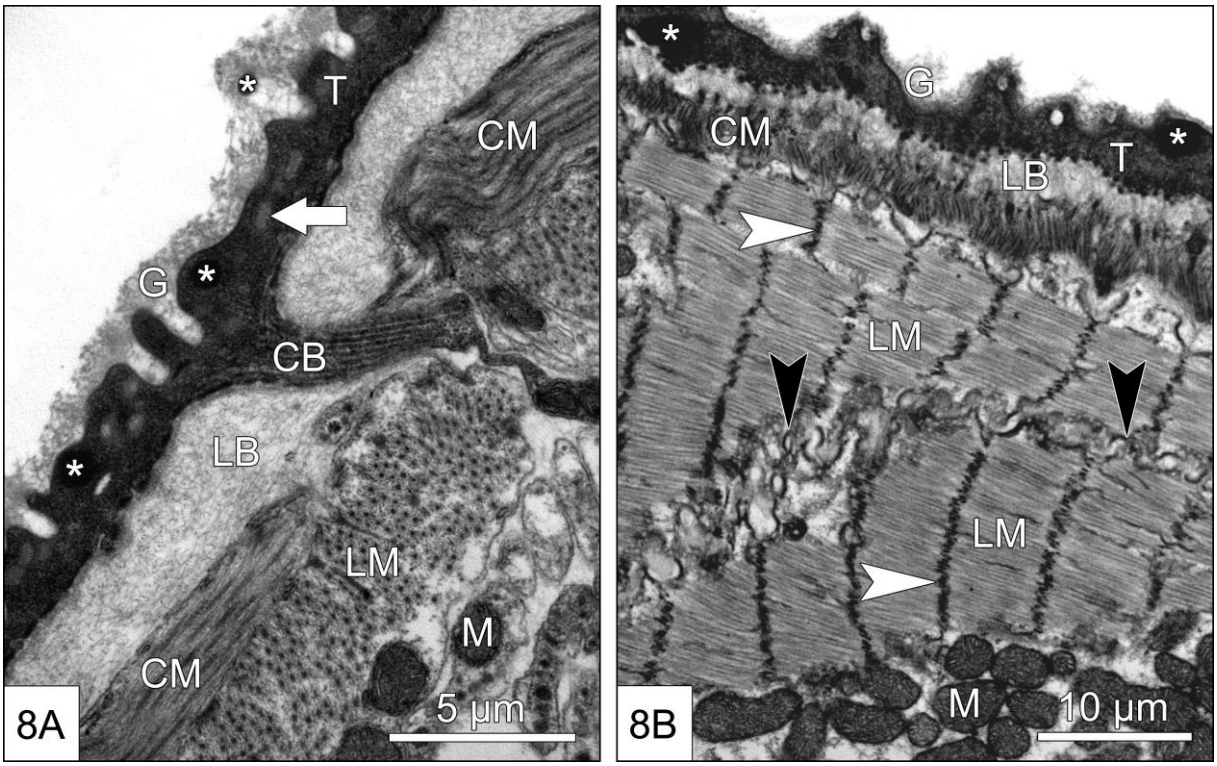
Function of head organ is supported by a huge cone with highly developed and complexly arranged musculature (fig. 13A) which has its own layer of lamina basalis (for more details see Bulantová *et al*. 2011). This layer is necessary for attachment of muscles and apparently separates head organ (future oral sucker) from the rest of cercarial body (Bulantová *et al.* 2011).

The same arrangement of lamina basalis is also present in the main organ of attachment of cercaria, the ventral sucker (acetabulum). As in the case of head organ, huge musculature of *T. regenti* cup-shaped acetabulum is attached to lamina basalis which joins

ventral sucker and the body of cercaria. External part of acetabulum is equipped with circular and longitudinal musculature as in the case of *S. mansoni* (Cousin *et al.* 1995, Mair *et al*. 2003), internal muscles are aggregated to radially organized muscle bundles. Tight integration of acetabulum into the cercarial body is mediated also by wide muscle bundles, outgoing from acetabular rim laterally (3 pairs) and posteriorly (1pair) (fig. 13B). These muscles lead subjacently to the cercarial body wall musculature (Mair *et al*. 2003, Collins *et al.* 2011) and serve as "rooting" muscles which are responsible also for protruding and retracting movements of acetabular cup. (Bulantová unpublished).

Tail musculature of schistosomes is highly adapted to rapid movement. That is visible on its mass, arrangement and ultrastructural peculiarities (Lumsden and Foor 1968, Mair *et al*. 2003, Bulantová *et al*. 2011, Collins *et al*. 2011). Circular muscles of the tail stem resemble those of cercarial body. Longitudinal musculature comprises dorsal and ventral doublets of non-striated myofibers. Remaining muscles in the subtegumental sheath are formed from striated myofibers (fig. 8B) with U or L shaped appearance in cross section. Tail furcae contain only sparse net of individual circular and bundled longitudinal muscles. Opposite sides of each furca are connected with numerous short fibers. No equivalents of these fibers were observed in the tail stem (Dorsey *et al.* 2002, Mair *et al.* 2003, Bulantová *et al.* 2011).

Presence of striated musculature exclusively in the cercarial tail stem is known from cercariae of many trematode species (Lumsden and Foor 1968). This special type of musculature is predetermined for rapid and sustained movements, and is not present in any other part of cercarial body or any other ontogenetic stage of schistosomes. Lateral position of striated musculature in cercarial tail stem indicates that the main swimming movements are performed from left to right. It corresponds also with posture of furcae, plains of which are also laterally oriented. Dorsal and ventral bundles of longitudinal muscles in the tail stem are non-striated, and could be responsible for minor tail activity, e.g., for bending of tail in direction required for oriented swimming of cercariae or regulation of mutual position between two branches of tail furka.




**<u>Fig. 8A</u> – Body wall of *T. regenti* cercarial body, TEM.**

Cercarial tegument **(T)** contains discoid bodies **(white arrow)** and spines **(white asterisks)**. It is coated with layer of glycocalyx **(G)** and connected to subtegumental cell bodies by cytoplasmic bridge **(CB)**. Labels: **LB** = lamina basalis, **CM** = circular muscles, **LM** = longitudinal muscles, **M** = mitochondria.

**<u>Fig. 8B</u> – Body wall of *T. regenti* cercarial tail stem, TEM.**

Tegument **(T)** with spines **(white asterisks)** is coated with thin layer of glycocalyx **(G)**. Striation of longitudinal muscles is labeled with **white arrowheads**, the border between individual muscle cells is designated by **black arrowheads**. Labels: **LB** = lamina basalis **CM** = circular muscles, **M** = mitochondria.

### 1.2.6. Schistosomulum

Cercariae which penetrate vertebrate host change to the stage of schistosomulum. Schistosomula enter blood vessels or migrate through the host tissues to places of definitive localization of adult worms. They continually change their appearance. First of all, just penetrated schistosomula transform their cercarial surface membrane from single trilaminate to double heptalaminate. This is essential for adaptation to the conditions inside the vertebrate host, evasion of host immunity and ability to acquire nutrition from the host (Hockley and McLaren 1973, Pearce *et al.*1986, Sobhon and Upatham 1990, Skelly and Shoemaker 1996). The main part of membrane transformation is completed in several hours, but parts of the already transformed surface membranes are continuously changing for the rest of worm life. Additional changes as growth, corrugation of enlarged tegumental mass and development of internal organs gradually occur in migrating schistosomula (Hockley and McLaren 1973, Sobhon and Upatham 1990).

Heptalaminate surface membrane consists of two parallel trilaminate membranes which differ in their biophysical properties (Kusel and Gordon 1989). This unusual surface covering was detected not only in the intravertebrate stages of schistosomes, but also in other flukes inhabiting bloodstream (Sanguinicolidae of fishes and Spirorchiidae of turtles). It indicates that there exists connection between intravascular localization and formation of heptalaminate surface membrane (McLaren and Hockley 1977).

