Department of Parasitology, Faculty of Science, Charles University in Prague

BIRD SCHISTOSOMES:

DEVELOPEMENT OF SCHISTOSOMULA WITH FOCUS ON *TRICHOBILHARZIA* SPP.

Marta Chanová

PhD. thesis

Prague 2009

Supervisor: Prof. Petr Horák, PhD.

The present thesis consists of two parts: a review of literature data and original papers published in peer-reviewed journals.

The research work presented here was performed at the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic.

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 used in the review cited from literature are referred to in the list of references.

Prague, 2009 Marta Chanová

Ďakujem Petrovi Horákovi za cenné rady a pripomienky k mojej práci počas celej jej tvorby, počnúc uvedením do problematiky vtáčích schistozóm, prezentáciami prvých výsledkov, cez rukopisy článkov až po konečnú verziu dizertačnej práce. Ďakujem mu i za nekonečnú trpezlivosť a za vytvorenie ideálnych pracovných podmienok.

Spolu s ním ďakujem Liborovi Mikešovi, Honzovi Dvořákovi, Katke Dolečkovej, Martinovi Kašnému a Lucke Lichtenbergovej za skvelú pracovnú i mimopracovnú spoločnosť, a za mnohé rady do práce i do života. Ďakujem všetkýchm ostatním členom helmintologického tímu za vytváranie príjemnej pracovnej atmosféry.

Ďakujem celej svojej rodine, ktorá neprestala veriť, že raz naozaj doštudujem.☺

TABLE OF CONTENTS

ABSTRACT

Schistosomulum is the first stage developing in definitive host body, affecting various body parts and in the case of bird schistosomes present in host tissues for longest period. The aims of the present thesis are to summarize recent knowledge of bird schistosomula migration, development and pathogenic impact on host tissues and complete the details for two model species (*Trichobilharzia szidati* and *T. regenti*) with different life strategy. The other aim was to introduce and test the method for *in vitro* cultivation of schistosomula.

Schistosomulum is formed by transformation of cercaria in the host skin at the time of penetration. The process is preceded by cercarial tail detachment and includes emptying of penetration glands and extensive surface changes. All this take place also under defined *in vitro* conditions.

Transformed schistosomula migrate towards the target organ in host body. Depending on the species schistosomula migrate via the circulatory system or nervous tissues and the migration is directed either to intestinal or nasal area (visceral or nasal species, respectively). Specific migratory pattern for lung passage of *T. szidati* and migratory route of *T. regenti* through the nervous system, unique among schistosomes and including intra- and extra vascular location, are obligatory for bird schistosomula.

Schistosomula feed on host red blood cells, except for *T. regenti* ingesting either red blood cells or particles of nervous tissue in certain phases of migration.

Pathological consequences of bird schistosome infections caused by schistosomula are of high significance (contrary to mammalian schistosomes with low pathogenic impact of migrating larvae). Schistosomula of the neurotropic *T. regenti* cause degenerative changes of the nervous tissues, and a leg paralysis and balance disorders develop. Lung stage schistosomula of visceral species cause an inflammatory reaction with infiltrates formed around the blood vessels and in the gas-exchange tissues, followed by pneumonia, oedema and hemorrhages development.

Bird schistosomula are also able to penetrate abnormal (mammalian) host. Depending on the host immune status (previously unexposed or sensitized host) penetrating cercariae either transform to schistosomula and migrate and develop for certain period, or cause specific cutaneous immune response (cercarial dermatitis) and become trapped in the skin.

INTRODUCTION

Schistosomes (Schistosomatidae) cover 13 genera, from which 8 (of approximately 58 species totally) use birds as definitive hosts. The bird schistosome genus *Trichobilharzia*, including more than 40 species, is the most abundant among schistosomes (Blair and Islam 1983).

The problem of human schistosomiasis caused by few particular species of the genus *Schistosoma* and affecting hundreds of milions people puts the bird schistosomes into the shade. Veterinary impact of bird schistosomiasis did not attract the scientists' attention in the past. However, bird schistosomes are known to cause human cercarial dermatitis, recently a re-emerging disease of worldwide occurrence. Local outbreaks of this disease, called also schistosomal dermatitis or swimmer's itch, led several authors to focus on the causative agent – the cercariae. Their description and determination, epidemiology of cercarial dermatitis, and presence, distribution and spreading of infected intermediate and definitive hosts became the most studied areas within bird schistosome research. Later also biochemical and immunological studies related to human cercarial dermatitis progressed.

The ontogeny of parasite and influence on the bird host were of minor interest. This started to change when the experimental studies revealed that bird schistosomes invading previously unexposed mammals are able to migrate and develop for certain periods (e.g. Olivier 1953; Bacha et al. 1982; Haas and Pietsch 1991; Horák and Kolářová 2000). Cumulation of migrating bird schistosomula in the lungs of mammals led to the hypothesis that some of unexplained pulmonary disorders in humans may be caused by bird schistosomes (Bayssade-Dufour et al. 2001).

The description of a new species, *Trichobilharzia regenti* (Horák et al. 1998a), a nasal schistosome unique among schistosomes in its neurotropism, migration via the nervous tissue and ingestion of nervous tissue components confirmed also in abnormal murine host enhanced the interest in host-parasite interactions in bird schistosomiasis lately. In context of these findings, the study of schistosomula migration, development and pathogenic impact on the host may be of high importance.

The objectives of the present thesis are to summarize recent knowledge about bird schistosome schistosomula biology and complete the data for unknown phases of their development. To describe the details on migratory routes of schistosomula through the host

body two *Trichobilharzia* species (*T. szidati*, a visceral species migrating via blood vessels, and *T. regenti*, a nasal species migrating via nervous tissue) were studied.

As the number of experiments focused particularly on schistosomula stage of bird schistosomes increases, a high quantity of various developmental stages is required. Unfortunately, those stages developing inside a host are not available in sufficient amounts and/or quality. For this purpose, the artificially transformed and *in vitro* grown schistosomes are necessary.

Therefore, the other aim was the implementation of appropriate *in vitro* cultivation techniques based on methods used for human schistosomes and, subsequently, the mass *in vitro* production of schistosomula for further developmental, immunological and biochemical studies.

I. LIFE CYCLE OF BIRD SCHISTOSOMES

Bird schistosomes (as well as mammalian ones) have a two-host life cycle. Most of them, including the genus *Trichobilharzia*, use freshwater pulmonate snails as intermediate and birds (waterfowl) as definitive hosts. Some species use marine or freshwater Opisthobranchia or Prosobranchia. Those of *Austrobilharzia* and *Ornithobilharzia* develop in marine caenogastropod snails. The specificity for intermediate host is questionable. Formerly, bird schistosomes were considered to use a narrow spectrum of molluscan species - one species should usually parasitize one or several closely related snail species (e.g. Farley 1971; Blair and Islam 1983). In spite of this conclusion recent observations revealed, that the bird schistosomes may adapt to different snail host under certain circumstances (Aldhoun et al. 2009).

Specificity for definitive host differs among genera. Birds of several unrelated orders may serve as definitive hosts for bird schistosomes of *Trichobilharzia*, *Gigantobilharzia* and *Bilharziella* (e.g. Horák et al. 2002; Jouet et al. 2009, Kolářová et al. 1997). On the other hand, bird schistosomes of *Dendritobilharzia* sp. are usually found in birds of only one order – Anseriformes (Vande Vusse 1980).

Free swimming ciliated larva, **miracidium**, hatches from the egg in the water, or in the definitive host tissues exposed to the water environment (nasal mucosa in the case of *T. regenti*; Horák et al. 1998a).

Miracidium actively penetrates the snail in the area of head-foot region, looses surface ciliated plates and forms **mother sporocyst**. After asexual multiplication, **daughter sporocysts** (moving to the hepatopancreas) and, subsequently, motile bifurcated cercariae are produced (Horák et al. 2002). Cercariae leave the host by penetration of its body wall. The multiplication is repeated and continuous, the snail becomes a source of infection for the rest of its life. Therefore, a single miracidium that successfully penetrate a snail host may become a source of thousands of cercariae (Sluiters et al. 1980).

Free swimming **cercariae** express host-finding and host-recognition behavior (with strategies different from those of human schistosomes). Specific stimuli are responsible for different activities (e.g. shadow for changes in swimming movements from surface towards deeper water layers, warmth for host identification, ceramides and cholesterol for atachment to host skin, free fatty acids for skin penetration; for review see Haas 1994).

Cercaria infects the definitive host by penetration of the skin. Once cercariae attach, they search for suitable penetration site. Then, the larva attaches oral sucker to the skin and disrupts the penetration site by spiny oral sucker movements and action of penetration gland content (Haas and Van de Roemer 1998). Complete penetration occurs within several minutes; cystein peptidases (especially cathepsin B-type) from penetration glands probably play significant role in host skin protein hydrolysis (Kašný et al. 2007).

Experimentally, drinking of water containing cercariae or ingestion of infected snails led also to infection establishment (e.g. Macy et al. 1955), but no exact data are available to support the role of peroral infection in spreading of bird schistosomiasis. The oral administration of cercariae probably leads to worm penetration through the upper part of the digestive tract and the infection by ingested cercariae may be excluded (Blažová, unpublished). Similarly, experimental peroral infections with mammalian schistosomes cause parasite penetration through the mucosa of lips and oral cavity (e.g. Giver et al. 1999) and the swallow of cercariae (excluding the penetration in oral cavity) does not establish schistosomiasis (Oleaga and Ramajo 2004).

In the skin cercariae transform to **schistosomula** and start to migrate to deeper skin layers. The migration through the host body continues via circulatory or nervous systems, involving obligatory phases spent in various host organs/tissues (e.g. Bourns et al. 1973; Hrádková and Horák 2002; Chanová et al. 2007; Chanová and Horák 2007).

Schistosomula develop and mature to **adult male and female worms** which reproduce sexually. According to the definitive location of adult worms and egg-laying place, visceral and nasal species of schistosomes are distinguished. Either **eggs** containing fully developed miracidia in the feces (e.g. *T. szidati*) or free (already hatched) miracidia (*T. regenti*) are released to the water environment.

Bird schistosomes can also attack mammals as accidental (abnormal) hosts. The migration and maturation is incomplete and the infection fails in such cases. However, at least the initial phase of migration through the mammalian body occurs in case of both visceral and nasal species (Horák et al. 2002).

II. OVERVIEW OF THE INTRAVERTEBRATE STAGES

Cercaria-schistosomulum transformation starts in the host skin at the moment of penetration. The process is preceded by cercarial tail detachment and includes emptying of penetration glands, surface rebuilding (loss of glycocalyx, and formation of surface double

membrane), and accompanying physiological and biochemical changes (Horák et al. 1998b).

The tail is released, contrary to observations on *S. mansoni* (Whitfield et al. 2003), prior to full penetration of cercarial body. This seems to employ the muscle sphincter close to the body-tail junction (Haas and Van de Roemer 1998).

Subsequent ultrastructural changes are similar to those described in schistosomula of human schistosomes (e.g. Hockley and McLaren 1973; Stirewalt 1974). Cytoplasmic connections between subtegumental nucleated cells and surface syncytial tegument serve for the transport of electron-dense granula and membraneous bodies into the tegument. The bodies fuse and replace the original simple (trilaminate) tegumental membrane with the newly formed double (heptalaminate) one, which has transiently a pentalaminate appearance (Horák et al. 1998b; Bulantová, unpublished). Simultaneously, surface tegumental structures become rebuilt. The initial surface changes are most apparent in "scar" region from where cercarial tail was detached. Therefore, the "scar" is considered to be the area where the transformation begins (Bulantová, unpublished). In contrast, lectin staining methods applied on transforming *T. szidati* showed the first changes of surface lectin-binding being localized at the head region; this led to a postulate that glycocalyx shedding starts at this site (Horák *et al*. 1998b). The discrepances between ultrastructural and lectin-binding changes are known also for human schistosomes. Ultrastructural changes of surface membrane of transforming *Schistosoma mansoni* were firstly present in the posterior part, but the head region was the first area that became completely free of glycocalyx (Sobhon and Upatham 1990).

Transformed and developing schistosomula migrate either towards the visceral area (visceral species, e.g. *T. szidati*, *A. visceralis*, etc.) or to the nasal cavity (8 nasal species known, e.g. *T. regenti*; Horák et al. 1998a).

The schistosomula of visceral species leave the skin, enter the blood vessels, and start the vascular migration towards the target organs (Bourns et al. 1973). Migrating schistosomula only transiently leave the blood system during the lung passage (for details see chapter III-1.1.).

As the only known exception within the family Schistosomatidae, the nasal schistosomes of *T. regenti* move from the skin to the peripheral nerves and use nervous tissue (including CNS) instead of blood system as migratory route (Horák et al. 1999). Except for this particular species no data are available on migration of other nasal bird schistosomes, neither on mammalian *S. nasale*.

Adult worms of visceral species are localized either intravascularly (e.g. *T. parocellata*, Islam and Copeman 1986) or extravascularly (e.g. *T. szidati*, Neuhaus 1952). Intravascularly localized worms (except for arterial specialists of *Dendritobilharzia* spp.) inhabit venous system (Platt and Brooks 1997). Adults of nasal schistosomes were found in blood vessels (Fain 1955a, 1956a, 1956b; Blair and Islam 1983; Islam 1986), except for *T. regenti*, localized mostly extravascularly in soft nasal tissues (Chanová and Horák 2007).

The worms in the target organ/tissue begin to reproduce sexually. Adults of human schistosomes form pairs with a female kept in ventral gynaecophoric canal of a male. Pairing is essential for the development of female reproductive system and the transcription of female-specific genes (Grevelding et al. 1997); separation from male leads to reversible degeneration of female reproductive tract (Clough 1981).

In spite of this influence on females confirmed for human schistosomes, no worms were seen in copula in case of *T. ocellata* (Bourns et al. 1973) or *T. parocellata* (Islam and Copeman 1986). No exact observation has confirmed pair formation of *T. regenti*. In addition some species of bird schistosomes (e.g. *Dendritobilharzia* sp.) have a reduced or no gynaecophoric canal and pair formation is therefore excluded. Such a difference in female development controllability between human and bird schistosomes remains unexplained. However, findings of Morand and Müller-Graf (2000) show that reduction of male gynaecophoric canal in some schistosome species correlates with increase of number of testis, and that two male adaptations occur (either a well developed musculature of gynaecophoric canal to hold one female, or numerous testis to fertilize a number of females).

Single uterine egg is present in the body of fertilized female of bird schistosomes. As the eggs of visceral species are usually deposited in the intestinal wall tight to the lumen (e.g. *T. ocellata*, Bourns et al. 1973), most of them are able to leave the host body easily. Within the eggs in faeces, mostly fully developed miracidia are present. In the water environment the egg shells break and miracidia escape.

The eggs of *T. regenti* in nasal cavity are localized in the connective tissue close to the cartilage of nasal conches, and subsequently with growing abundance in all over the nasal mucosa. After full development of eggs, miracidia hatch *in situ* (Chanová and Horák 2007). This allows miracidia to become flushed out from the host nasal cavity.

The periods of migration, maturation and survival of bird schistosomes in the definitive host take a shorter time than those of mammalian schistosomes (except for *Schistosomatium douthitti*; for review see Loker 1983). While human schistosomes of *S.*

mansoni reach the target organs and develop to adults within more than 6 weeks and reside in host body for a long period (estimations vary from 5.5 to more than 30 years in humans; Vermund et al. 1983), the maturity of bird schistosomes and egg production occur as soon as 2-3 weeks post infection. Shortly after the egg laying the worms disappear.

According to Bourns et al. (1973) such a rapid maturation is considered to be a result of an adaptation to external ecological factors, rather than a consequence of host-parasite interaction (e.g. reflecting short period of intermediate and definitive hosts persistence at the same place). Among mammalian schistosomes, species parasitizing shortly living hosts (*Schistosomatium douthitti*, *Schistosoma rodhaini*) produce eggs faster than the others (Loker 1983). This can also be the case of bird schistosomes.

III. STAGE OF SCHISTOSOMULA

1. Development *in vivo*

1.1 Migration

Two different strategies of migration of bird schistosomes in host body have been described.

The visceral species (at least those for which migration was studied until now) migrate via the blood system, like human schistosomes of the genus *Schistosoma*. The lungs are the first place of accumulation of these larvae in the host body (Haas and Pietsch 1991). After several days, the parasites continue systemic migration via blood vessels to the liver and subsequenly to the intestinal area. In the case of *T. ocellata* schistosomula, the same direction as that of human schistosomes (against the blood flow via hepatic portal system) was confirmed (Bourns et al. 1973) at this phase.