Surprisingly, despite of localization of *T. regenti* schistosomula in the nervous tissue outside the bloodstream (Horák *et al.*1999, Hrádková and Horák 2002), intravertebrate stages of this parasite undergo the same process of surface transformation as is usual for typical blood-dwelling schistosomes. (Chanová *et al.*2009).

Transformation of cercariae to schistosomula is a complex process described in detail by many authors (Hockley and McLaren 1973, Wiest *et al.*1989, McLaren and Hockley 1976, Sobhon and Upatham 1990, Horák *et al.*1998b, Chanová *et al.*2009). It begins with emptying of cercarial glands onto the host skin to enable its degradation and penetration of transforming schistosomula to the subjacent layers of host tissue. During this process, cercarial tail is lost. Scar arising after tail abruption and containing interrupted collecting ducts of excretory system invaginates to be closed by contraction of muscular rim, and by covering the place with tegument (fig. 9A, 9B, 10A, 10B).

Also granula from cercarial head gland are released into the tegument in the early phase of cercaria/schistosomulum transformation. In *S. mansoni*, this process is believed to

contribute to reconstruction of the anterior end in case of any damage during penetration into the host skin. Some of dense homogenous bodies of cercarial head gland change to membraneous bodies, and these bodies presumably participate in future reconstruction of surface membrane from trilaminate to heptalaminate (Dorsey 1976).

In the same time, cytoplasmic bridges of subtegumental cells open and allow transport of various dense granula and membraneous bodies from subtegumental cells to the tegument, where they are noticeable at about 30 minutes after the skin penetration. The most numerous membraneous bodies apparently serve for substitution of the simple membrane by heptalaminate one (Smith *et al.*1969, Hockley 1973, Hockley and McLaren 1973). At first, membraneous bodies fuse together inside the tegument and form multilamellar vacuoles, tubular channels or sheets, which are preassembled in the tegument and added to the former surface membrane as a large unit (Hockley and McLaren 1973, Sobhon and Upatham 1990). Parts of cercarial simple membrane with glycocalyx are in *S. mansoni* rejected in the form of long microvilli and simultaneously replaced by blocks of heptalaminate membranes from multilamellar vacuoles (Hockley and McLaren 1973, Wiest *et al.*1989, McLaren and Hockley 1976, Horák *et al.*1998b, Chanová *et al.*2009). In the case of oriental schistosomes, rejection of surface membranes was observed in the early phase as a rapid formation of microvilli, cytoplasmic droplets, membrane puffs and large blebs. In addition, occurrence of dense-core vesicles created from simple surface membrane with glycocalyx and pieces of spines was recorded (Sobhon and Upatham 1990).

In the case of bird schistosomes, processes similar to both different ways of transformation were recorded in various species. *Trichobilharzia szidati* and *T. ocellata* using blood vessels for migration show presence of blebs or bubbles on surface of transforming schistosomula, as known for oriental schistosomes. Surprisingly, these blebs of shed surface material were apparent already under the light microscope (Howell and Bourns 1974, Chanová *et al.*2009). Contrary to that, no apparent blebs or bubbles were observed on the surface of *T. regenti* migrating through the nervous tissue; only occasional occurrence of small structures resembling dense-core vesicles was recognized by SEM on the surface of tegumental spines. Briefly, early transformation of *T. regenti* shows attributes of that in *S. mansoni* (simple membrane rejection in the form of microvilli without blebs and bubbles), but also of that in oriental schistosomes (presence of possible dense-core vesicles). In the next phase of transformation, changes of surface membrane are followed by modifications of tegumental topography. The tegumental surface between spines of *T. regenti* starts to form

small microtubercules with top invaginations (Chanová *et al.*2009) (fig. 11A), and just this resembles changes known from *S. mansoni* (Bogitsh and Carter 1979).

The main wave of surface membrane replacement lasts for about 3 hours in *S. mansoni*, and takes from 6 to 12 hours in oriental schistosomes. After this phase, the tegument still contains membraneous bodies, but also large multilamellar vacuoles and discoid (elongate) bodies. Glycocalyx is noticeably reduced and the surface membrane has heptalaminate or pentalaminate appearance, the latter caused by close proximity of two trilaminate membranes (Hockley and McLaren 1973, Chanová *et al.*2009).

Immediately after the change of surface membrane, schistosomula come to lag-phase, when no food ingestion and only minute growth are observed. Also turnover rate of the surface membranes is much slower than in older schistosomula or adults. This phenomenon was documented for human schistosomes as well as for *T. regenti* (Bogitsh and Carter 1979, Blažová and Horák 2005, Chanová *et al.*2009), and it is believed to serve probably for conditioning of schistosomula to the new intravertebrate environment, and rebuilding of metabolic pathways after the rapid and demanding transformation of the tegumental membrane.

Nevertheless, we hypothesize that there could exist another reason for the presence of lag-phase in the development of schistosomula. The lag-phase could be connected with the development of oral sucker. As seen in some older articles, head organ of cercariae is sometimes called oral sucker (e.g. Hockley 1973), although oral opening is in cercariae present only in a form of small aperture on the ventral site of head organ (fig. 12A). Also organization of cercarial head organ (presence of head gland, ducts of penetration glands, arrangement of musculature) importantly differs from structures usually designated as suckers. Development of oral sucker from head organ (fig. 13A, 13B, 13C) usually takes approximately tens of hours (Bogitsh and Carter 1979, Blažová and Horák 2005). In *T. regenti*, time needed for creation of oral sucker basement closely corresponds with termination of lag-phase, when schistosomula start to eat and rapidly grow.

Immediately after the lag-phase, schistosomula continue in development, probably thanks to food ingestion. This is connected with rapid growth and changes of the tegument and internal organs. Increase of tegumental mass and submersion and/or disintegration of tegumental spines in middle body part were observed (Bogitsh and Carter 1979, Sobhon and Upatham 1990). Cytoplasm of tegument still contains sporadic mitochondria, membraneous and discoid bodies. Additionally, numerous dense homogenous bodies emerging from subtegumental cells appear in tegument (Smith *et al.*1969, Hockley and McLaren 1973).

Dense homogenous bodies recognized in the stage of mid-developed schistosomulum of *S. mansoni* disappear in adult worms (Hockley and McLaren 1973). Similar bodies with dense content were observed in large amounts also in tegument of schistosomula and adult worms of *T. regenti*. We can hypothesize that (a) the content of homogenous bodies is used inside the tegument for e.g. addition of material needed for increase of tegumental mass, (b) the granula contain highly immunogenic compounds which are released out of the tegument (see Chanová *et al.* 2012), or (c) the granula without multilamellar membraneous content can represent only a temporary stage and can change to the membraneous bodies after their maturation inside the tegument, as described e.g. for bodies released by the head gland (see above).

Subsequently, tegument gradually forms extending protrusions and invaginations. These changes in topography continue to form tegument of several days old schistosomulum which comprises tegumental ridges, pits, wholes and channels (fig. 11B). Also internal tegumental membrane bound to lamina basalis by hemidesmosomes corrugates (Hockley and McLaren 1973, Bogitsh and Carter 1979, Sobhon and Upatham 1990) and finally, it copies/lines subjacent fibers of circular musculature (Bulantová *et al.*2011). These changes lead to expansion of surface area which is in direct contact with internal milieu of vertebrate host, in order to enable more intensive interaction with the host. Tegument in the middle part of schistosome body usually lacks tegumental spines, and these are preserved only in the most anterior (internal surface of oral sucker) and posterior (vicinity of excretory porus) ends (fig. 12B).