On the other hand, *T. regenti* migrates through the peripheral and central nervous tissues in certain migration phases. The affinity of *T. regenti* schistosomula to the nervous tissue was also confirmed *in vitro* (Hrádková and Horák 2002).

For visceral bird schistosomes (as well as for mammalian schistosomes) different migratory patterns were described. They vary mostly in time spent at certain localizations and/or in the exact target location (for reviews see Blair and Islam 1983; Loker 1983).

Details of the migration of model organisms used in our experiments are described below.

T. szidati migration

Schistosomula pass the skin layers, reach the blood vessels and become established in the lungs within 2 days p.i.

According to our findings, the schistosomula of *T. szidati* use a specific migratory route for the lung passage within their natural hosts; this route includes a period spent extravascularly in the free air space of the lungs (Chanová et al. 2007). The direction from pulmonary blood vessels to the lung tissue of bronchial walls, than to airspace of 2°bronchi and subsequently of parabronchi seems to be obligatory for migrating schistosomula.

Maximum number of schistosomula found in the lungs of ducks was under 15 % of the total number of cercariae used for infection. Corresponding ratio varies between 10% and 30% in the case of 5 species of mammalian schistosomes parasitizing experimentally infected mice (Rheinberg et al. 1998). The pattern of lung passage described for *T. szidati* (peak abundance 2 days p.i.; Chanová et al. 2007) resembles the passage of *S. japonicum* in the mice host (Rheinberg et al. 1998). There are no exact data available for adults' number relation to infection dose or to the number of lung schistosomula of bird schistosomes. However, the rate of cercariae able to develop to adults varies among different species and reflects the lung passage pattern in the case of mammalian schistosomes (Rheinberg et al. 1998). Based on the authors' hypothesis, in the case of *S. japonicum* (and potentially also of *T. szidati*) a short stay in the lungs may reduce the risk of parasite elimination and enable higher number of worms developing to adults than that of other mammalian schistosomes.

T. regenti migration

Early schistosomula leave the skin and as soon as 1.5 day p.i. they enter the peripheral nerves of legs. The nerves *nervus ischiathicus* and *n. femoralis* represent the main route to the spinal cord (Hrádková and Horák 2002).

The worms appear first in synsacral and thoracic, and later also in cervical part of the spinal cord (Hrádková and Horák 2002). They are located in meninges, grey and white matter of synsacral part, and mainly in meninges of thoracic part (Kolářová et al. 2001). From the spinal cord schistosomula migrate to *medulla oblongata* and continue to the brain (Horák et al. 1999; Hrádková and Horák 2002). In the brain, schistosomula are present mostly in meninges (Kolářová et al. 2001, Chanová and Horák 2007). Finally, the pre-adult

schistosomula move to the nasal cavity. About 50% of schistosomula, however, remain in the spinal cord in the later phases of infection (Hrádková and Horák 2002).

The route between CNS and nasal cavity was not clearly described. The low number of worms being localized in hemispheres, *bulbus olfactorius*, ocular lobes and nerves found in previous studies (Horák et al. 1998a, Hrádková and Horák 2002) led the authors to suppose, that the route between CNS (brain) and nasal cavity could be realized via nervous tissue (e.g. cranial nerves). However, parasites or lesions caused by parasites were neither recorded in *bulbus olfactorius*, nor in the nerves connecting brain and nasal cavity (*n. olfactorius* and *n. opticus*) in the period prior to worm presence in nasal cavity (Chanová and Horák 2007). Meninges represent the location in the brain where the worms were regularly detected during this phase of migration. Most of the worms are localized extravascularly, but several worms are also seen in blood vessels. Based on these findings, the hypothesis on migratory route through the cranial nerves was rejected, and a different route (probably via blood stream) connecting brain meninges and nasal cavity is preferred in hypotheses on this phase of migration.

1.2 Growth and ontogenesis

The growth of schistosomula is usually only longitudinal and the width is reduced under the width of cercariae. Comparing the two schistosome species with known data on the development, *T. ocellata* (Bourns et al. 1973) and *T. regenti* (Blažová and Horák 2005), the growth of the latter is slower. The length of early schistosomula (19 hours p.i.) of *T. ocellata* is smaller than the length of cercarial body; the length of cercarial body is exceeded on day 2 p.i., more than quadrupled on day 4 p.i. and reaches the definitive length 7 days p.i. The length of cercarial body of *T. regenti* is quadrupled between 6 and 9 days p.i. and schistosomula reach only half of the length of adults on day 12 p.i. Then a rapid growth occurs at the time schistosomula reach the nasal cavity. The length of *T. regenti* late schistosomula being still present in the spinal cord is smaller than that of worms continually migrating, and differs among the worms from various parts of the spinal cord.

There is a similarity in the transformation process of the two model *Trichobilharzia* species used in the present study (described in Chapter II). Later development of the tegument includes lamina basalis corrugation and change of spiniferous surface to surface

with pits, holes and canals (Bulantová, unpublished), similarly to the surface changes of *S. mansoni* (Hockley and McLaren 1973).

The tegumental development of *T. szidati* schistosomula is faster than that of *T. regenti*. The surface of *T. regenti* reaches the appearance of 5 days old *T. szidati* as late as 10 days p.i. (Bulantová, unpublished).

The changes of body wall musculature in developing schistosomula of *T. regenti* were observed by Bulantová (unpublished). Muscle fibers of all types (circular, longitudinal, radial and diagonal) develop with the growth of schistosomula. Diagonal muscle fibers expand from the anterior body part to the hindbody, their number grows and their distribution changes. Circular muscle fibers ascend to the outer layers of corrugated lamina basalis. Longitudinal fibres fuse into ligaments forming a continuous muscle layer.

The development of digestive system includes oesophageal and intestinal wall changes (Bulantová, unpublished), and growth with further morphological changes of intestine (Blažová and Horák 2002).

The development of reproductive system of males starts with testes and seminal vesicle formation, continues with gynaecophoric canal and further testes development, and terminates with sperm production (Bourns et al. 1973, Blažová and Horák 2005). In females, firstly uterus is seen, later other reproductive organs (last of them seminal receptacle) develop in *T. ocellata* (Bourns et al. 1973). Conversely, vitellaria and ovary were developed prior to uterus formation in *T. regenti* (Blažová and Horák 2005)

According to the above mentioned authors, the developmental changes of intestine undergo more rapidly in *T. ocellata* schistosomula; also the initial development of reproductive organs was seen as soon as 4 days p.i. in *T. ocellata* schistosomula and 5 days later in *T. regenti*.

Slower development of *T. regenti* comparing to visceral *Trichobilharzia* species may reflect different migratory routes of these species, requiring different time to reach the final location (the intra-vertebrate development of *T. regenti* is approximately 5 days longer than that of *T. szidati* and *T. ocellata*). Also the different source of food (see below) may play a role. As potential benefit of the delay in *T. regenti* growth and maturation, minor host damage through the CNS migration and increase of host survival rate is supposed (Blažová and Horák 2005).

1.3 Digestion

Schistosomes feed on red blood cells. The only known exception is *T. regenti* ingesting particles of CNS while localized in the spinal cord (Horák et al. 1999).

Schistosomula of *T. ocellata* start to feed 1 day post infection (p.i.; Bourns et al. 1973), those of *T. szidati and T. regenti* at the latest 2 and 3 days p.i., respectively (Chanová et al. 2007, Blažová and Horák 2005) in the definitive host body.

The presence of dark granules in the intestinal content of all worms migrating through the lungs (*T. szidati*; Chanová et al. 2007) and liver (*T. ocellata*; Bourns et al. 1973) of ducks indicates their feeding on red blood cells in all (including extravascular) locations. Once the pre-adult worms of *T. ocellata* reach the host intestine and leave the circulatory system, no blood cells ingested were observed in their gut (Bourns et al. 1973). This is not in agreement with data on *T. szidati* (Horák, pers. communication) and human schistosomes which do not leave the vascular system and remain feeding on blood cells also in adulthood (Bogitsh 1989).

A unique situation among schistosomes exists in *T. regenti*. The first material being apparent in the intestine of migrating schistosomula (extracted from the host spinal cord) is represented by nervous tissue particles; less than 5% of worms seemed to digest blood (pigment present in the intestinal content) while localized in the nervous tissue (Blažová and Horák 2005). As late as *T. regenti* worms reach the meninges, there is often a presence of dark brown granules apparent. These granules are different from the intestinal content of schistosomula migrating through CNS (Horák et al. 1999) and are probably of blood origin. This indicates another food source at the end of prepatent period when the worms localized in meninges probably started to take up and digest blood/red blood cells (Chanová and Horák 2007). Besides these findings, further *in vitro* studies (see Chapter III - 2.2), confirmed blood, but not NS particles, digestion by early schistosomula (Chanová et al. 2009). According to these observations, migrating *T. regenti* might switch at least twice from one to another source of food (with blood as initiate source of food).

Schistosomes digest host blood proteins by employment of gut-associated peptidases, mainly cysteine and aspartic peptidases for hemoglobin breakdown. The predominant digestive peptidase of human schistosomes of *S. mansoni* is cathepsin B1 (SmCB1; for review see Kašný 2009). Orthologues of cathepsins B1 being localized in the gut of *T. regenti* schistosomula (TrCB1 isoforms) possibly fulfill a similar role in host protein hydrolyzation (Dvořák et al. 2005). Digestion of host proteins other than blood by

T. regenti schistosomula is unique among schistosomes. Cathepsin B1 isoforms (TrCB1.1 and TrCB1.4) may represent adaptation to the nervous tissue degradation; they poorly hydrolyze hemoglobin, but they do it efficiently with myelin basic protein. Such a substrate specificity of two cathepsin B1 isoforms may increase the ability to hydrolyze host proteins more specifically and effectively (Dvořák et al. 2005).

1.4 Pathogenic effect on host organism

Significant pathologies of bird schistosome infections are caused by migrating schistosomula, contrary to mammalian schistosomes for which the pathogenic impact of migrating larvae is of low importance.

Migrating larvae of the neurotropic *T. regenti* cause degenerative changes of the nervous tissues and, as a consequence, leg paralysis and balance disorders develop (Horák et al. 1999). Pathology is a result of host immune cell reactions against parasite presence. Cell infiltrates composed mainly of eosinophils and heterophils are formed around the worms. Inflammatory reaction occurred also perivascularly and around the central canal of spinal cord. In addition, schistosomula might release a component acting as a neurotoxin, because occasionally dystrophic and/or necrotic neurons in the spinal cord are found. In common, the spinal cord is more injured than the brain, and tissue damage is stronger at site with numerous parasites (Kolářová et al. 2001).

Neurological symptoms are occasionally described also for infections by human schistosomes. They may develop as a result of neuroschistosomiasis. This is, however, due to the eggs and/or adult worms ectopically disseminated by blood stream to CNS, mostly spinal cord. Moreover, although such egg/adult localization is the second most often reported for *S. mansoni* infection, an asymptomatic form is far more frequent than symptomatic one (for review see Nascimento-Carvalho and Moreno-Carvalho 2005).

Also schistosomula of *T. szidati* migrating through the host lungs in the acute phase of infection cause significant pathological changes of host tissues. The infection results in inflammatory reaction with nodules composed of infiltrated lymphocytes, heterophils, eosinophils and macrophages formed around the blood vessels and in the gas-exchange tissues. Later, an extensive inflammation of secondary bronchi and parabronchi with oedema and haemorrhage in their lumen takes place. Pneumonia, alveolar hyperplasia and haemorrhages in peripheral parabronchi persist for several days (Chanová et al. 2007).

Similar impact of migrating larvae of mammalian schistosomes on the host lungs was never described. The main pathological change of host tissues caused by human schistosomes is formation of a granuloma around accumulated eggs in target or other visceral organs, mediated by CD4+ Th cells specific for egg antigens (e.g. Wynn et al. 2004). Also the reports describing "pulmonary schistosomiasis" caused by mammalian schistosomes in natural hosts are usually focused on the postpatent period and the lesions induced by eggs deposited in the lungs (e.g. Souza Vidal et al. 1993).

1.5 Interaction with non-compatible host (mammal)

Bird schistosomes are adapted to find and invade the leg skin of birds (contrary to non-living objects, cold-blooded organisms, or feathered body parts of birds), but not to avoid invasion of mammals (Haas 1994). The presence of penetration stimuli in mammalian skin leads bird schistosomes to infect also abnormal hosts. Moreover, cercariae of *T. szidati* penetrate the human skin more efficiently than *S. mansoni* does (Haas and Haeberlein 2009). Cercariae penetrating various mammals undergo the transformation, schistosomula start to migrate and feed similarly to those in the bird host. However, the migration is incomplete, the growth and maturation of schistosomula is retarded.

As well as in birds, schistosomula of visceral species migrate from the skin to the lungs in various mammals including primates; however, contrary to bird infections, these are almost exclusively the only sites of parasite presence in mammalian body (e.g. Olivier 1953; Bacha et al. 1982; Bayssade-Dufour et al. 2001). In the lungs of experimentally infected mammals schistosomula survive for several days up to the several months (155 days for *Austrobilharzia variglandis* in the lungs of gerbil; Bacha et al. 1982). After this period the worms disappear (Olivier, 1953, Haas and Pietsch, 1991, Horák and Kolářová, 2000). In our study, the number of schistosomula found in the mice lungs was under 3% of the total number of cercariae used for infection (unpublished). The lung schistosomula of *T. szidati* are localized extravascularly in the alveolar walls of mice (Chanová et al. 2007). The migration similar to that in the lungs of ducks does not occur.

Schistosomula of nasal *T. regenti* exhibit neurotropic behaviour also in murine host. The larvae were detected in peripheral nerves, spinal cord and brain (*medulla oblongata* and *cerebellum*) of BALB/c mice and also in hemispheres in the case of immunosupressed SCID mice (Hrádková and Horák 2002). In comparison with bird host, the migration is

faster and the brain is reached earlier, but this seems to be caused by differences in host body size.

In spite of non-compatibility of bird schistosomes and mammals as the final hosts, the worms are able to feed on mice tissues. In our study, approximately 50% of *T. szidati* worms found in the lungs 2 and 3 d.p.i. did not show any evidence of food ingestion, similarly to results of Olivier (1953). However, the rest of these worms and all the worms found 4 days p.i. had apparently ingested blood, as the typical dark brown granules were present in their intestine. Based on this observation and on the significant decrease in abundance after 3rd day p.i. we suppose that only those schistosomula that have ingested red blood cells survived. Ingestion of mammalian blood cells might support parasite survival. However, some other nutritional or stimulatory host factors may be absent and thus cause the development inhibition, as reported by Hrádková and Horák (2002).

The pathogenic impact on mammalian host was also confirmed for bird schistosomula. The most serious injuries are caused by neurotropic *T. regenti* worms. An inflammatory reaction with edema, vasodilatation and tissue infiltrates occurs in the skin of mice infected with *T. regenti*. Subsequently, migrating schistosomula cause neuronal inflammation in deeper dermis and subcutis (Kouřilová et al. 2004b). Once present in spinal cord, schistosomula initiate an inflammatory cell reaction with a granuloma formation and increased proliferation of astrocytes in grey and white matter; distrophic and necrotic changes of neurons are present around parasites (Kolářová et al. 2001, Lichtenbergová, personal communication). The effect on infected animals range from asymptomatic infection to leg paralysis (Horák et al. 1999) and the intensity of pathogenic changes of nervous tissues is related to the host immune status (Kourilova et al. 2004b).

Contrary to these results, no cellular response develops in the skin of mammals (rabbits) infected by *T. ocellata* and *T. stagnicolae* (Olivier et al. 1953). The skin reaction was not evaluated for *T. szidati* infection; no specific inflammatory cell reaction to this parasite present in the lungs of mice occurred. Anyway, numerous hemorrhages, alveolar wall congestion, edema and lymphocyte infiltrates appeared in the lungs (Chanová et al. 2007). The impact of *T. regenti* schistosomula occasionally found in the lungs of immunosupressed (SCID) mice remains to be evaluated.