There is an apparent interspecific variability in the time required for surface membrane transformation, and also in the presence of various structures within the tegument or on the surface membrane. This disparity was supposable in the case of bird schistosomes of the genus *Trichobilharzia* where one of the species migrates via blood vessels (*T. szidati*) and the other through the nervous tissue (*T. regenti*). Nevertheless, a level of diversity was also described for human schistosomes which all belong to the blood-dwelling parasites. This phenomenon remains to be investigated.

Transformation of free living cercariae to the intravertebrate schistosomula is apparent also in changes of musculature. The only one part of cercariae with striated musculature, the tail, is lost and the scar invaginates deeply to form future excretory porus (fig. 9A, 9B).

Muscular cone of the head organ becomes flattened and changes to the basis of future oral sucker (fig. 13B). The latter is formed from the invaginated anterior end and subterminal oral opening of cercariae (fig. 12A) (Bulantová *et al.*2011). Internal and external walls of oral

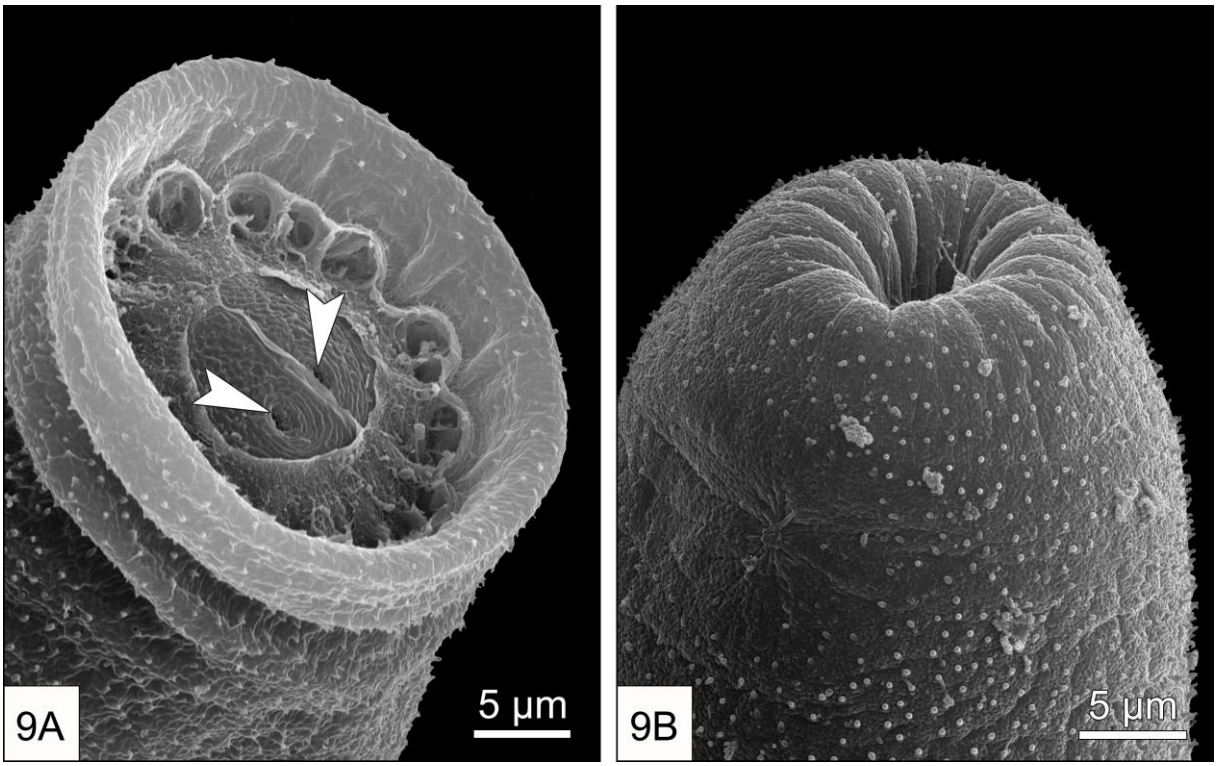
sucker become connected by strengthening fibers of radial muscles. Finally, the developed oral sucker represents a firm cup-like muscular organ (fig. 13C) which is separated from the rest of the body by subjacent layer of connective tissue (lamina basalis).

Musculature of acetabulum is still well developed after the transformation of cercaria to schistosomulum. Anyway, its fate highly depends on migratory routes of schistosomula, and also definitive localization of adult worms. In the case of schistosomes inhabiting major blood vessels, ventral sucker expands and changes to the nearly flat round structure with a short stem, serving as an effective anchor against blood flow inside vessels. In opposite, species living extravascularly have only small or fully atrophied suckers (Cousin *et al.*1995, Loker and Brant 2006).

In *T. regenti* migrating extravascularly through the spinal cord, acetabulum becomes smaller in relation to the rest of the body during development, shows slightly transversally elongated shape (Skírnisson *et al.* 2012), and it is less prominent if compared to human schistosomes.

Some other species of the genus *Trichobilharzia* with a known life cycle migrate through the blood vessels similarly as human schistosomes do or finish their development extravascularly. Their filiform body shape enables migration and subsequent definitive localization of both sexes in small capillaries with a low blood flow or directly in host tissue e.g., close to the intestinal epithelium (Bourns *et al.*1973). There anchoring function of acetabulum is not needed.

During schistosomula development, increase in number of circular muscles, and length and thickness of longitudinal muscles was observed in body wall musculature. Also the diagonal muscle fibers expand considerably, and additional crossed pairs of doubled muscle fibers are formed with the growth of schistosomula. Within a short time, a sparse net of diagonal musculature can be observed under the entire body surface. Radial muscle fibers sporadically observed in cercariae become denser in schistosomula, and form abundant connections of body wall with internal organs (mainly digestive tract) (Bulantová *et al.*2011).