The situation is different in the sensitized mammalian hosts. After repeated contact with cercariae, a specific cutaneous immune response against transforming larvae occurs in mammals (including humans). It is called cercarial dermatitis (or swimmer's itch) and manifested by oedematous and erythematous maculopapular skin eruption accompanied by

intense itching (Horák et al. 2002). The lesions were characterized as lymphocytic vasculitis with a predominant infiltration of CD3, CD4 and CD8 T-lymphocytes in the vessel walls (Vuong et al. 2002). The lesions occur only on body parts exposed to water containing cercariae (Appleton 1984) and persist from days to several weeks. The time course and the severity of reaction depend on host immune status and number of exposures (Olivier 1949); massive infection of sensibilized host may initiate also fever, lymphadenopathy, nausea, or diarrhea (e.g. Berg and Reiter 1960, Chamot et al. 1998). Cercarial dermatitis is Th2–associated reaction, initially type I immediate hypersensitivity response, later the late phase inflammatory reaction against the larvae (Kouřilová et al. 2004a, Lichtenbergová et al. 2008). The most antigenic components recognized by antibodies are cercarial glycocalyx shed by transforming larva, and released content of penetration glands (Horák et al. 1998b; Mikeš et al. 2005). A protein of 34kDa in cercarial secretions is considered to be the molecule acting as a major immunogen initiating the Th2-immune response (Lichtenbergová et al. 2008).

The symptoms of cercarial dermatitis caused by various *Trichobilharzia* species are similar, and more severe than those of cercarial dermatitis caused by human schistosomes (Basch 1991).

The diagnosis is based on clinical and epidemiological data. Experimentally, serodiagnostic methods using antigens prepared from *T. szidati* cercariae reveal antibodies in sera of patients as soon as 3 days p.i. (Kolářová et al. 1994). The treatment is symptomatic, using antipruritic preparations and systemic antihistaminics or corticosteroids in case of massive infestation (Chamot et al. 1998).

Human cercarial dermatitis caused by bird schistosomes is reported worldwide (for review see Horák et al. 2002). It becomes a serious health, and also, if it occurs in tourist areas, an economical problem (de Gentile et al. 1996). As a useful protection against bird schistosome penetration into a man, sunbathing cream enriched by a low-dose of niclosamide (Yomesan^{TM}) was defined (Wulff et al. 2007).

2. Development *in vitro*

In the research of human schistosomes, the methods of *in vitro* cultivation are established for considerable part of parasite life cycle. Under defined conditions, schistosomula grow to adults (Clegg 1965; Basch 1981), and miracidia transform to mother sporocysts, giving rise to daughter sporocysts with cercariae (Ivanchenko *et al.* 1999; Coustau and Yoshino 2000). Nowadays, the *in vitro* transformed schistosomula of human schisosomes cultivated for different periods are routinely used for molecular, biochemical, immunological and developmental studies (e.g. Mountford et al. 1995; Dalton et al. 1997; Harrop et al. 1999; Jenkins et al. 2005).

Increasing interest in experiments with bird schistosomes requires in vitro production of schistosomula as well. At least two main objectives of the experiments on *in vitro* cultivation of bird schistosome have been stated in the present study: 1) optimalization of the cultivation for intravertebrate developmental stages, based on the methods used routinely for human schistosomes of *S. mansoni* and 2) comparison of *in vitro* grown schistosomula with those developing *in vivo*.

2.1. Cultivation methods

The methods simulating natural conditions for schistosomula development should match several requirements. Stimuli for the tail detachment, penetration gland emptying and initiation of transformation process of cercariae should be present, and pH, osmotic, temperature and nutritional requirements of developing schistosomula should be covered.

The initial cultivation of human schistosomes used schistosomula transformed *in vivo*. After penetration through the host skin, the worms developing in the host body for different periods were extracted from corresponding tissues (e.g. Clegg 1965). Alternatively, the penetration through the isolated host skin or through artificially prepared membranes was used (e.g. Stirewalt and Uy 1969). Later, the penetration through the membrane was replaced by chemical stimuli derived from the host skin components (Gilber et al. 1972) or by mechanical stimuli (Basch 1981, Basch and O'Toole 1982). The media for schistosomula cultivation ranged from commercially available basal media to complex mixtures, including e.g. sera.

The methods for mass *in vitro* cultivation were first established for human schistosomes of *S. mansoni* by Clegg (1965) and Basch (1981). The cultivation of bird schistosomes of *T. ocellata* (Howell and Bourns 1974) was based on methods for *S.*

mansoni, using *in vivo* transformed schistosomula (Clegg 1965); for transformation of bird schistosomes of *Austrobilharzia terrigalensis* the schistosomula acquired after passage through the hardened gelatin membrane enriched with host skin lipids were used (Clegg 1969).

For our initial experiments (performed mainly as a proof of elementary conditions for survival and maintenance) we have used schistosomula extracted from the host tissues (the lungs for *T. szidati* and the spinal cord for *T. regenti*, both 3 and 6 days post transformation, p.t.). *In vivo* transformed schistosomula were placed in RPMI 1620 cultivation media. They survived, developed, grew and ingested blood, and no obvious difference between experiments performed with 3 and 6 days old schistosomula was recorded (unpublished).

Subsequently, cercariae stimulated to transform by mechanical means (repeated passages through syringe needle instead of penetration of cercariae into the living host) were used. The effect of different cultivation media, temperatures and CO_2 concentrations was evaluated (Chanová et al. 2009).

Contrary to the former results the cultivation of *in vitro* transformed schistosomula failed in RPMI 1620. Schistosome culture medium 169 (SCM 169) defined by Basch (1981), supplemented with duck RBC's and appropriate antimycotics and antibiotics at 37°C and 5% CO_2, is the only medium supporting full *in vitro* transformation and early development of bird schistosomes. The observed developmental changes are reported below. Unfortunately, even this medium and/or conditions used do not support full development and maturation of worms, and their further modification for a long-term cultivation is necessary.

In spite of the failure of later schistosomula development our results show that early schistosomula are comparable with those from living definitive host and may, therefore, be used in further experiments. Recently they are routinely produced and used for immunological and biochemical studies recently.

2.2 Transformation and schistosomula development

The tails are detached by cercariae immediately after mechanical stimulation. The tail detachment does not result in release of the gland content. The penetration glands of worms cultivated in media free of stimuli leading to penetration gland emptying described by Haas et al. (1997) are emptied 1 day p.t. (circumacetabular glands) and 2 days p.t. (postacetabular glands).

The loss of ligands of the lectins defined as cercaria–schistosomulum transformation markers for *T. szidati* and *T. regenti* (Horák et al. 1998, Blažová and Horák 2005) is pronounced also on *in vitro* transformed schistosomula (Chanová et al. 2009). Different binding of these lectins to cercarial and schistosomular surfaces allows estimation of the carbohydrate changes on the surface. Subsequent surface changes were compared for early schistosomula developed *in vivo* and *in vitro* in the present study. Partially formed double surface membranes are present on 3 hours old schistosomula of both species isolated from the duck skin as well as on *in vitro* schistosomula. Complete removal of glycocalyx and formation of double surface membrane of *T. szidati* (either pentalaminated or heptalaminated) on the entire surface appears within the 1st day p.t.; this is in accordance with the findings of Horák et al. (1998b), who characterized *in vivo* developed *T. szidati* schistosomula 12 hours p.i. The surface membrane transformation in our experiments is therefore considered to be completed.

The structures which may precede the extensive changes of tegument (described in chapter II) were reported by Chanová et al. (2009) as top invaginations of the tegumental tubercles. They are similar to those reported on *S. mansoni* surface, and are also identical on *T. regenti* and *T. szidati* schistosomula from both, *in vivo* and *in vitro* conditions. We suppose that also these surface changes of *in vitro* transformed schistosomula fully follow the changes taking place *in vivo* in the period under study.

The surface of oesophagus undergoes similar processes as that of tegument during transformation. Double (heptalaminate) membrane of oesophagus was observed in both *in vivo* and *in vitro* conditions.

Both early and late (3 and 6 days p.t.) schistosomula of bird schistosomes ingest RBC's in the culture after extraction from the host (unpublished). Schistosomula of both species being cultivated without previous transformation *in vivo* (Chanová et al. 2009) started to feed on red blood cells (RBC's) 3 days p.t. Although both RBC's and nervous tissue homogenate were provided (separately or in combination) for the neurotropic schistosomula of *T. regenti*, there is no evidence that the cultured worms do ingest CNS. *In vivo*, hematin is rarely seen in the gut of *T. regenti* (less than 5% of schisosomula migrating in the CNS) and hemoglobin is considered to become necessary nutrition only after maturation of worms (Horák et al. 1999). According to our results, the duck RBC's seem to be the adequate source of food for *T. szidati*; the same stands for *T. regenti* in this period. The RBC's availability is described as an essential factor for stimulation of intestinal and body growth and development of human schistosomes (Bogitsh & Carter

1977; Bogitsh 1993, 1997). Probably, the availability of RBC's in culture medium is also necessary to initiate feeding of *T. regenti*.