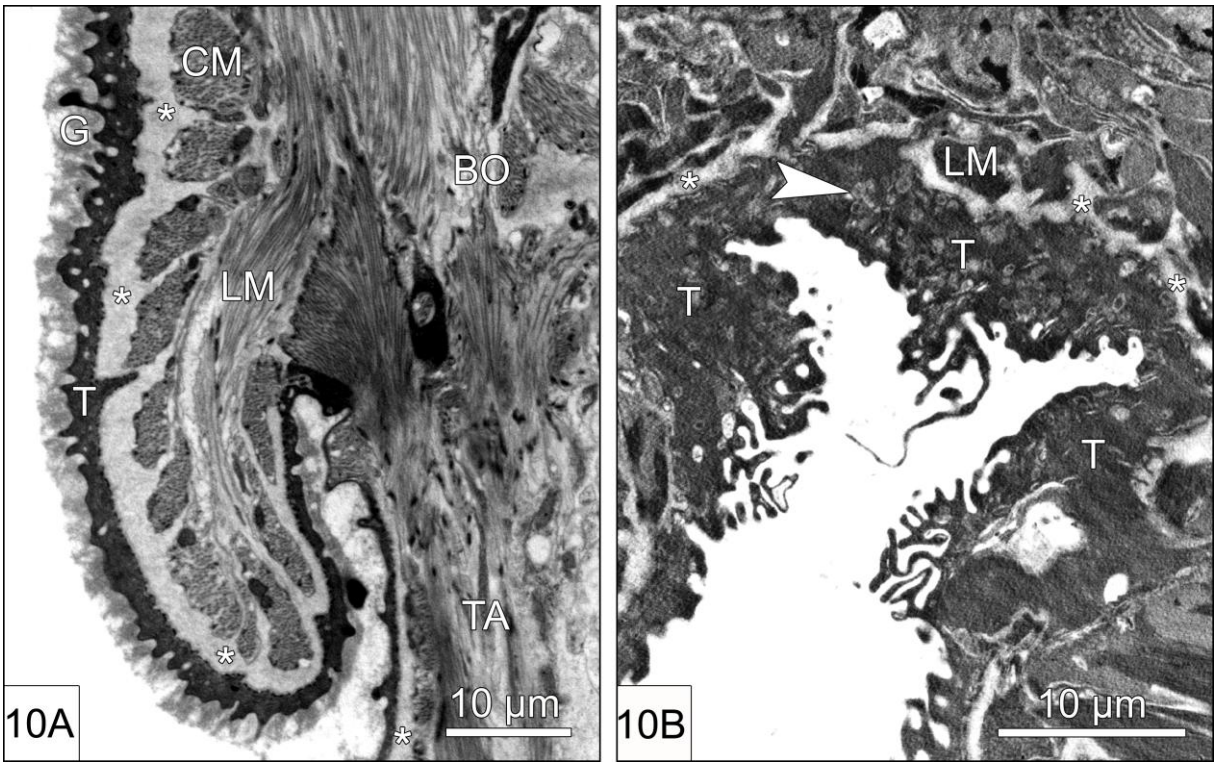



**<u>Fig. 9A</u> – Scar after tail abruption in *T. regenti* cercaria, SEM.**

Note the high collar of cercarial body margin with minimum of tegumental spines, and a pair of interrupted collecting ducts of the excretory system **(white arrowheads)**.

**<u>Fig. 9B</u> – Scar after tail abruption in newly transformed schistosomulum of *T. regenti* 3 hours p.i., SEM.**

Scar margins with collar invaginate and form excretory pore. Note blunt tegumental spines on the surface.

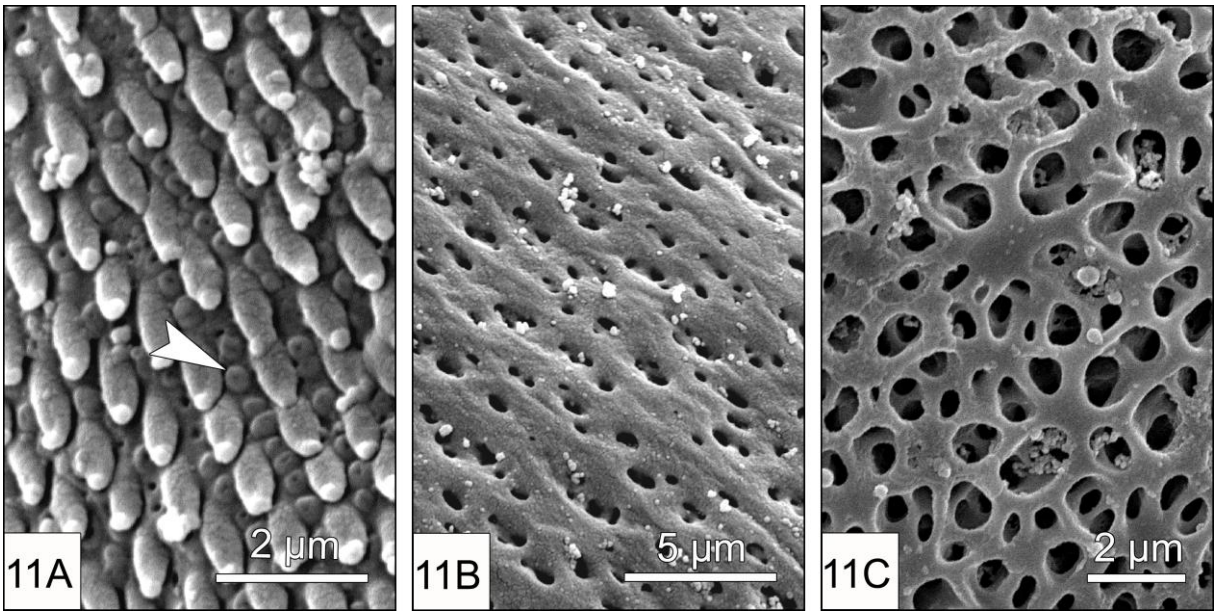



**<u>Fig. 10A</u> – Cercarial body of *T. regenti* before tail abruption, TEM.**

Labels: **BO** = cercarial body, **TA** = cercarial tail, **G** = glycocalyx, **T** = tegument, **asterisks** = lamina basalis, **CM** = circular muscles, **LM** = longitudinal muscles.

**<u>Fig. 10B</u> – Schistosomulum of *T. regenti* 3 hours p.i., TEM.**

Note the enlarged tegumental layer inside the invaginated scar after tail abruption. The layer is filled with numerous membraneous bodies **(white arrowhead)**. Labels: **T** = tegument, **asterisk** = lamina basalis, **LM** = longitudinal muscles.




**<u>Fig. 11A</u> – Surface of newly transformed schistosomulum of *T. regenti* 3 hours p.i., SEM.**

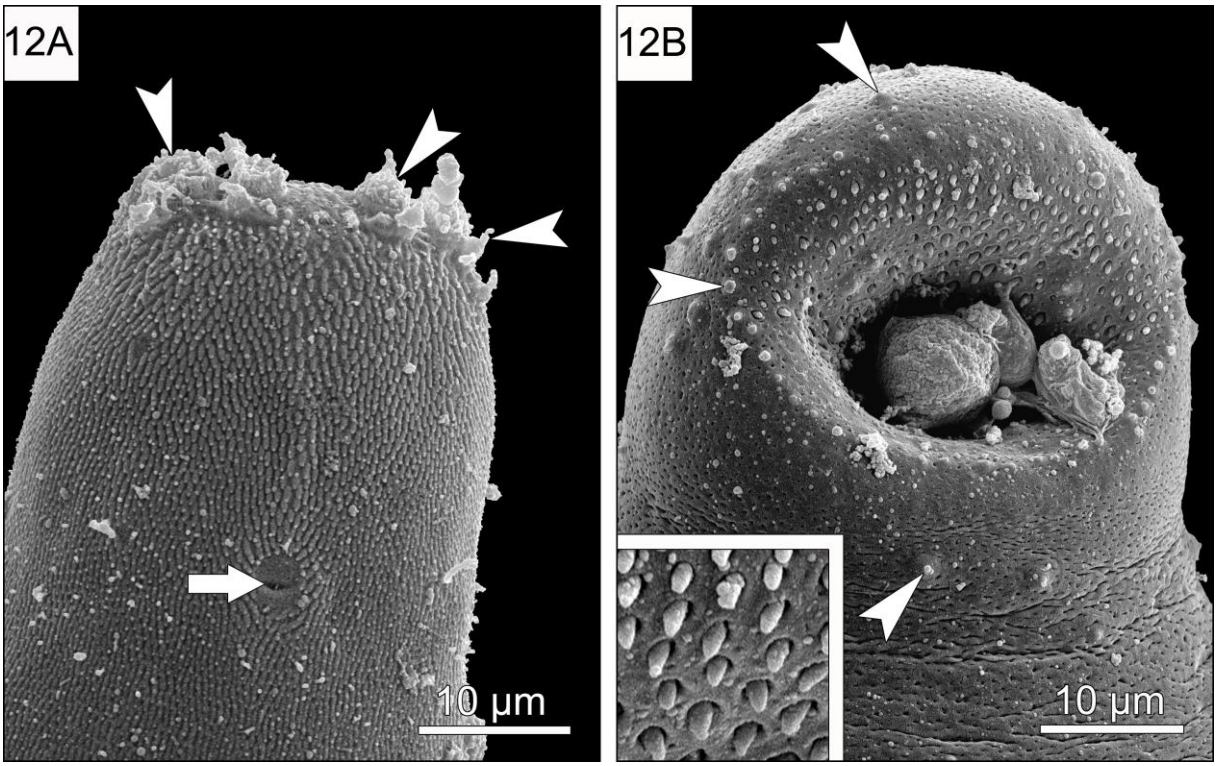
Note small tubercles with apical invagination **(white arrowheads)** in the space between tegumental spines.

**<u>Fig. 11B</u> – Surface of developed schistosomulum of *T. regenti* from the duck spinal cord 10 days p.i., SEM.**

Tegument has numerous holes and invaginations, no spines are visible.

**<u>Fig. 11C</u> – Surface of adult *T. regenti* from the nasal cavity of duck 20 days p.i., SEM.**

Deep invaginations form complex net of wide channels.

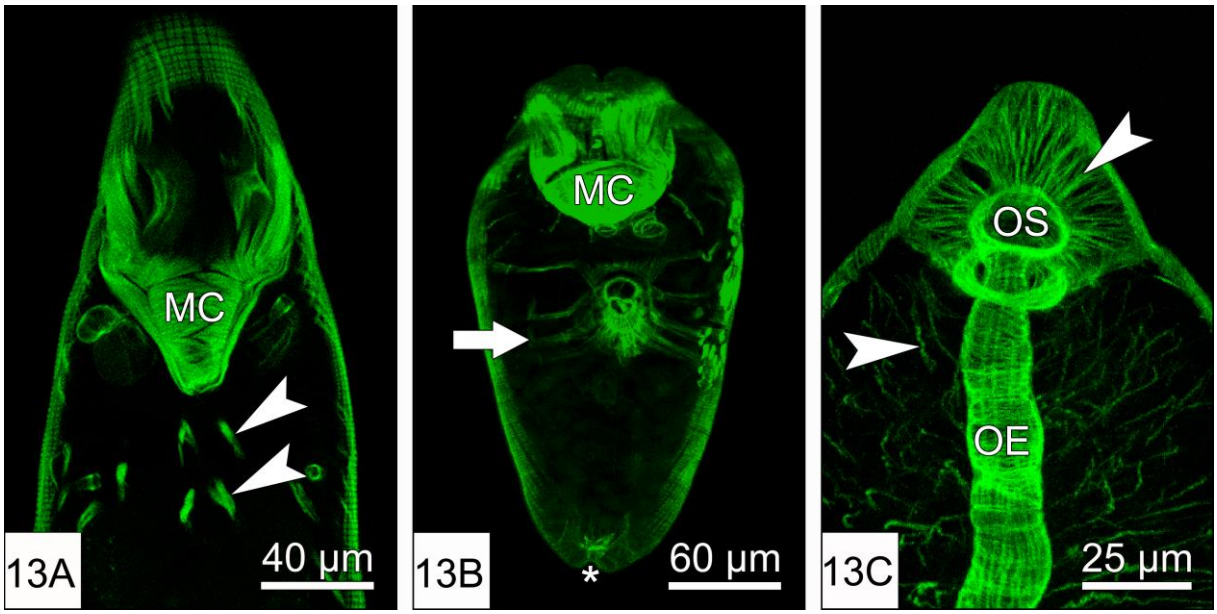



**<u>Fig. 12A</u> – Ventral view of cercarial head organ of *T. regenti*, SEM.**

Note the absence of tegumental spines around small future oral opening **(white arrow)** and apical part of cercaria with numerous surface receptors and openings of penetration glands **(white arrowheads)**.

**<u>Fig. 12B</u> – Oral sucker of *T. regenti* schistosomulum 10 days p.i., SEM.**

Transformed tegument is free of spines, except for the inner surface of oral sucker. Note numerous small papillae surrounding the rim or oral opening, and other types of papillae around the anterior end **(white arrowheads)**. Frame: Detail of tegumental spines on the inner surface of oral sucker.




**<u>Fig. 13A</u> – Anterior part of *T. regenti* cercarial body, CLSM, FITC labeled F-actin in muscle fibers.**

Note muscular cone **(MC)** of head organ. Sparse radial muscle fibers are designated with **white arrowheads**.

**<u>Fig. 13B</u> – Schistosomulum of *T. regenti* 3 days post transformation *in vitro*, CLSM, FITC labeled F-actin in muscle fibers.**

Apical part of head organ invaginates, bottom of muscular cone **(MC)** becomes wider. Note also acetabulum with trinity of lateral rooting muscles **(white arrow)** and accumulation of muscle fibers in the area of the excretory pore **(white asterisk)**.

**<u>Fig. 13C</u> – Schistosomulum of *T. regenti* from the spinal cord of duckling 5 days p.i., CLSM, FITC labeled F-actin in muscle fibers.**

Note the flat base of oral sucker **(OS)** which is created from the former muscular cone of cercaria. Numerous radial muscle fibers **(white arrowheads)** are apparent in the oral sucker and body where they lead towards oesophagus **(OE)**.

### 1.2.7. Adult worm

Development of migrating schitosomula leads to their maturity and reproduction in the place of definitive localization. Architecture of adult schistosomes arises as a gradual outcome of ongoing processes in the stage of schistosomulum, thus late schistosomulum and young adult worm resemble each other in organization of tegument and body musculature. Growth of adult worms is terminated, but maintenance processes as repetitive surface membrane replacements stay preserved.

The family Schistosomatidae shows many unusual biological features that are exceptional among other digenetic trematodes. One of the most interesting peculiarities is gonochorism, i.e., the presence of separated sexes in all schistosomes (Basch 1991). Gender of many schistosomes (e.g. the genera *Heterobilharzia*, *Macrobilharzia* and *Schistosoma*) can often be distinguished at first sight according to their size and shape. Apparently bigger males have muscular body with canalis gynecophorus at their ventral side. This structure serves for embrace of one or several longer, but slender females. The other group of schistosomes (e.g. the genera *Dendritobilharzia*, *Gigantobilharzia*, *Trichobilharzia*) show low level of sexual dimorphism. Both sexes have similar size and body shape and their gender can be recognized by a closer microscopical examination (Loker and Brant 2006).

Ultrastructurally, males and females of schistosomes with highly developed sexual dimorphism differ in tegumental topography (e.g. Silk *et al.* 1969, Silk *et al.* 1970, Miller *et al.* 1972, Sobhon and Upatham 1990, Gobert *et al.* 2003). These differences start to be apparent already in the stage of late schistosomulum.While females have densely pitted, moderately corrugated flat tegument with numerous holes and channels, the tegument of adult males is considerably more corrugated by rows of tall-undulated and branched ridges. In some species (e.g. *S. mansoni*, *S. haematobium*) males have additional large tegumental tubercles bearing small tubercular spines which differ from tegumental spines of cercariae and schistosomula, and are probably synthesized de novo in the adult worm. Sensoric papillae and tegumental spines are sometimes preserved from the stage of schistosomulum. They vary in distribution between individual species, but also intraspecifically between males and females (Sobhon and Upatham 1990).

Support and nourishment of females by males is probably the main reason for extraordinariness in topography of “male type” tegument. Males are usually essential for the development of eggs inside females. Intimate contact of females with males allows insemination, but also interchanges of soluble materials necessary for nourishment of females,

or hormonal interactions between both partners. Additional role of males consists in physical protection of females and their stimulation by muscular contractions. Males with well developed musculature are also able to transport females closer to the sites suitable for oviposition (Basch 1991, Loker and Brant 2006).

Not all schistosomes have so pronounced sexual dimorphism. In some species, males and females are nearly uniform in size and shape of the body. In many of them, gynecophoric canal is reduced or completely absent (Loker and Brant 2006). In the genus *Trichobilharzia*, both sexes have a filiform body shape and often also spatulate posterior end. Both sexes are of almost uniform width, and canalis gynecophorus of males is usually short (Horák *et al.* 1998a, Horák *et al.* 2002).