The length of early schistosomula of both species kept *in vitro* is shorter than that *in vivo*, and the growth was considered not sufficient (Chanová et al. 2009). These results are similar to those obtained with mammalian schistosomes. The *in vitro* growth and development is slower than that of schistosomula from the host body, even if *in vivo*, or artificially transformed schistosomula are cultivated (Clegg 1965, Basch 1981, Basch and O'Toole 1982).

Bird schistosomes survived for maximum of 11 days p.t. in our study. No sexual development was recorded. The absence of sexual maturation, poor growth and short period of survival *in vitro* might reflect the situation with schistosomula in the non-specific mammalian host. Deficiency in some essential nutritional or stimulatory host factors might be the cause of developmental failure in the non-specific host (Blažová & Horák 2005) and also of cultured worms. Moreover, an inadequate stimulation of transformation might also negatively influence long-term cultures *in vitro*.

THE AIMS OF EXPERIMENAL PART OF THE PRESENT THESIS

The purpose of experimental work was to elucidate the development of schistosomula of two *Trichobilharzia* species with different life strategies.

Three phases of their life cycle were in focus:

- **Early schistosomula phase of *T. regenti* and *T. szidati*** developed *in vitro*
- **Lung phase of *T. szidati* migration** (passage through the lungs from early schistosomula establishment to withdrawal of late schistosomula)
- **Terminal phase of *T. regenti* migration** (passage from CNS to the nasal cavity)

The study of *in vitro* development was preceded by introduction of mass *in vitro* cultivation method.

Particular aims of the experimental work were as follows:

Experiments *in vivo*: schistosomula in the definitive (bird) or abnormal (mice) hosts

- Characterization of the migratory route of ***T. szidati*** schistosomula through the lungs of experimentally infected birds
- Evaluation of the pathogenic effect of migrating ***T. szidati*** schistosomula on the bird host during the lung passage
- Comparison of migratory route and pathogenic effect of ***T. szidati*** lung schistosomula in birds and experimentally infected abnormal mammalian hosts
- Identification of ***T. regenti*** schistosomula location in the brain of experimentally infected birds
- Characterization of migratory route of *T. regenti* from the brain to the nasal area.
- Identification of ***T. regenti*** location in the nasal cavity
- Evaluation of pathogenic effect of ***T. regenti*** worms in the nasal mucosa

Experiments *in vitro*

- Introduction of methods for mass *in vitro* production of early schistosomula of bird schistosomes, with potential use of these worms in further experiments
- Comparison of *in vitro* developing schistosomula with those growing in definitive host body

LIST OF ORIGINAL PUBLICATIONS

1. CHANOVÁ M, VUONG S, HORÁK P. (2007): ***Trichobilharzia szidati*: the lung phase of migration within avian and mammalian hosts.** Parasitol Res. 100(6): 1243 - 1247

2. CHANOVÁ M**.,** HORÁK P. (2007): **Terminal phase of bird schistosomiasis caused by *Trichobilharzia regenti* (Schistosomatidae) in ducks (*Anas platyrhynchos* f. domestica).** Folia Parasitol 54(2): 105 - 107

3. CHANOVÁ M, BULANTOVÁ J, MÁSLO P, HORÁK P (2009): ***In vitro* cultivation of early schistosomula of nasal and visceral bird schistosomes (*Trichobilharzia* spp., Schistosomatidae).** Parasitol Res 104(6): 1445 - 1452

1. *Trichobilharzia szidati*: the lung phase of migration within avian and mammalian hosts

Parasitol Res. (2007) 100: 1243 – 1247

Marta Chanová, Sarra Vuong, Petr Horák

2. Terminal phase of bird schistosomiasis caused by *Trichobilharzia regenti* (Schistosomatidae) in ducks (*Anas platyrhynchos* f. domestica)

Folia Parasitol (2007) 54: 105 - 107

Marta Chanová, Petr Horák

3. In vitro cultivation of early schistosomula of nasal and visceral bird schistosomes (*Trichobilharzia* spp., Schistosomatidae)

Parasitol Res (2009) 104:1445–1452

Marta Chanová, Jana Bulantová, Petr Máslo, Petr Horák

CONCLUSIONS

The results of experimental part of the present thesis extend the knowledge about the biology of bird schistosome schistosomula. The details in development of two model *Trichobilharzia* species (representing visceral and nasal schistosomes) were provided.

Here are the most important results of our experimental work:

***In vitro* cultivation**

- Schistosomula transformed *in vivo* (extracted from the duck tissues) are able to survive and develop under *in vitro* conditions kept in cultivation medium (RPMI 1620) supplemented with red blood cells (37°C, 5% CO_2).
- RPMI 1620 medium is not useful for *in vitro* transformation and subsequent development of bird schistosomes. SCM 169, a medium designed for human schistosome cultivation, supplemented with duck RBC's and appropriate antimycotics and antibiotics is a usable medium for *in vitro* transformation and early development of bird schistosomula.
- The developmental changes observed on early schistosomula transformed and cultured *in vitro* correspond to those of early schistosomula developing in the hos body.

Lung phase of *T. szidati* migration in the host

- The schistosomula of *T. szidati* use a specific migratory route for the lung passage within their natural hosts, including an obligatory period spent extravascularly in the free air space of the lungs.
- This type of migratory route does not occur within the abnormal murine host.

- Lesions of the lung tissue induced by schistosomula are apparent in both bird and murine hosts. Bird schistosomes of *T. szidati* represent therefore a potential risk of lung injury not only for the natural hosts, but also for the accidentally infected mammals.

Terminal phase of *T. regenti* migration in the host

- Schistosomula that have reached the brain after the passage through the spinal cord are mainly localized in the brain meninges. Some of them are intravascularly localized. After reaching this site, schistosomula leave the CNS. Local blood vessels are probably used for transport of worms to the nasal area.
- In the brain meninges migrating parasites start to feed on food source different from nervous tissue (probably on the red blood cells).
- The subadult worms reach the nasal area. They are localized extravascularly, in the connective tissue between cartilage of the nasal conchae and the glandular epithelium of the nasal mucosa, and continue to feed on blood.
- No immune cell reaction occurs around the worms.

REFERENCES

4. ALDHOUN JA, KOLÁŘOVÁ L, HORÁK P, SKÍRNISSON K (2009): Bird schistosome diversity in Iceland: molecular evidence. J Helminthol 83(2):173-180
5. APPLETON CC (1984): Schistosome dermatitis - an unrecognized problem in South Africa? S Afr Med J 65(12): 467-469
6. BACHA WJ, ROUSH R, ICARDI S (1982): Infection of the gerbil by the avian schistosome *Austrobilharzia variglandis* (Miller and Northup; Penner 1953). J Parasitol 68(3): 505-507
7. BASCH PF (1991): Schistosomes. Development. Reproduction and Host Relations. Oxford University Press, Oxford, 248 pp.
8. BASCH PF, O'TOOLE ML (1982): Cultivation *in vitro* of *Schistosomatium douthiti* (Trematoda: Schistosomatidae). Int J Parasitol 12(6): 541-545
9. BASCH PF (1981): Cultivation of *Schistosoma mansoni In vitro*. I. Establishment of cultures from cercariae and development until pairing. J Parasitol 67(2): 179-185
10. BAYSSADE-DUFOUR C, MARTINS C, VUONG PN (2001): Histopathologie pulmonaire d'un modele mammifere et dermatite cercarienne humaine. Méd Mal Infect 31: 713-722
11. BERG K, REITER HFH (1960): Observations on schistosome dermatitis in Denmark. Acta Derm Venerol 5: 369–380
12. BLAIR D, ISLAM KS (1983): The life cycle and morphology of *Trichobilharzia australis* n. sp. (Digenea: Schistosomatidae) from the nasal blood vessels of the black duck (*Anas superciliosa*) in Australia, with a review of the genus *Trichobilharzia*. Syst Parasitol 5: 89–117
13. BLAŽOVÁ K, HORÁK P (2005): *Trichobilharzia regenti*: The developmental differences in natural and abnormal hosts. Parasitol Int 54: 167-172
14. BOGITSH BJ (1989): Observations on digestion in schistosomes or "Blood and Guts". Tran Am Micr Soc 108(1): 1-5
15. BOGITSH BJ (1993): The feeding of A type red blood cells *in vitro* and the ability of *Schistosoma mansoni* schistosomules to acquire A epitopes on their surfaces. J Parasitol 79(6): 946-948