As observed in *T. regenti*, also tegumental topography is similar in both sexes, and resembles more or less the "female type" of surface of schistosomes with highly developed sexual dimorphism (fig. 11C, 14B). That corresponds with different mating behavior of schistosomes with low degree of sexual dimorphism. No long-term intimate contact is necessary for successful reproduction. Both sexes live independently inside the host tissue and meet each other only for a short time during copulation. With the exception of insemination, the males probably do not provide any other support for females. Therefore, they do not need any special type of tegument, allowing care and nourishment of the embraced female as was described for human schistosomes.

Surface of bird schistosomes of the genus *Trichobilharzia* can be covered by various tubercles, spines and sensoric papillae mainly in the area of oral sucker, acetabulum, canalis gynaecophorus, male opening of reproductive system and gonad region, and tail of both sexes (Horák *et al.* 2002). Presence of spines in all of the above mentioned areas was described in detail also for *T. regenti* from natural infections in Iceland (Skírnisson *et al.* 2012).

However, in our SEM study of *T. regenti* adults from the laboratory strain originated from Czech Republic, short tegumental spines were observed in both sexes only in the area of oral sucker and acetabulum. No spines were visible in the area of gonad, tail and around genital opening of adult females. Canalis gynecophorus of males was not examined by SEM, but presence of spines in walls of ventral groove was previously described by use of light microscopy (Horák *et al.* 1998a). Surface receptors of several types were abundant mainly in the area of oral sucker; numerous papillae also formed two narrow longitudinal zones along both sides of worm body (fig. 14B).

Discrepancy in observation of tegumental spines on the surface of adult worms of *T. regenti* by light microscopy and SEM could have several reasons. (a) Worms from natural

infections in Iceland were markedly larger than those described from experimental infections in our laboratory, thus other minute differences cannot be excluded. (b) As it was already mentioned by Skirnisson et *al.* 2012, some features can be detected only in fresh material with using e.g. Nomarski contrast and can disappear during processing. (c) SEM allows to see only surface topography. Tegument of adult worms of *T. regenti* reaches thickness of about 5–7 μm and obliquely arranged spines could be completely sunken into the tegumental layer and invisible by SEM. This presumption could be verified in fresh material by use of light microscopy with Nomarski contrast and subsequent SEM and TEM observation.

As suggested for schistosomula of *S. mansoni*, tegumental spines on the body surface can be used for movement inside the blood vessels (Crabtree and Wilson 1986). Adults of bird schistosomes, including *T. regenti* migrating outside the blood vessels, also show spination on their surfaces, predominantly in the same areas as documented for human schistosomes (Müller and Kimmig 1994, Horák *et al.* 2002, Skírnisson *et al.* 2012). Despite of differences in the body shape, the way of life and the degree of sexual dimorphism of bird and human schistosomes, the main pattern of spine distribution is preserved in both groups, independently on the migratory routes.

Tegument of schistosomes documented by TEM is characterized by the presence of high and broad microvilli, pits and branched channels, which enlarge surface area to about tenfold if compared with a flat surface (fig. 14A) (Hockley 1973). Complexity of tegumental surface increases the area which is in direct contact with the host tissue or paired counterparts. This allows a highly effective exchange of various substances between host-parasite or male-female. Heptalaminate surface membrane formed previously by schistosomulum persists in the adult worm. Small pieces of surface with only trilaminate membranes were described, as well as small areas covered by additional membranes, forming eleven or fifteen layers (Hockley and McLaren 1973, Sobhon and Upatham 1990). These areas with unusual number of membranes could be the place of release of the content of numerous tegumental bodies, the membrane of which merge with that on the worm surface (see below).

Inner tegumental membrane is also apparently corrugated and usually follows contours of subjacent fibers of circular musculature (Hockley 1973, Bulantová *et al.* 2011). In human schistosomes, basal membrane additionally invaginates to the tegumental syncytium in the form of numerous long folds. They shorten the relative thickness of tegumental mass and allow a more easy passage of substances from the host to the parasite and *vice versa*. Invaginations of basal membrane may have a role also in the attachment of the tegument to

the subjacent lamina basalis, and flexibility of the tegument (Hockley 1973, Sobhon and Upatham 1990).

Tegumental cytoplasm of schistosomes is commonly filled with sporadic small mitochondria and numerous discoid bodies provided with a simple membrane. Presence of other structures may differ in various schistosomes. Usually, there are numerous membraneous bodies with heptalaminate membranes, serving mainly for repairing and turnover of surface tegumental membrane (Hockley 1973, Hockley and Mclaren 1973). Two types of membraneous bodies (loosely and tightly packed) are documented for *S. mansoni*. In *S. japonicum*, membraneous bodies and ring-like bodies were described by Sobhon and Upatham (1990), but only large bodies without membraneous content were observed by Gobert *et al.* (2003). Tegument of *S. haematobium* contains round membraneous bodies and additional dense bodies (Leitch *et al.* 1984), and for *S. mekongi* membraneous bodies and electron-lucent vesicles with small fragments of membranes were documented (Sobhon *et al.* 1984). All the mentioned types of tegumental bodies originate in subtegumental cells and their distribution varies within different areas of the worm tegument (Gobert *et al.* 2003).

Presence of heptalaminate membrane in schistosomes is believed to be associated with their intravascular localization (McLaren and Hockley 1977). Schistosomula of *T. regenti* migrate through the nervous tissue and usually mature extravascularly in the nasal tissue of ducks (Chanová and Horák 2007). Nevertheless, heptalaminate tegumental membrane was documented for this species as for previously studied blood-dwelling schistosomes (Chanová *et al.* 2009). Also content of the tegumental cytoplasm corresponds with that in other schistosomes. Cytoplasm of tegument contains mitochondria positioned near the basal membrane, numerous discoid bodies regularly distributed throughout the cytoplasm, and at least two types of round dark bodies. Despite of bad membrane preservation in samples of *T. regenti* for TEM, some of dark bodies are apparently bounded with a heptalaminate membrane, but the others seem to have only basic trilaminate membrane and homogenous dense content as described for homogenous bodies in the tegument of e.g. 7 days old schistosomula of *S. mansoni* (Hockley and McLaren 1973). Both types of bodies (membraneous and homogenous) are apparently stored in subtegumental cells and transported to the surface tegumental layer through cytoplasmic bridges. Surprisingly, homogenous bodies, function of which is discussed in schistosomulum (see chapter 1.2.6.), disappear from the tegument before adulthood of *S. mansoni*, but stay apparent in *T. regenti*.

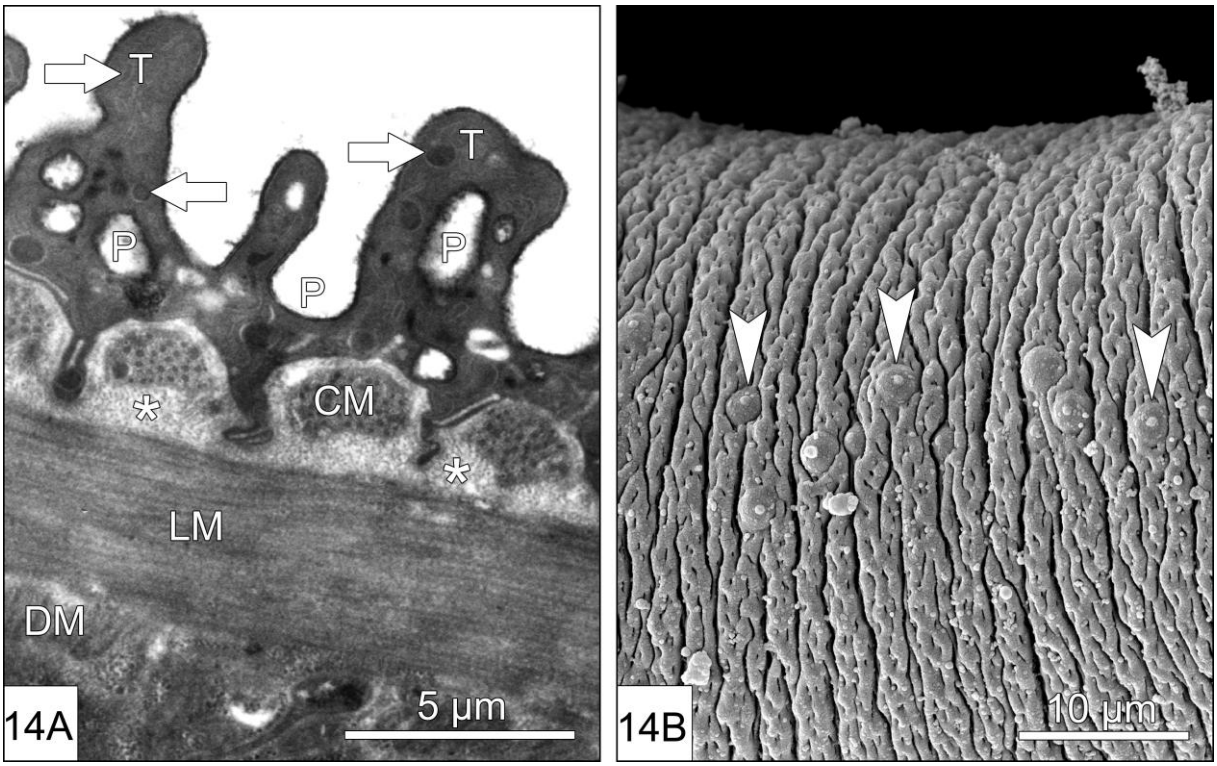
High interspecific variability in the appearance and distribution of tegumental membraneous bodies was observed among members of the genus *Schistosoma*. Apparent

differences were also evident between sexes of one species, or between different regions of individual worms (Gobert *et al.* 2003). Moreover, characteristics of tegumental bodies were dependent on sample processing and possible presence of artifacts. Identification of membranes which bound various tegumental bodies seems to be the most problematic issue. It could be resolved by use of e.g. appropriate contrasting reagents during primary fixation and/or by use of freezing methods for TEM.

Massiveness of body musculature reflects life strategy of various schistosomes, but also different tasks of males and females in species where sexual dimorphism is highly developed. Despite of variability in body shape of schistosomes, body wall musculature is organized very similarly in both sexes of all species. It comprises groups of muscles described already in the stage of schistosomulum. The outermost layer of musculature is formed by thin and densely arranged circular muscle fibers. Contrary to the situation in cercariae and early schistosomula, circular muscles of adult worms are fully sunken into lamina basalis, and are pushed up immediately under the highly furrowed basal membrane of the tegument (Hockley 1973, Sobhon and Upatham 1990, Bulantová *et al.* 2011). Therefore, the main layer of lamina basalis is situated between circular and longitudinal muscle fibers (fig. 14A).

Subjacent wide and spindle shaped myofibers of longitudinal musculature enlarge during development, and remain arranged in the shape of long ligaments, forming second layer of nearly compact muscular envelope (Mair *et al.* 2000, Bulantová *et al.* 2011). Diagonal musculature still preserves its appearance of a sparse net, created from doublets of regularly crossed muscle fibers. These fibers did not change their appearance and density from the stage of schistosomulum, but their number depends on the final length of the worm. Body shape and stability of internal organs is provided by radial muscle fibers. Number and density of these muscles gradually increase in schistosomula and reach maximum in adult worms (Bulantová *et al.* 2011).

Musculature of oral sucker and acetabulum does not differ from that of late schistosomulum. Walls of cup-like suckers are connected with numerous radial muscle fibers (Mair *et al.* 2000, Bulantová *et al.* 2011) which are important for maintenance of cup-like shape of suckers. Acetabulum is an important organ of attachment in schistosomes from stage of cercaria to adult worm. In cercariae, ventral sucker is used for locomotion and attachment, in schistosomula for migration and in adult worms it usually serves for anchoring of worms in the bloodstream (Cousin *et al.* 1995). However, the structure of acetabulum differs significantly in various groups of schistosomes according to their way of life and its future fate is apparent already during the development of schistosomula (see chapter 1.2.6.).




**<u>Fig. 14A</u> – Longitudinal section of *T. regenti* adult worm from duck 20 days p.i., TEM.**

Corrugated tegument **(T)** with deep tegumental pits **(P)** is filled with numerous tegumental bodies **(white arrows)**. Basal membrane copies subjacent circular muscle fibers **(CM)** which are sunken into the lamina basalis **(white asterisk)**. Labels: **LM** = longitudinal muscles, **DM** = diagonal muscles.

**<u>Fig. 14B</u> – Tegumental surface of *T. regenti* adult female from duck 20 days p.i., SEM.**

Surface papillae **(white arrowheads)** are apparently concentrated in narrow lateral zone along worm body.

## 2. AIMS OF THE THESIS

*Trichobilharzia regenti* represents extraordinary schistosome species which was studied mainly in relation to cercarial dermatitis of humans and neuropathological signs observed during migration of schistosomula through the nervous tissue of vertebrate hosts.

Bodies of cercariae, schistosomula and adult worms are known to contain various highly immunogenic molecules which are responsible for host immune reactions against parasitic worms. Nevertheless, till now, details about origin, localization and abundance of these immunogenic molecules on ultrastructural level were missing for *T. regenti*.

While cercariae of *T. regenti* can be obtained repeatedly in big amounts from infected snails, number of accessible schistosomula is limited by use of experimental vertebrate hosts. This limitation could be eliminated by introduction of confirmed cultivation methods which should allow mass production of early *T. regenti* schistosomula *in vitro* without necessity of vertebrate hosts infections.

Surface ultrastructure and arrangement of body musculature importantly differ in particular stages of parasitic worms according to external environment and role of appropriate stage in the life cycle. Mentioned characteristics were newly described and/or summarized for all developmental stages of *T. regenti* and other (especially human) schistosomes. These observations also represent the main body of introductory part of the thesis.

**Particular aims of the thesis**

- Localization of dominant antigenic structures on the surface and in the body of cercariae, schistosomula and adult worms of *T. regenti* using iTEM

- Evaluation of *in vitro* methods of *T. regenti* schistosomula cultivation, including ultrastructural characterization of surface changes during *in vitro* transformation of cercariae to schistosomula

- Characterization of surface ultrastructure and topography of *T. regenti* developmental stages by SEM and TEM

- Description of musculature of body wall and organs of attachment in particular stages of *T. regenti* by TEM and CLSM

- Comparison between *T. regenti* and other (especially human) schistosomes in terms of developmental changes of surface ultrastructure and arrangement of body musculature

## 3. LIST OF ORIGINAL PAPERS

As the original papers listed below are protected by copyright, they are presented in full version in the printed thesis only. The electronic (publicly/freely available) version of the thesis contains abstracts of the papers.

**3.1. Chanová M., Bulantová J., Máslo P., Horák P.** (2009): *In vitro* cultivation of early schistosomula of nasal and visceral bird schistosomes (*Trichobilharzia* spp., Schistosomatidae). *Parasitology Research* **104**: 1445–1452.

**3.2. Bulantová J., Chanová M., Houžvičková L., Horák P.** (2011): *Trichobilharzia regenti* (Digenea: Schistosomatidae): Changes of body wall musculature during the development from miracidium to adult worm. *Micron* **42**: 47–54.

**3.3. Chanová M., Lichtenbergová L., Bulantová J., Mikeš L., Horák P.** (2012): *Trichobilharzia regenti*: Antigenic structures of intravertebrate stages. *Central European Journal of Biology* **7**: 83–90.