16. BOGITSH BJ, CARTER OS (1977): Developmental Studies on the Digestive Tract of Schistosomules (*Schistosoma mansoni*) grown *in vitro*. I. Ultrastructure. Trans Am Micr Soc 96(2): 219-227
17. BOURNS, TKR, ELLIS JC, RAU ME (1973): Migration and development of *Trichobilharzia ocellata* (Trematoda: Schistosomatidae) in its duck hosts. Can J Zool 51: 1021-1030
18. CLEGG JA (1969): Skin penetration by cercariae of the bird schistosome *Austrobilharzia terrigalensis*: the stimulatory effect of cholesterol. Parasitology 59(4): 973-989
19. CLEGG JA (1965): *In vitro* cultivation of *Schistosoma mansoni*. Exp Parasitol 16: 133-147
20. CLOUGH ER (1981): Morphology and reproductive organs and oogenesis in bisexual and unisexual transplants of mature *Schistosoma mansoni* females. J Parasitol 67(4): 535-539
21. COUSTAU C, YOSHINO TP (2000): Flukes without snails: Advances in the *in vitro* cultivation of intramolluscan stages of trematodes. Exp Parasitol 94: 62-66
22. DALTON JP, CLOUGH KA, JONES MK, BRINDLEY PJ (1997): The cysteine proteinases of *Schistosoma mansoni* cercariae. Parasitology 114: 105-112
23. DE GENTILE L, PICOT H, BOURDEAU P, BARDET R, KERJAN A, PIRIOU M, LE GUENNIC A, BAYSSADE-DUFOUR C, CHABASSE D, MOTT KE (1996): La dermatite cercarienne en Europe: un problème de santé publique nouveau? Bull World Health Organ 74(2): 159-163
24. DVOŘÁK J, DELCROIX M, ROSSI A, VOPÁLENSKÝ V, POSPÍŠEK M, ŠEDINOVÁ M, MIKEŠ L, SAJID M, SALI A, MCKERROW JH, HORÁK P, CAFFREY CR (2005): Multiple cathepsin B isoforms in schistosomula of *Trichobilharzia regenti*: identification, characterisation and putative role in migration and nutrition. Int J Parasitol 35: 895-910
25. FAIN A (1955): Recherches sur les schistosomes d'oiseaux au Ruanda - Urundi. Découverte d'une nouvelle bilharziose aviaire: La richobilharziose nasale, et descrption de Schistosomes noveaux. Rev Zool et Bot Afr 51: 373-387
26. FAIN A (1956a): Les schistosomes d'oiseaux du genre *Trichobilharzia* Skr. et Zak. au Ruanda - Urundi. Rev Zool et Bot Afr 54: 147-178
27. FAIN A (1956b): Nasal trichobilharziasis: a new avian schistosomiasis. Nature 177(4504): 389

28. FARLEY J (1971): A review of the family Schistosomatidae: excluding the genus *Schistosoma* from mammals. J Helminthol 45(4): 289-320
29. GILBERT B, DA ROSA MN, BOROJEVIĆ R, PELLEGRINO J (1972): *Schistosoma mansoni*: *in vitro* transformation of cercariae into schistosomula. Parasitology 64(2): 333-339
30. GIVER H, JOHANSEN MV, CHRISTENSEN NO, BØGH H, NANSEN P (1999): Peroral infection of pigs with *Schistosoma japonicum* cercariae. Vet Parasitol 83(2): 161-165
31. GREVELDING CG, SOMMER G, KUNZ W (1997): Female-specific gene expression in Schistosoma mansoni is regulated by pairing. Parasitology 115 (6): 635-640
32. HAAS W, DIEKHOFF D, KOCH K, SCHMALFUSS G, LOY C (1997): *Schistosoma mansoni* cercariae: stimulation of acetabular gland secretion is adapted to the chemical composition of mammalian skin. J Parasitol 83(6): 1079-1085
33. HAAS W, HAEBERLEIN S (2009): S Penetration of cercariae into the living human skin: *Schistosoma mansoni* vs. *Trichobilharzia szidati*. Parasitol Res 105(4): 1061-1066
34. HAAS W, PIETSCH U (1991): Migration of *Trichobilharzia ocellata* schistosomula in the duck and in the abnormal murine host. Parasitol Res 77: 642-644
35. HAAS W, VAN DE ROEMER A (1998): Invasion of the vertebrate skin by cercariae of *Trichobilharzia ocellata*: penetration processes and stimulating host signals. Parasitol Res 84: 787-795
36. HAAS W (1994): Physiological analyses of host-finding behaviour in trematode cercariae: adaptations for transmission success. Parasitology 109(1): 15-29
37. HARROP R, COULSON PS, WILSON RA (1999): Characterization, cloning and immunogenicity of antigens released by lung-stage larvae of *Schistosoma mansoni*. Parasitology 118: 583-594
38. HOCKLEY DJ, MCLAREN DJ (1973): *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm. Int J Parasitol 3(1): 13-25
39. HORÁK P, KOLÁŘOVÁ L (2000): Survival of bird schistosomes in mammalian lungs. Int. J Parasitol 30: 65-68
40. HORÁK P, KOLÁŘOVÁ L, ADEMA CM (2002): Biology of the schistosome genus *Trichobilharzia*. Adv Parasitol 52: 155-233
41. HORÁK P, KOLÁŘOVÁ L, DVOŘÁK J (1998): *Trichobilharzia regenti* n. sp. (Schistosomatidae, Bilharzielinae), a new nasal schistosome from Europe. Parasite 5: 349-357

42. HORÁK P, KOVÁŘ L, KOLÁŘOVÁ L, NEBESÁŘOVÁ J (1998): Cercaria-schistosomulum surface transformation of *Trichobilharzia szidati* and its putative immunological impact. Parasitology 116: 139-147

43. HORÁK, P, DVOŘÁK J, KOLÁŘOVÁ L, TREFIL L (1999): *Trichobilharzia regenti*, a pathogen of the avian and mammalian central nervous systems. Parasitology 119: 577-581

44. HOWELL MJ, BOURNS TK (1974): *In vitro* culture of *Trichobilharzia ocellata*. Int J Parasitol 4(5): 471-476

45. HRÁDKOVÁ K, HORÁK P (2002): Neurotropic behaviour of *Trichobilharzia regenti* in ducks and mice. J Helminthol 76: 137-141

46. CHAMOT E, TOSCANI L, ROUGEMONT A (1998): Public health importance and risk factors for cercarial dermatitis associated with swimming in Lake Leman at Geneva, Switzerland. Epidemiol Infect 120: 305–314

47. CHANOVÁ M, VUONG S, HORÁK P (2007): *Trichobilharzia szidati*: the lung phase of migration within avian and mammalian hosts. Parasitol Res 100(6): 1243-1237

48. CHANOVÁ M, HORÁK P (2007): Terminal phase of bird schistosomiasis caused by *Trichobilharzia regenti* (Schistosomatidae) in ducks (*Anas platyrhynchos* f. domestica). Folia Parasitol 54(2):105-107

49. CHANOVA M, BULANTOVA J, MASLO P, HORAK P (2009): *In vitro* cultivation of early schistosomula of nasal and visceral bird schistosomes (*Trichobilharzia* spp., Schistosomatidae). Parasitol Res 104(6):1445-1452

50. ISLAM KS, COPEMAN DB (1986): The morphology and life-cycle of *Trichobilharzia parocellata* (Johnston & Simpson, 1939) Islam & Copeman, 1980 from the visceral blood vessels of Australian anatids. Syst Parasitol 8(1): 39-49

51. ISLAM KS (1986): The morphology and life-cycle of *Trichobilharzia arcuata* n. sp. (Schistosomatidae: Bilharziellinae) a nasal schistosome of water whistle ducks (D*endrocygna arcuata*) in Australia. Syst Parasitol 8(2): 117-128

52. IVANCHENKO MG, LERNER JP, MCCORMICK RS, TOUMADJE A, ALLEN B, FISCHER K, HEDSTROM O, HELMRICH A, BARNES DW, BAYNE CJ (1999): Continuous *in vitro* propagation and differentiation of cultures of the intramolluscan stages of the human parasite *Schistosoma mansoni*. Proc Natl Acad Sci USA 96: 4965-4970

53. JENKINS SJ, HEWITSON JP, FERRET-BERNARD S, MOUNTFORD AP (2005): Schistosome larvae stimulate macrophage cytokine production through TLR4-dependent and -independent pathways. Int Immunol 17(11): 1409–1418

54. JOUET D, FERTÉ H, HOLOGNE C, KALTENBACH ML, DEPAQUIT J (2009): Avian schistosomes in French aquatic birds: a molecular approach. J Helminthol 83(2): 181-189.

55. KAŠNÝ M, MIKEŠ L, DALTON JP, MOUNTFORD AP, HORÁK P (2007): Comparison of cysteine peptidase activities in *Trichobilharzia regenti* and *Schistosoma mansoni* cercariae. Parasitology 134(11):1599-609

56. KAŠNÝ M, MIKEŠ L, HAMPL V, DVOŘÁK J, CAFFREY CR, DALTON JP, HORÁK P (2009): Chapter 4. Peptidases of trematodes. Adv Parasitol 69: 205-297

57. KOLÁŘOVÁ L, HORÁK P, ČADA F (2001): Histopathology of CNS and nasal infections caused by *Trichobilharzia regenti* in vertebrates. Parasitol Res 87: 644-650

58. KOLÁŘOVÁ L, HORÁK P, SITKO J (1997): Cercarial dermatitis in focus: schistosomes in the Czech Republic. Helminthologia 34: 127-139

59. KOUŘILOVÁ P, HOGG KG, KOLÁŘOVÁ L, MOUNTFORD AP (2004a): Cercarial dermatitis caused by bird schistosomes comprises both immediate and late phase cutaneous hypersensitivity reactions. J Immunol 172(6): 3766-3774

60. KOUŘILOVÁ P, SYRŮČEK M, KOLÁŘOVÁ L (2004b): The severity of mouse pathologies caused by the bird schistosome *Trichobilharzia regenti* in relation to host immune status. Parasitol Res 93: 8-16

61. LICHTENBERGOVÁ L, KOLBEKOVÁ P, KOUŘILOVÁ P, KAŠNÝ M, MIKEŠ L, HAAS H, SCHRAMM G, HORÁK P, KOLÁŘOVÁ L, MOUNTFORD AP (2008): Antibody responses induced by *Trichobilharzia regenti* antigens in murine and human hosts exhibiting cercarial dermatitis. Par Immunol 30(11-12): 585-95

62. LOKER ES (1983): A comparative study of the life-histories of mammalian schistosomes. Parasitology 87(2): 343-369

63. MACY RW, MOORE D J, PRICE NS (1955): Studies on dermatitis producing schistosomes in the Pacific Northwest, with special refetence to *Trichobilharzia oregonensis*. Trans Am Micr Soc 74: 235-251

64. MIKEŠ L, ZÍDKOVÁ L, KAŠNÝ M, DVOŘÁK J, HORÁK P (2005): In vitro stimulation of penetration gland emptying by *Trichobilharzia szidati* and *T. regenti* (Schistosomatidae) cercariae. Quantitative collection and partial characterization of the products. Parasitol Res 96: 230-241

65. MORAND S, MÜLLER-GRAF CD (2000): Muscles or testes? Comparative evidence for sexual competition among dioecious blood parasites (Schistosomatidae) of vertebrates. Parasitology 120(1): 45-56

66. MOUNTFORD AP, HARROP R, WILSON RA (1995): Antigens derived from lung-stage larvae of Schistosoma mansoni are efficient stimulators of proliferation and gamma interferon secretion by lymphocytes from mice vaccinated with attenuated larvae. Infect Immun 63(5): 1980-1986

67. NASCIMENTO-CARVALHO CM, MORENO-CARVALHO OA (2005): Neuroschisosomiasis due to *Schistosoma mansoni*: A review of pathogenesis, clinical syndromes and diagnostic aproaches. Rev Inst Med Trop S Paulo 47(4): 179-184

68. NEUHAUS W (1952): Biologie und Entwicklung von *Trichobilharzia szidati* n. sp. (Trematoda, Schistosomatidae), einem Erreger von Dermatitis beim Menschen. Z Parasitenkd 15(3): 203-266 Neuhaus W (1952) Z Parasitenkd 15:203–266

69. OLEAGA A, RAMAJO V (2004): Efficiency of the oral, intramuscular and subcutaneous routes for the experimental infection of hamster and sheep with *Schistosoma bovis*. Vet Parasitol 124(1-2): 43-53

70. OLIVIER L (1949): Sschistosome dermatitis, a sensitization phenomenon. Am J Epidemiol 49: 290-302

71. OLIVIER L (1953): Observations on the migration of avian schistosomes in mammals previously unexposed to cercariae. J Parasitol 39(3): 237-243

72. OLIVIER L, WEINSTEIN PP (1953): Experimental schistosome dermatitis in rabbits. J Parasitol 39(3): 280-291

73. RHEINBERG CE, MONÉ H, CAFFREY CR, IMBERT-ESTABLET D, JOURDANE J, RUPPEL A (1998): *Schistosoma haematobium*, *S. intercalatum*, *S. japonicum*, *S. mansoni*, and *S. rodhaini* in mice: relationship between patterns of lung migration by schistosomula and perfusion recovery of adult worms. Parasitol Res 84(4): 338-342

74. SLUITERS JF, BRUSSAARD-WÜST CM, MEULEMAN EA (1980): The relationship between miracidial dose, production of cercariae, and reproductive activity of the host in the combination *Trichobilharzia ocellata* and *Lymnaea stagnalis*. Z Parasitenkd 63(1): 13-26

75. SOUZA VIDAL MRF, BARBOSA AA, ANDRADE ZA (1993): Experimental pulmonary schistosomiasis: Lack of morphological evidence of modulation in schistosomal pulmonary granulomas. Rev Inst Med trop São Paulo 35: 423-429

76. SOBHON P, UPATHAM ES (1990): The tegument of cercariae and schistosomula. In: Snail Hosts, Life-cycle, and Tegumental Structure of Oriental Schistosomes. UNDP/WORLD BANK/WHO special programme for research and training in tropical diseases. Geneva, Switzerland. 112–151

77. STIREVALT MA (1974): *Schistosoma mansoni*. Cercaria to Schisosomule. Adv Parasitol 12: 115-182

78. STIREWALT MA, UY A (1969): *Schistosoma mansoni*: cercarial penetration and schistosomule collection in an *in vitro* system. Experimental Parasitology 26(1): 17-28

79. VANDE VUSSE FJ (1980): A review of the genus *Dendritobilharzia* Skrjabin and Zakharow 1920 (Trematoda: Schistosomatidae). J Parasitol 66(5): 814-822

80. VERMUND SH, BRADLEY DJ, RUIZ-TIBEN E (1983): Survival of *Schistosoma mansoni* in the human host: estimates from a community-based prospective study in Puerto Rico. Am J Trop Med Hyg 32(5): 1040-1048

81. VUONG, PN, BAYSSADE-DUFOUR C, MARTINS C, BONÈTE, R, PRIGENT F, BALATON A (2002): Dermatite cercarienne: étude histologique et immuno-histochimique d`un cas humain. Méd Mal Infect 32: 284-293

82. WHITFIELD PJ, BARTLETT A, KHAMMO N, BRAIN AP, BROWN MB, MARRIOTT C, CLOTHIER R (2003): Delayed tail loss during the invasion of human skin by schistosome cercariae. Parasitology 126(2): 135-140

83. WULFF C, HAEBERLEIN S, HAAS W (2007): Cream formulations protecting against cercarial dermatitis by *Trichobilharzia*. Parasitol Res 101(1): 91-97

84. WYNN TA, THOMPSON RW, CHEEVER AW, MENTINK-KANE MM (2004): Immunopathogenesis of schistosomiasis. Immunol Rev 201: 156–167