## 3.1. *IN VITRO* CULTIVATION OF EARLY SCHISTOSOMULA OF NASAL AND VISCERAL BIRD SCHISTOSOMES (*TRICHOBILHARZIA* SPP., SCHISTOSOMATIDAE).

**Chanová M., Bulantová J., Máslo P., Horák P.**

*Parasitology Research* 104: 1445–1452.

2009

**Abstract**

Cercariae of bird schistosomes (*Trichobilharzia szidati* and *Trichobilharzia regenti*) were mechanically stimulated to transform to schistosomula and kept in different cultivation media supplemented with duck red blood cells and/or homogenized nervous tissue. The development under *in vitro* conditions was compared with that *in vivo*, using the following characters: emptying of penetration glands, surface changes, food uptake, and growth of early schistosomula. The results show that the cultivation medium routinely used for human schistosomes is also suitable for mass production of early schistosomula of bird schistosomes, including the unique nasal species – *T. regenti*. The changes observed resemble those present in worms developing *in vivo*; therefore, the *in vitro* produced early schistosomula might be used for further studies of host-parasite interactions.

## 3.2. *TRICHOBILHARZIA REGENTI* (DIGENEA: SCHISTOSOMATIDAE): CHANGES OF BODY WALL MUSCULATURE DURING THE DEVELOPMENT FROM MIRACIDIUM TO ADULT WORM.

**Bulantová J., Chanová M., Houžvičková L., Horák P.**

*Micron* 42: 47–54

2011

## Abstract

*Trichobilharzia regenti* (Schistosomatidae, Digenea), a parasite of birds, exhibits a unique strategy among schistosomes, having affinity to the nervous system of vertebrate hosts. Migration of parasitic stages within hosts and/or swimming of non-parasitic larvae in water environment depend on the action of body wall muscles which were studied with confocal and electron microscopy. In all stages, body wall musculature is comprised of differently organized circular and longitudinal muscles. During the development, an extensive change of musculature characteristics and/or formation of new muscle structures were recorded; cercariae, schistosomula and adult worms produce additional underlying diagonal muscle fibers and inner plexus of radial musculature. Substantial changes of the outer environment during penetration of a host (osmotic values of water vs. host tissues) are accompanied by surface transformation of miracidia/mother sporocysts and cercariae/schistosomula. Contrary to that, changes of body musculature in these stages are characterized only by growth and re-organization of existing structures, and never by formation of new components of body musculature. Future studies in this field may contribute to a better knowledge of morphology and function of trematode muscles, including those of schistosomes that are important pathogens of humans and animals.

## 3.3. *TRICHOBILHARZIA REGENTI*: ANTIGENIC STRUCTURES OF INTRAVERTEBRATE STAGES.

**Chanová M., Lichtenbergová L., Bulantová J., Mikeš L., Horák P.**

*Central European Journal of Biology* 7: 83–90

2012

**Abstract**

Like several other bird schistosomes, neurotropic schistosome of *Trichobilharzia regenti* can invade also mammals, including humans. Repeated infections cause cercarial dermatitis, a skin inflammatory reaction leading to parasite elimination in non-specific mammalian hosts. However, in experimentally primo-infected mice, the worms escape from the skin and migrate to the central nervous system. In order to evade host immune reactions, schistosomes undergo cercaria/schistosomulum transformation accompanied with changes of surface antigens. The present study is focused on localization of the main antigens of *T. regenti*; cercariae, schistosomula developed under different conditions and adults were compared. Antigens were localized by imunofluorescence and ultrastructural immunocytochemistry using sera of mice repeatedly infected with *T. regenti*. Detected antibody targets were located in glycocalyx and penetration glands of cercariae and in tegument of cercariae, schistosomula and adults. Shedding of cercarial glycocalyx significantly reduced surface reactivity; further decrease was reported during ongoing development of schistosomula. Spherical bodies, probably transported from subtegumental cell bodies to worm surface, were identified as the most reactive tegumental structures. Based on similar results for schistosomula developed in specific, non-specific hosts and *in vitro*, it seems that the ability of *T. regenti* to decrease the surface immunoreactivity during ontogenesis is independent on the host type.

# 4. CONCLUSIONS

Surface and body musculature of *T. regenti* were characterized in terms of their arrangement, ultrastructure, immunoreactivity and changes accompanying development of *T. regenti* during life cycle. Observed characteristics were then compared to that of other schistosomes.

Immunogenic molecules were ultrastructurally confirmed i.a. in various tegumental bodies which originate in subtegumental cells of *T. regenti* cercariae, schistosomula and adult worms. These informations could be used during characterization of content of mentioned bodies or subsequent elucidation of purpose of these bodies for host/parasite interactions. Successful cultivation of early schistosomula of bird schistosomes including *T. regenti* allowed production of these intravertebrate stages in a large amount, without use of experimental animals. Our research on *T. regenti* body musculature showed some new aspects of the development, organization and function of schistosome muscles.

Comparison of observed characteristics between *T. regenti* and other schistosomes revealed peculiarities of *T. regenti*, and also some neglected features which are probably typical for the family Schistosomatidae as a whole.

**The most important findings of the thesis can be sorted into three categories:**

**Localization of immunogenic structures**

- Sera of animals infected with *T. regenti* strongly reacted with glycocalyx of cercariae. The other structures with a lower immunoreactivity were penetration glands of cercariae and tegumental bodies inside tegument and subtegumental cells of cercariae, schistosomula and adult worms.

- Comparison of immunoreactivity of *T. regenti* cercariae, schistosomula and adult worms with host sera showed a gradual decrease in antigen abundance during intravertebrate development.

**Transformation and cultivation *in vitro***

- Cultivation media commonly used for human schistosomes were also suitable for bird schistosomes with migration through the vascular (*T. szidati*) or the nervous (*T. regenti*) systems of vertebrate hosts.

- Contrary to *T. regenti*, surface changes during the *in vitro* transformation of *T. szidati* cercariae to schistosomula were accompanied by formation of surface "bubbles" of shed cercarial membranes.

**Observation of surface and body musculature**

- Egg shell pores known for human schistosomes were not detected in *T. regenti* eggs by conventional TEM techniques, although their presence is probable due to a strong immune reaction around mature eggs, and growth of the ageing eggs.

- Number and arrangement of surface receptors differed between *T. regenti* and *T. szidati* miracidia and represented, therefore, valuable criteria for species determination.

- Head organ of *T. regenti* cercariae with musculature separated from the body by lamina basalis transformed to a cup-shaped oral sucker of schistosomula.

- Sparse fibers of radial musculature were observed for the first time in the stage of cercaria. The number of radial muscle fibers increased with development of intestine and reproductive organs of schistosomula and adult worms. That corresponds with expected function of radial musculature which probably serves for maintenance of a stable position of internal organs of the worms.

- In relation to the body mass of intravertebrate stages, acetabulum of *T. regenti* schistosomula and adult worms were smaller than that of human schistosomes. This could be related to threadlike body shape and different migratory route of *T. regenti* which probably do not use ventral sucker for anchoring the worms in the bloodstream as human blood-dwelling schistosomes.

- Contrary to human schistosomes, tegumental ultrastructure of *T. regenti* adult worms was similar in both genders. It confirmed a low degree of sexual dimorphism.

## 5. ABBREVIATIONS

**DAPI –** 4',6-diamidino-2-phenylindole

**FS –** freeze substitution

**FITC –** fluorescein isothiocyanate

**HPF –** high pressure freezing

**iTEM –** immuno transmission electron microscopy

**p.i. –** post infection

**SEM –** scanning electron microscopy

**TEM –** transmission electron microscopy

## 6. REFERENCES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